

**The Role of Delta Sarcoglycan in Dystrophin-Glycoprotein Complex
Function in Cardiac Muscle**

by

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To Shelby
my strength, my life, my love

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Chapter 1

Introduction to Mutations in the DGC Causing Muscle Disease

The Components and Structure of the DGC

The dystrophin-glycoprotein complex (DGC) is a large multi-member complex with high expression and conservation located at the sarcolemma of cardiac, skeletal, and smooth muscle cells. The DGC is composed of both membrane bound, and membrane associated proteins. Two of the most highly conserved members of the DGC are the dystroglycans. These two proteins are transcribed from a single gene and are proteolytically cleaved to create α and β dystroglycan¹⁻⁷. Alpha dystroglycan (α DG) is made up of two large globular domains connected via a long stalk⁸⁻¹¹, that is proteolytically cleaved in the N-terminal domain after translocation to the plasma membrane¹². Alpha-dystroglycan undergoes complex glycosylation that is required for its function as an extracellular matrix receptor¹²⁻¹⁶. The carboxyl-terminal domain of α DG binds the amino-terminal of β -dystroglycan (β DG) on the extracellular side of the sarcolemma^{17, 18}. Beta dystroglycan is a single pass type-I transmembrane protein and is an integral component to the core of the DGC. The carboxyl-terminal domain of β DG binds directly to multiple domain sites of the dystrophin or utrophin within the cytosol¹⁹⁻²⁴.

Dystrophin is a large 427-kD protein made up of four domains: the carboxyl-terminus domain, cysteine-rich domain, rod domain, and amino-

terminus domain²⁵. The dystrophin protein binds cytoskeletal F-actin²⁶⁻²⁸ with a tight stoichiometry of 24 actin monomers polymerized and bound laterally to the dystrophin protein at the amino-terminus and a number of spectrin repeats within the rod domain at a length of 65 nm²⁹⁻³². The cysteine-rich domain of dystrophin binds to the carboxyl terminus of β -dystroglycan²¹ establishing a linkage between the cytoskeleton of muscle cells to the membrane bound portion of the DGC and thus the laminin-containing extracellular matrix through the linkage to α DG which is a high affinity laminin receptor. Additionally α -syntrophin^{33, 34} and α -dystrobrevin³⁵ bind at the carboxyl-terminus of dystrophin and serve as accessory proteins that may help link signaling molecules to the DGC.

In addition to dystrophin and the dystroglycans the DGC is composed of at least 5 other membrane-bound proteins, the sarcoglycans and sarcospan. Sarcospan is a quadruple-pass transmembrane protein of the tetraspanin superfamily. The interaction of sarcospan with the other members of the DGC appears to be indirect but appears important for expression of dystrophin and utrophin³⁶. The sarcoglycans exist within the DGC as a tetrameric subcomplex of proteins. There have been six sarcoglycans identified and all are transcribed from different genes: alpha-sarcoglycan³⁷ (α SG), beta-sarcoglycan^{38, 39} (β SG), gamma-sarcoglycan⁴⁰ (γ SG), delta-sarcoglycan⁴¹ (δ SG), epsilon-sarcoglycan⁴² (ϵ SG), and zeta-sarcoglycan⁴³ (ζ SG). Together the sarcoglycan complex, dystroglycans, sarcospan, dystrophin, and syntrophins form the DGC, a large complex that appears to serve as an important link between the cytoskeleton and the basement membrane around muscle cells.

The Function of the DGC in Muscle

The critical roles of the DGC in muscle are still debated but the DGC has been implicated in a number of cellular functions within muscle. The DGC has a proposed role in cellular signaling that is actively pursued but many conflicting reports have arisen that call into question whether the DGC truly plays a role in active signaling. Some of the earliest evidence suggesting a signaling role for the DGC was the fact that neuronal nitric oxide synthase (nNOS) copurifies with the DGC from skeletal muscle.⁴⁴ Neuronal nitric oxide synthase has a role in cardiac myocyte contraction⁴⁵, calcium cycling^{46, 47}, and left ventricular function⁴⁸. The primary nNOS signaling mechanism in cardiac myocytes is through phosphorylation of phospholamban causing increased calcium uptake into the sarcoplasmic reticulum^{49, 50}. Neuronal nitric oxide synthase is associated with the DGC through tight binding with α_1 -syntrophin and the PDZ domain of α_1 -syntrophin binds dystrophin⁵¹. Originally the association of nNOS with the DGC led to the hypothesis that knockout would cause a dystrophic phenotype similar to that seen in many other mutations of the DGC. However, knockout of nNOS does not cause a noticeable muscular dystrophy⁵² nor does knockout of α_1 -syntrophin⁵³, β_2 -syntrophin or both α_1 and β_2 -syntrophin⁵⁴. Although alteration of the nNOS-syntrophin complex within the DGC does not cause muscular dystrophy disruption of nNOS does appear to alter other pathways such as muscle regeneration⁵⁵ and muscle inflammation⁵⁶. Despite association with the DGC and the role of nNOS in muscle signaling pathways, exactly how the DGC regulates action of nNOS is currently unknown.

In addition to signaling pathways involving nNOS it is proposed that β -dystroglycan (β DG) has a direct signaling role through the binding of the adaptor protein growth factor receptor 2 (Grb-2). Cells bind extracellular matrix proteins, including laminin- α 2, triggering phosphorylation directly on tyrosine residue 892 of β DG that binds the SH3 domain of Grb-2⁵⁷, a protein involved in the organization of the cytoskeleton. Oddly this site is also where β DG binds to dystrophin suggesting that phosphorylation at this site in β DG may somehow cycle the interaction of β DG with either Grb2 or dystrophin. Preventing phosphorylation of β DG improves the dystrophic phenotype of dystrophin deficient mice⁵⁸ further supporting the hypothesis dystrophin binding to β DG helps stabilize the complex.

There is quite a bit of evidence suggesting that the DGC is not only involved in cell signaling but is also partially responsible for stabilizing the sarcolemma during mechanical strain (Figure 1-2). The dystrophin protein and thus DGC is enriched at the costamere aligning to the z-discs in skeletal muscle^{59, 60}. The costameres are hypothesized to be partially responsible for lateral force transmission in both skeletal muscle⁶¹ and cardiac muscle⁶² suggesting that the DGC plays some role in force transmission and perhaps protection of the sarcolemma during mechanical events. Sarcolemma membrane stability is decreased when dystrophin is lost in patients⁶³, mouse muscle^{64, 65}, or cultured mouse myotubes⁶⁶. Additionally, knockout of dystroglycan⁶⁷ results in compromised sarcolemmal permeability. When membrane stability is altered the sarcolemma becomes increasingly permeable to extracellular ions including

calcium^{65, 66, 68, 69} and this phenotype is further exacerbated through exercise and activation of the muscle⁷⁰ but can be improved through immobilization^{71, 72} indicating that the mechanical action of muscle stretch and contraction is at least partially responsible for sarcolemmal damage that occurs in muscle with disrupted DGC.

Mutations in the DGC and Related Diseases

The importance of the DGC in muscle is indicated by the fact that mutations in many of its components lead to inherited forms of muscle disease. There have been many mutations identified within the genes responsible for transcribing the proteins of the DGC and within genes responsible for some modulations of the DGC. Most well known are mutations within the dystrophin gene that cause Duchenne muscular dystrophy^{73, 74} (DMD) or less severe Becker Muscular Dystrophy²⁵ (BMD). DMD/BMD are X-linked disorders, DMD affects 1 in 3500 live male births and is characterized by severe skeletal muscle wasting and weakening leading ultimately to loss of ambulation and early death. In conjunction with severe skeletal phenotypes individuals with DMD are at a high risk for cardiovascular complications including progressive dilated cardiomyopathy (DCM), congestive heart failure (CHF), arrhythmias, and sudden cardiac death⁷⁵. BMD affects approximately 1 in 19,000 live male births and results in a similar, but much less severe phenotype than that of DMD. Mutations in the dystrophin gene cause a total loss of the dystrophin protein⁷⁶⁻⁷⁸ (DMD) or a reduction of dystrophin protein⁷⁹ (BMD) at the plasma membrane in muscle.

Despite the integral role played by α and β DG in overall DGC function only one disease causing mutation has been identified in the gene that codes for the two proteins. A T192M mutation in a single patient was identified that presented with limb-girdle muscular dystrophy and cognitive impairment⁸⁰. This mutation results in hypoglycosylation of α DG and a decrease in laminin-binding activity. This primary dystroglycanopathy actually resembles a number of other mutations that have been identified in genes responsible for post-translational modification of α DG that are pathogenic resulting in several forms of mild to severe muscular dystrophies. Glycosyltransferases are enzymes responsible for imparting sugar moieties to proteins. There are eight known glycosyltransferases necessary for proper glycosylation of α DG: POMT1, POMT2, POMGnT1, LARGE, FKR, IPSD. Mutations in many of these glycosyltransferases is sufficient to disrupt normal binding of α DG to laminin- α 2 causing a dystrophic phenotype⁸¹⁻⁸⁷.

The majority of mutations in the DGC causing muscle disease all have a common theme – disruption of the functional linkage between the cytoskeleton in muscle and the extracellular matrix through the DGC. Two connections are responsible for this DGC protection during mechanical events: 1) The link to the extracellular matrix via glycosylated α DG and 2) the link to the cytoskeleton via dystrophin. If either of these two links is disturbed it results in a mild to severe muscular dystrophy and a greater prevalence for cardiac disease suggesting the role of mechanical protection by the DGC is similar in both skeletal and cardiac muscle.

The Sarcoglycans are Integral Components of the DGC

The sarcoglycan complex is a specific subcomplex of four sarcoglycans that exists at the plasma membrane and associated with the DGC. There have been six sarcoglycan genes identified and each transcodes a separate sarcoglycan protein. There have been a number of mutations identified within the sarcoglycans. The most common mutations are those in the sarcoglycans expressed in striated muscle that cause limb-girdle muscular dystrophy. Examining the literature on these sarcoglycans individually allows insight into the role that a single sarcoglycan may play within the complex as a whole.

Alpha-sarcoglycan

Alpha sarcoglycan (α SG), originally named adhalin, is a 50-kD member of the sarcoglycan complex^{37, 88, 89}. α SG is expressed highly in skeletal muscle and in lower levels in cardiac muscle. The cardiac form of α SG is a shorter alternatively spliced form of the same α SG gene product⁹⁰. α SG is single-pass type-I transmembrane protein composed of three primary domains: a large extracellular domain, transmembrane domain, and a cytoplasmic domain. α SG contains two putative sites for N-linked glycosylation at residues 174 and 246 and is glycosylated in vivo⁹¹ in skeletal muscle.

Alpha sarcoglycan is transcribed by the SGCA gene located on chromosome 17. There have been at least 28 naturally occurring variants and mutations identified in α SG and many of these mutations in the SGCA gene cause limb-girdle muscular dystrophy type 2D (LGMD2D) of differing severity^{89, 92-95}. Interestingly, all of the known LGMD2D occur within the α SG's extracellular

domain. The bulk of the α SG protein is the extracellular domain and contains a cadherin-like domain from residues 27-131⁹⁶. This domain has been hypothesized to be important for interactions between the cadherin-like domain within α DG. Additionally, it is possible that the cadherin-like domain within α SG may be important for interaction with other sarcoglycans or adhesion to unidentified binding partners within the extracellular matrix. The potential for the cadherin-like domain to bind proteins within the ECM is even more intriguing when it is noted that 16 of the known mutations causing LGMD2D map to this domain in α SG. However, there are at least another 12 LGMD2D causing mutations within α SG that map to the extracellular domain but not within the cadherin-like domain. It is possible that mutations outside the cadherin-like domain may still alter protein structure enough to disrupt α SG stability sufficient to cause loss of the protein and subsequent muscular dystrophy. Limb-girdle muscular dystrophy 2D causing mutations in α SG likely affect protein stability resulting in protein loss from the sarcolemma. It is likely that mutations within the extracellular domain of α SG exert their effects by disrupting interactions of α SG with the other sarcoglycans leading to degradation of α SG through endoplasmic reticulum associated degradation (ERAD).

A mouse model of LGMD2D has been generated in which the *Sgca* gene has been knocked out. This mouse recapitulates the degenerative skeletal muscle phenotype seen in patients with LGMD2D⁹⁷. Similar to LGMD2D patients these mice show no apparent cardiac muscle defects. This is likely due to compensation by ϵ SG in cardiac tissue. ϵ SG is expressed at lower levels in the

skeletal and cardiac muscle and in high levels in smooth muscle^{98, 99}.

Overexpression of ϵ SG in striated muscle of mice lacking α SG ameliorates skeletal muscle dystrophy¹⁰⁰ supporting the hypothesis of ϵ SG compensation in α SG deficiency. Furthermore, double knockout of α and ϵ SG in mice results in cardiac fibrosis and increased membrane permeability exacerbated by exercise¹⁰¹.

Beta-sarcoglycan

Beta sarcoglycan (β SG) is a 43-kD member of the sarcoglycan complex highly expressed in both skeletal and cardiac muscle^{38, 39, 102-105}. β SG is a single-pass type II transmembrane protein composed primarily of three domains: a cytoplasmic domain, a transmembrane domain, and an extracellular domain. β SG contains a putative N-linked glycosylation site at residue 258 and two known glycosylation sites at 158 and 211¹⁰⁶. It should be noted that experimental evidence identifying the two known N-linked glycosylation residues, 158 and 211, was only demonstrated in human liver; the precise glycosylation of β SG in muscle is still undetermined. Additionally, sequence analysis reveals possible sites for disulfide bonds in β SG between residues 288 and 314, and between 290 and 307.

β SG is transcribed by the SGCB gene located on chromosome 4. There have been at least 13 naturally occurring variants and mutations identified in β SG, and many of the mutations in the SGCB gene cause limb-girdle muscular dystrophy type 2E^{38, 39, 94, 107-109} (LGMD2E). Only one of the identified mutations in β SG occurs within its cytoplasmic domain. A glutamine to glutamic acid

substitution at residue 13 in the cytoplasmic domain causes severe DMD-like LGMD2E. It is unclear what the functional consequences of this transformation are but this particular mutation and subsequent LGMD2E supports the hypothesis that the δ/β sarcoglycan core interacts directly with dystrophin¹¹⁰ within the cytoplasmic domain of β SG. The remaining mutations in β SG occur within the extracellular domain and likely disrupt formation of the δ/β sarcoglycan core resulting in complete sarcoglycan complex loss. Oddly despite a large extracellular domain, residues 87-314, all known disease causing mutations fall between residues 91 and 184. This may be because mutations outside of this region of the extracellular domain do not disrupt β SG protein interactions resulting in loss of protein and thus do not cause disease. A mouse model of LGMD2E has been generated in which the *Sgcb* gene has been knocked out. These mice develop severe muscular dystrophy and cardiomyopathy¹¹¹. This phenotype is remarkably similar to patients with LGMD2E although global muscular dystrophy in these mice is more severe than that seen in patients suggesting that pathology in humans differs slightly from those in mice.

Gamma-sarcoglycan

Gamma sarcoglycan (γ SG) is 35-kD member of the sarcoglycan complex highly expressed in both skeletal and cardiac muscle⁴⁰. γ SG is a single-pass type II transmembrane protein composed primarily of three domains: a cytoplasmic domain, transmembrane domain, and extracellular domain. γ SG contains one putative site for N-linked glycosylation at residue 110. Additionally, sequence

analysis reveals possible sites for disulfide bonds in γ SG between residues 265 and 290, and between residues 267 and 283.

γ SG is transcribed by the SGCG gene located on chromosome 13. There have been at least 5 naturally occurring variants and mutations identified in γ SG and many of the mutations in the SGCG gene cause limb-girdle muscular dystrophy type 2C^{40, 112-115}. All but one mutation identified in γ SG occurs within the extracellular domain. Point mutations in this domain and truncations lacking the C-terminus likely disrupt formation of the sarcoglycan complex. Oddly a leucine to proline mutation has been identified at residue 53 within the transmembrane domain of γ SG¹¹². This mutation causes mild LGMD2C, and although the sarcoglycans were reduced in muscle biopsies they are still present at higher levels than more severe limb-girdle muscular dystrophies. Mutations within the extracellular domain of γ SG probably disrupt interaction with the δ/β sarcoglycan core and full sarcoglycan complex assembly. The L53P mutation within the transmembrane domain of γ SG likely disturbs sarcoglycan complex integration into the plasma membrane explaining why the sarcoglycans are present but reduced at the plasma membrane. A mouse model of LGMD2C has been generated in which the *Sgcg* gene has been knocked out. These mice develop muscular dystrophy, cardiomyopathy, and smooth muscle necrosis⁹¹.

Delta-sarcoglycan

Delta sarcoglycan (δ SG) is a 35-kD member of the sarcoglycan complex highly expressed in skeletal and cardiac muscle^{41, 116}. δ SG is a single-pass type II transmembrane protein composed primarily of three domains: a cytoplasmic

domain, transmembrane domain, and large extracellular domain. δ SG contains three putative sites for N-linked glycosylation at residues 60, 108, and 284. Additionally sequence analysis reveals possible sites for disulfide bonds between residues 263 and 288, and between 265 and 281. δ SG is also phosphorylated at serine residue 194, and threonine residue 196 in human platelets¹¹⁷. There have been at least 8 naturally occurring variants and mutations identified in δ SG leading to limb-girdle muscular dystrophy 2F and/or cardiomyopathy. Mutations and natural variants of δ SG are discussed in further detail in the section, "Mutations in Delta Sarcoglycan and Related Diseases."

Epsilon-sarcoglycan

Epsilon sarcoglycan (ϵ SG) is a 45-kD member of the sarcoglycan complex ubiquitously expressed in human tissue⁴². Although ϵ SG is ubiquitously expressed it is lower in overall abundance than α , β , γ , and δ SG. ϵ SG is a single-pass type I transmembrane protein composed primarily of three domains: a cytoplasmic domain, transmembrane domain, and large extracellular domain. Residue 200 in ϵ SG is a site of N-linked glycosylation within liver tissue¹⁰⁶.

ϵ SG is transcribed by the SGCE gene on chromosome 7. There have been at least 15 naturally occurring variants and mutations identified in ϵ SG and many of the mutations in the SGCE gene cause myoclonus dystonia¹¹⁸⁻¹²⁶ (DYT11). Myoclonus dystonia disorders are heterogeneous in presentation but generally are characterized by involuntary jerks of the arms, neck, and trunk. Additionally, patients frequently develop psychological abnormalities. The molecular basis for myoclonus dystonia is still not well understood but patients

exhibit abnormal EEG in the subcortical region of the brain suggesting DYT11 is neurological in regard to pathology¹²⁴. It is unclear why mutations in ϵ SG cause disease dissimilar to mutations in the other sarcoglycans but suggests the ϵ SG has a functional role in brain that is clearly unique from the role that the sarcoglycan complex plays in striated muscle.

Zeta-sarcoglycan

Zeta sarcoglycan (ζ SG) is a 40-kD member of the sarcoglycan complex expressed primarily in striated muscle and highly in smooth muscle cells⁴³. ζ SG is a single-pass type II transmembrane protein composed primarily of three domains : a cytoplasmic domain, transmembrane domain, and extracellular domain. ζ SG contains two putative glycosylation sites at residues 62 and 110. Additionally sequence analysis reveals possible sites for a disulfide bond between residues 273 and 289.

Sarcoglycan Organization

Despite variation among the mutations, disease pathology among many of these mutations is remarkably similar. One explanation for this phenomenon is that many of the point mutations within single sarcoglycans are null mutations that completely destabilize the assembly of the sarcoglycan complex. The level of severity among the many mutations in the sarcoglycan complex may be completely related to the degree to which the sarcoglycan complex is destabilized. For instance, mutations within the extracellular domain of β SG

cause severe skeletal muscle dystrophy as well as cardiomyopathy, whereas mutations in α SG are not as severe.

The six sarcoglycans vary in tissue expression. In cardiac and skeletal muscle the most highly expressed sarcoglycans are β , δ , γ , and α . The homology among a number of sarcoglycans suggests that some of the sarcoglycans may have arisen from gene duplication events during vertebrate evolution. Global sequence alignments produce three distinct groups among the sarcoglycans. β SG is about one third homologous to three other sarcoglycans, but its homology is relatively low compared to the high homology among other sarcoglycans. The second group is the α/ε sarcoglycans. These two sarcoglycans are greater than 50 percent homologous to one another and show very low homology with all other sarcoglycans. The third and final group is the $\gamma/\delta/\zeta$ sarcoglycans. These three sarcoglycans all have greater than 70 percent homology with each other and low homology with all other sarcoglycans. See table 1.2 for sarcoglycan protein family homology details.

Although the sarcoglycans show homology with one another each individual sarcoglycan in the complex is required for proper assembly and localization to the plasma membrane¹²⁷. It is hypothesized within skeletal and cardiac muscle that the stoichiometry of the tetrameric sarcoglycan complex composed of α , β , γ , and δ is 1:1:1:1¹¹⁶, and that assembly of the sarcoglycans and subsequent association with sarcospan and the dystroglycans follows a strict process through the secretory pathway. The first step of sarcoglycan synthesis and assembly in muscle is the association of a sarcoglycan core composed of δ

and β SG^{128, 129}. The second and third steps are not completely defined, but follow one of two likely scenarios. The first possible scenario is that γ SG is added to the δ/β core and then associates directly with α SG. The second possible scenario is that α SG directly interacts with the δ/β core rather than being recruited to the complex by γ SG^{91, 130, 131}. Of all the interactions within the sarcoglycan complex, the association of α to the remaining sarcoglycans is the weakest, supporting the idea that it is added last and with the lowest overall binding affinity¹²⁸ (Figure 1-3).

Regardless of the remaining order the most essential step of sarcoglycan complex assembly is the association of the δ/β core¹³⁰. Mutation or loss of δ , β , or both sarcoglycans disrupts complex assembly and localization to the plasma membrane is halted. If this occurs all sarcoglycan components are completely undetectable at the plasma membrane^{91, 111}. Conversely, when γ SG is lost there are still detectable, albeit reduced, levels of the other sarcoglycans at the plasma membrane in muscle⁹¹. Additionally, there are residual levels of δ , β , and γ SG detectable in skeletal muscle when α SG is lost and the entire complex appears to be expressed normally in cardiac muscle⁹⁷. This may be due to other sarcoglycans, including ϵ SG, compensating for the mutation or loss of α SG.

Sarcoglycan compensation is observed in cardiac muscle where ϵ SG can compensate for the loss of α SG⁹⁷. The high homology between α and ϵ SG is likely what allows compensation without noticeable deleterious effects. This hypothesis is further supported by the fact that mutations resulting in loss of β SG generally have no compensatory mechanism and there are no sarcoglycans with

high enough homology to replace β SG. However, mutations within δ SG are particularly pathogenic and there is no apparent compensation by other sarcoglycans despite sharing high homology with γ and ζ SG. This may be due to the crucial assembly of the δ/β sarcoglycan core and that no compensation can occur during this critical step of sarcoglycan complex assembly.

It is possible that the interaction of β and δ to form the sarcoglycan core may be the only necessary ordered step in sarcoglycan complex assembly, and that interaction of γ and α SG with the complex may occur in any manner thereafter. There is another possibility suggested by a handful of groups that δ , β , and γ SG can also form a trimeric complex, distinct in function from the tetrameric complex^{110, 128, 132}. The exact ordering of the sarcoglycan associations is still unclear, but what is known is that the initial interactions of the sarcoglycans occur within the ER where the complex is formed and then transported to the golgi and finally to the plasma membrane¹³³. The sarcoglycans exit the golgi as a complex and are associated with sarcospan and the rest of the DGC either at the plasma membrane or at some point in the secretory pathway between the golgi and the plasma membrane¹³³ (Figure 1-4).

The Function of the Sarcoglycan Complex

The role of the sarcoglycan complex both in isolation and as a member of the DGC is not well defined. It is assumed that the sarcoglycan complex stabilizes the DGC at the plasma membrane in some manner. Loss of the sarcoglycan complex results in increased sensitivity of β DG to cleavage by a

matrix metalloproteinase (MMP) disrupting the linkage of the cytoskeleton to the extracellular matrix¹³⁴⁻¹³⁶. Muscular dystrophies that retain the sarcoglycan complex, like those caused by defects in glycosyltransferases, do not have similar cleavage of β DG¹³⁵ suggesting that physical access to the cleavage site on β DG by the MMP may be necessary and the presence of the sarcoglycan complex inhibits this access¹³⁶.

More evidence pointing toward a role in DGC stabilization is a strong association between the sarcoglycan complex and the dystroglycans and dystrophin protein^{24, 128, 137-139}. It is unknown whether the sarcoglycan complex binds directly to α DG, β DG, or both but in muscle that has lost dystrophin at the membrane the sarcoglycan complex is drastically reduced¹⁴⁰. Additionally, the C-terminus of dystrophin appears to directly interact with δ and β SG in vitro in COS-1 cells although this interaction is rather weak¹¹⁰. It is possible that in vivo dystrophin binds a fully assembled sarcoglycan complex with greater affinity. However, the association of dystrophin to the remaining members of the DGC is not contingent upon sarcoglycan expression because loss of the sarcoglycan complex results in only diminished expression of dystrophin at the plasma membrane⁹⁷ or no change in dystrophin expression^{91, 111, 141}. The apparent role of dystrophin in membrane stabilization^{63, 66}, and the close association of the sarcoglycans with dystrophin, suggests that the role of the sarcoglycan complex in stabilization of the DGC also extends to a mechano-protective role. When the sarcoglycan complex is lost, cell membrane permeability is compromised^{91, 97, 141-144}. However, there is evidence to suggest that the exact mechanical role of the

sarcoglycan complex may be distinct from the role that dystrophin plays¹⁴⁵. In addition to DGC stabilization, the sarcoglycan complex may have a direct role in linking the extracellular matrix to the cytoskeleton. Both γ and δ SG interact with the actin binding protein γ -filamin¹⁴⁶. On the extracellular side disruption of either α or γ SG reduces cell adhesion mediated via $\alpha_5\beta_1$ integrin¹⁴⁷.

There is also data to suggest the sarcoglycan complex is involved in cell signaling pathways via the DGC. The sarcoglycan-sarcospan complex can associate directly with the N-Terminal region of the α -dystrobrevins¹⁴⁷. Despite binding the dystrobrevins it has not been established that the sarcoglycans actually alter signaling activity. It may be that the sarcoglycans bind dystrobrevin and form a scaffold for signaling through dystrophin rather than directly modulate activity of the dystrobrevin-syntrophin-nNOS complex. The type-II sarcoglycans δ , β , and γ all contain epidermal growth factor (EGF) receptor-like domains in the C-terminus¹⁴⁸ suggesting these sarcoglycans may be receptors for unknown signaling mechanisms. One sarcoglycan signaling mechanism that is known is phosphorylation of γ SG and subsequent upregulation of the ERK signaling pathway following eccentric contractions in skeletal muscle¹⁴⁹. The function of this phosphorylation is unknown but suggests that the sarcoglycan complex may act as a signaling sensor when muscle is mechanically stimulated. The sarcoglycan complex is also implicated in ATP signaling via α SG. Alpha sarcoglycan contains an ATP-binding site in the extracellular domain and hydrolyzes ATP^{150, 151}. ATP signaling via this pathway appears protective for

myocytes during contraction¹⁵² suggesting that loss of this enzymatic function may play a role in muscular dystrophy pathology.

The sarcoglycan complex is implicated in diverse roles in cellular function, however parsing the exact role of each sarcoglycan is confounding for a number of reasons. 1) Organization of the sarcoglycan complex is contingent upon proper expression of all sarcoglycans. 2) Compensation that occurs between sarcoglycans in disease states is not complete and appears tissue specific. 3) Muscular dystrophies caused by mutations in the sarcoglycans have similar pathogenic outcomes suggesting the sarcoglycans may function primarily as a complex. Mutations in the sarcoglycans have so far provided the most insight into both the function of individual sarcoglycans and the sarcoglycan complex as a whole.

Mutations in Delta Sarcoglycan and Related Diseases

Delta sarcoglycan is a crucial sarcoglycan that has not been discussed yet that is both critical for sarcoglycan complex formation and function and is also unique in the way mutations apparently affect pathology. Similar to mutations in α , β , and γ SG recessively inherited mutations in δ SG cause limb-girdle muscular dystrophy type 2F^{112, 116, 153-156} (LGMD2F). The original mutation linking δ SG to LGMD2F was a deletion of nucleotide 656 in the coding region of the gene that produces a premature truncated protein lacking the C-terminus and cysteine rich region of the δ SG protein¹⁵⁶. A second LGMD2F patient was identified resulting from a truncated δ SG protein¹⁵⁴. This mutation, R165X, causes a severe

muscular dystrophy suggesting any number of mutations in δ SG may cause LGMD2F. Since the initial discovery of δ SG mutations leading to LGMD2F there have been at least three other mutations in δ SG identified that cause LGMD2F of varying severity: E262K¹⁵⁵, G76C¹¹², and A131P¹⁵³. Truncations of the δ SG protein expressed in COS-1 cells have suggested that the region of the extracellular domain of δ SG nearest the plasma membrane is the site of interaction with β SG¹¹⁰ during sarcoglycan complex assembly. All five known mutations that cause LGMD2F occur within the extracellular domain of δ SG supporting the hypothesis that this region is critical for δ SG function.

In addition to causing LGMD2F some mutations in δ SG have been linked to dilated cardiomyopathy (DCM) without an accompanying skeletal muscle dystrophy. Using candidate gene screening, patients with a dominant familial pattern of DCM inheritance were identified that had mutations within δ SG^{157, 158}. One mutation identified was a deletion of lysine residue 238 (Δ K238). Following heart transplant of the patient immunostaining of the affected myocardium showed a reduction, but not complete loss of the sarcoglycan complex at the plasma membrane. It was not identified whether the other DCM-linked δ SG mutations followed a similar pattern of expression within the myocardium. Transgenic mice overexpressing DCM-linked δ SG mutant S151A develop DCM and have a subsequent early mortality. Oddly δ SG mutant S151A proteins did not localize to the plasma membrane and were instead retained in discrete nuclear foci costained with nuclear proteins lamin A/C and emerin¹⁵⁹. Additionally nuclear foci stained with mutant δ SG S151A show partial staining for β and γ SG.

These results suggested that S151A is a dominant negative mutant affecting trafficking and not necessarily sarcoglycan complex assembly or function. The S151A mutation does not appear to follow the same pattern of expression as Δ K238 further confounding the debate about whether DCM-linked mutations in δ SG are the result of a dominant negative effect or haploinsufficiency. A report of a consanguineous family expressing both an LGMD2F causing mutation (A131P) and DCM-linked S151A mutation in δ SG showed that individuals with both mutations develop LGMD2F but not DCM¹⁵³ calls into question whether the S151A mutation in δ SG actually causes DCM. The heterogeneous presentation of DCM causing mutations in δ SG raises an important question about the mechanism of pathology. Dominantly inherited mutations do not appear to act as null mutations leading to the hypothesis that either these mutants act as dominant negative proteins or haploinsufficiency causes disease progression.

The Structure of Delta Sarcoglycan

The native structure of δ SG has not been completely elucidated. Delta sarcoglycan possesses a short cytosolic tail at the N-terminus from residues 1-35. A hydrophobic region from residues 36-56, likely forming an α -helix (based on sequence analysis), comprises the transmembrane domain. Finally, the majority of the δ SG protein is the extracellular domain from residues 57-289. The extracellular domain contains a conserved cysteine rich motif in its final 30 residues prior to the C-terminus. Deletion of the protein prior to the cysteine rich motif results in drastic reduction of the sarcoglycan complex at the plasma

membrane suggesting this region of the protein and particular motif are necessary for proper localization following secretory pathway processing¹¹⁰. The residues in the extracellular domain between 57 and 92 appear necessary for the interaction with β SG to form the δ/β sarcoglycan core¹¹⁰. This region of the sarcoglycan protein contains one of the sites of putative N-linked glycosylation although the role of glycosylation for the interaction with the sarcoglycan complex or proper processing through the secretory pathway is unclear. Finally the intracellular tail of δ SG appears to interact directly with the C-terminus of dystrophin¹³⁹ however this interaction only occurs when δ and β SG are associated¹¹⁰ suggesting that not only is the δ/β sarcoglycan core essential for complex assembly but also for some functional linkage to the cytoskeleton via dystrophin. The δ SG protein forms multiple interactions with members of the DGC. What is not completely clear is how the post-translational modifications of δ SG affect these interactions and or the function of δ SG.

The sarcoglycan complex appears to operate as a unit. Mutations and/or loss of any one sarcoglycan normally expressed in muscle destabilizes the entire complex causing muscular dystrophy and commonly cardiomyopathy. There is some evidence to suggest that despite the sarcoglycan complex operating as “a unit” some sarcoglycans have singular functions as well. The role of α SG in cell signaling, and the intracellular connection established between δ/β sarcoglycan and the protein dystrophin suggests that, much like the DGC as a whole, the sarcoglycan complex forms many interactions via the various sarcoglycan proteins. Most intriguing is the ability of δ SG mutations R97Q and R71T to

behave differently than mutations already identified that cause limb-girdle muscular dystrophy 2F. This in addition to the apparent dominant pattern of inheritance suggests that the mechanism of δ SG dysfunction is unique among sarcoglycan mutations. Oddly disease causing mutations in δ SG all occur within the extracellular domain suggesting that this region and its post-translational modifications are essential for proper function of the δ SG protein. Additionally, how different mutations in δ SG present with entirely different disease pathology has not been addressed. The lack of disease pathology in skeletal muscle caused by DCM-linked mutations in δ SG suggests that these mutations either cause haploinsufficiency in cardiac tissue or act as dominant negative proteins when integrated into the sarcoglycan complex.

Rationale and Experimental Approach

One of the most complex issues concerning δ SG is that mutations normally result in the destabilization of the entire sarcoglycan complex. The molecular mechanisms for the subsequent development of muscular dystrophy and cardiomyopathy are confounding because of this. The identification of dominantly inherited mutations in δ SG linked to DCM suggests that some mutations in δ SG may exert their effect in a dominant negative manner on sarcoglycan complex function. Why these mutations result in only cardiomyopathy and no skeletal muscle dystrophy is a mystery. Additionally the extracellular domain of δ SG appears to be the region of the protein most necessary for δ SG interactions and cellular function. Not by coincidence this

region of the protein undergoes heavy post-translational modifications suggesting that, similar to glycosylation of α DG, the post-translational modifications of δ SG likely have functional roles beyond simple processing through the secretory pathway.

The overall working hypothesis of this dissertation is the following:

dominant mutations in the extracellular domain of delta sarcoglycan alter protein structure or post-translational modifications causing DGC dysfunction and lead to disrupted mechanical stability of cardiac

myocytes. This hypothesis was tested with the following specific aims:

Specific Aim 1) Determine if sarcolemmal integrity is impaired in cardiac myocytes expressing DCM mutant δ SG proteins.

The purpose of this set of experiments, described in Chapter 2, was to investigate functional defects of cardiac myocytes caused by DCM mutant δ SG. The model for this study was isolated rat cardiac myocytes transduced with adenoviral vectors expressing wild type or mutant δ SG R97Q or R71T driven by the CMV promoter and engineered with a C-terminus myc tag. I tested the hypothesis that DCM-linked δ SG mutant proteins act in a dominant negative manner to destabilize sarcolemmal integrity in cardiac myocytes. Using a live cell-labeling assay shows that mutant δ SG proteins localize and integrate into the plasma membrane with the same efficiency as wild type proteins suggesting that the mechanism for mutant pathology is not the same as LGMD2F. In order to test sarcolemmal integrity cardiac myocytes were incubated with the membrane impermeable dye propidium iodide and stretched for 24 hours in a physiological

range. After mechanical strain cardiac myocytes expressing mutant δ SG R97Q or R71T have increased dye uptake indicating that expression of mutant δ SG is sufficient to cause sarcolemmal instability. Additionally cardiac myocytes expressing mutant δ SG show an increase in cell contracture post-stretch that suggests plasma membrane integrity is compromised enough to alter cell morphology. Furthermore, cell rounding may actually be an indication of increased cell death in a population of cardiac myocytes expressing mutant δ SG. Unexpectedly cardiac myocytes expressing mutant R71T display an abnormal expression pattern when observed via western blot. Exposure of mutant δ SG R71T to endoglycosidase EndoH or PNGaseF reveals this abnormal expression to be aberrant glycosylation. This was not expected but is easily explained. The alteration of residue 71 from an arginine to a threonine creates an ectopic consensus site for N-linked glycosylation. This study demonstrates that DCM-linked δ SG mutants act in a dominant negative manner to destabilize the sarcolemma during mechanical strain. Additionally these results suggest a functional role for post-translational modifications of the extracellular domain of δ SG

Specific Aim 2) Determine if glycan residues on δ SG are necessary to stabilize the sarcolemma during mechanical strain.

To further identify the functional role of N-linked glycosylation in δ SG protein function adenovirus was generated expressing mutant δ SG lacking the putative glycosylation consensus sites at residue 60, 108, and 284. I tested the hypothesis that glycan residues on the δ SG protein are necessary for protein

function and stabilize δ SG protein interactions within the DGC. These results, described in Chapter 3, demonstrate that the only residues glycosylated on δ SG in cardiac myocytes are residue 108 and 284. Oddly residue 108 appears to be glycosylated on all δ SG proteins whereas residue 284 appears to be occupied on approximately half of the proteins based on western blot visualization. Loss of glycosylation at either site does not affect δ SG localization to the plasma membrane as previously suspected. To test whether these glycan residues are necessary for sarcolemmal stabilization cardiac myocytes expressing glycan deficient δ SG were submitted to mechanical strain. Mutant N108Q expressing cardiac myocytes detach from the laminin coated plate after mechanical strain. Together these results suggest that the glycan at residue 108 is necessary for DGC interactions with the extracellular matrix during mechanical strain.

Chapter 4 will summarize the findings of this dissertation and explain how these results fit into current understanding of DGC dysfunction and the functional role δ SG plays in the overall DGC in cardiac myocytes. Finally, this dissertation will conclude with unanswered questions and logical future directions to answer these questions. Ultimately a better understanding of the functional role of δ SG within the DGC will hopefully provide insight into therapeutic targets for patients that have mutations in δ SG that cause either muscular dystrophy or lethal dilated cardiomyopathy.

Acknowledgements

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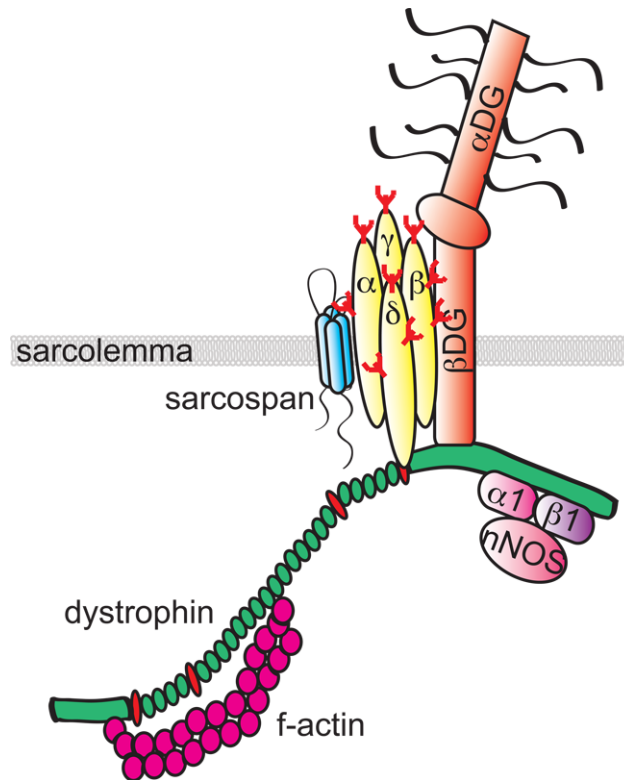


Figure 1-1 Composition of the DGC in striated muscle. Membrane bound proteins are the dystroglycans, sarcoglycans, and sarcospan. The dystroglycans and sarcoglycans undergo heavy post-translational modifications in the form of O and N-linked glycosylation. Glycan residues on α DG are necessary for binding to laminin. Singaling is accomplished primarily through nNOS and the syntrophin complex (α 1 and β 1) that interacts directly with dystrophin. Sarcolemma stabilization occurs through the linkage of β DG and δ/β sarcoglycan to dystrophin.

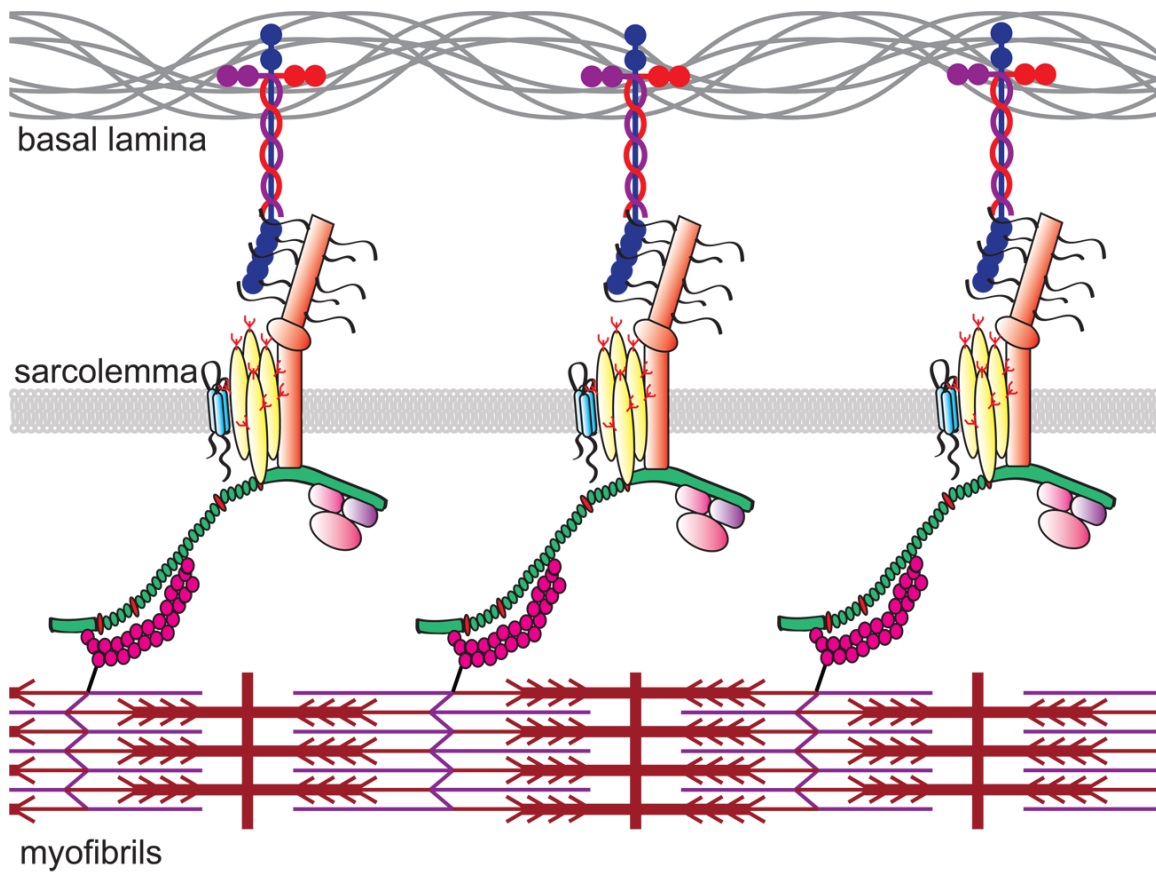


Figure 1-2 The DGC acts as a functional linkage between the basal lamina and myofibrils in striated muscle. This linkage stabilizes the sarcolemma during muscle contraction.

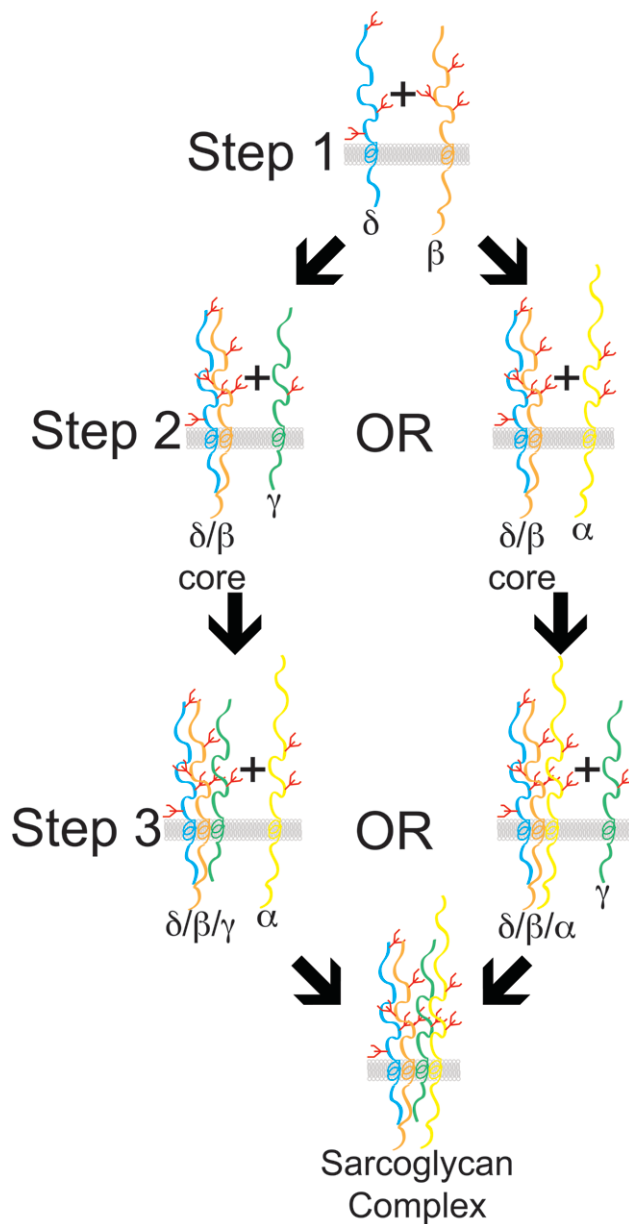


Figure 1-3 Assembly of the sarcoglycan complex in the secretory pathway. The sarcoglycans are first synthesized in the ER and N-glycosylated. Sarcoglycan complex assembly then occurs in multiple steps in the endoplasmic reticulum. **1)** δ and βSG associate to form the δ/β sarcoglycan core. **2)** Either γSG or αSG is recruited to the δ/β sarcoglycan core. **3)** The final sarcoglycan either α or γ is recruited to and associates with the intermediate tricomplex to form the full sarcoglycan complex. Full sarcoglycan assembly is normally a prerequisite for continued processing through the secretory pathway to the golgi apparatus.

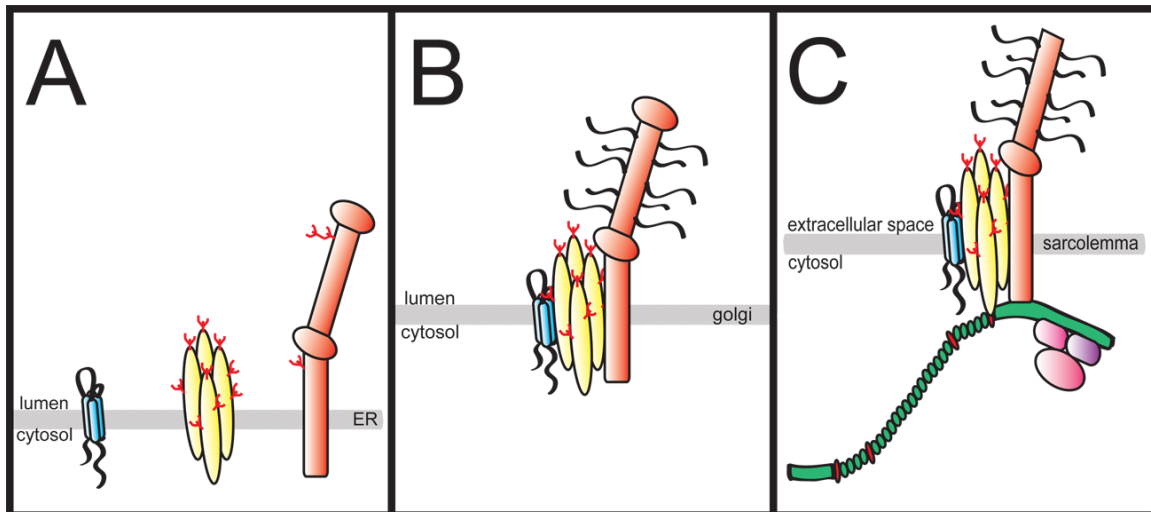


Figure 1-4. Overall assembly & post-translational modifications of the DGC

A, The primary membrane bound portion of the DGC is synthesized in the endoplasmic reticulum. The sarcoglycans are N-glycosylated in the ER and assemble to form the sarcoglycan complex. The dystroglycans are N-glycosylated in the ER. Sarcospan, the sarcoglycan complex, and the dystroglycan exit the ER unassembled and arrive at the golgi. **B**, At the golgi α DG is O-glycosylated. While still in the golgi the dystroglycans, sarcoglycan complex, and sarcospan fully assemble and are transported to the sarcolemma. **C**, Dystrophin, the syntrophins and linkage to the basal lamina through laminin likely occurs after proper localization and insertion into the sarcolemma.

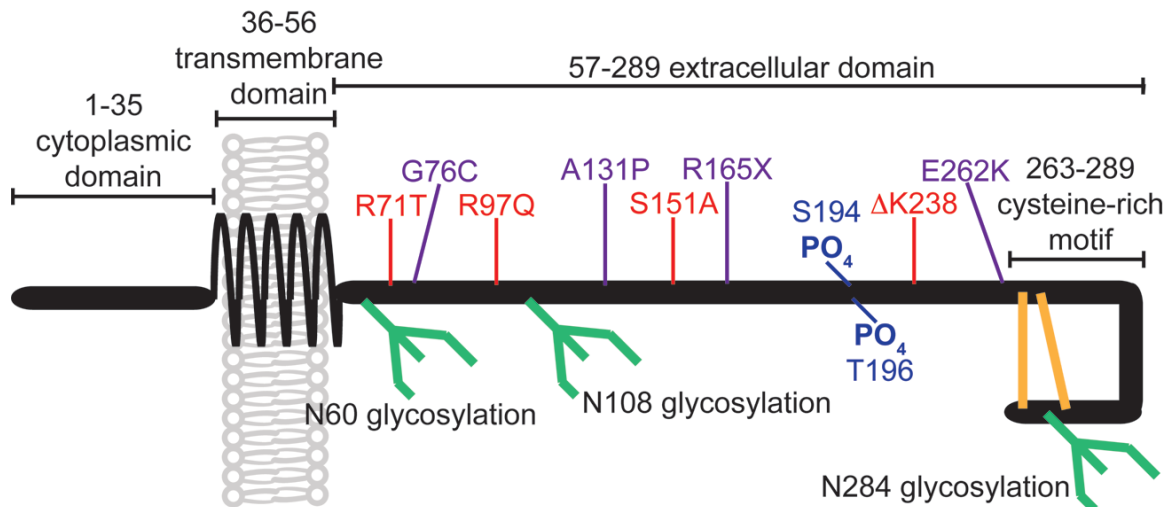


Figure 1-5 Domain structure of delta sarcoglycan including post-translational modifications and disease causing mutations. N-glycosylation (green) occurs at three putative sites that have been determined through sequence analysis. Mutations causing limb-girdle muscular dystrophy 2F (purple) and linked to DCM (red) occur throughout the extracellular domain. The cysteine-rich motif exists at the C-terminus and forms a possible short hairpin via disulfide bridges (orange). Phosphorylation (blue) occurs at two sites but has so far only been identified in human platelets.

Sarcoglycan Family Member	Mutations and Associated Disease(s)	Cardiovascular Involvement	Skeletal Muscle Involvement
α-sarcoglycan	Limb-Girdle Muscular Dystrophy 2D	Uncommon	Progressive muscle wasting and weakening
β-sarcoglycan	Limb-Girdle Muscular Dystrophy 2E	Frequent development of cardiomyopathy	Progressive muscle wasting and weakening from mild to severe DMD-like
γ-sarcoglycan	Limb-Girdle Muscular Dystrophy 2C	Frequent development of cardiomyopathy	Progressive muscle wasting and weakening
δ-sarcoglycan	Limb-Girdle Muscular Dystrophy 2F, Dilated Cardiomyopathy	Frequent development of cardiomyopathy in individuals with LGMD2F, DCM caused by dominantly inherited mutations	Progressive muscle wasting and weakening form mild to severe DMD-like
ε-sarcoglycan	Myoclonus Dystonia	n/a	n/a
ζ-sarcoglycan	n/a	n/a	n/a

Table 1-1 Mutations in the sarcoglycans and corresponding striated muscle phenotypes.

Sarcoglycan Protein	Gene	UniprotKB/Swiss-Prot #	Homology w/ Alpha	Homology w/ Beta	Homology w/ Gamma	Homology w/ Delta	Homology w/ Epsilon	Homology w/ Zeta
Alpha	SGCA	Q16586.1	-	12.8	8.5	8.5	54.5	8.0
Beta	SGCB	Q16585.1	12.8	-	37.5	36.0	8.1	37.3
Gamma	SGCG	Q13326.4	8.5	37.5	-	70.8	8.4	74.6
Delta	SGCD	Q92629.2	8.5	36.0	70.8	-	1.4	75.9
Epsilon	SGCE	O43556.6	54.5	8.1	8.4	1.4	-	2.3
Zeta	SGCZ	Q96LD1.1	8.0	37.3	74.6	75.9	2.3	-

Table 1-2 Sequence alignment of sarcoglycan family members. Percent

Homology was calculated using EMBOSS Pairwise Sequence Alignment Tool for proteins (www.ebi.ac.uk/Tools/psa/emboss_needle/) Alignment reveals three groups of homologous sarcoglycans 1) δ , γ , and ζ -sarcoglycan; 2) α and ε -sarcoglycan 3) β -sarcoglycan.

Chapter 2

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

ABSTRACT

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 delta sarcoglycan cause limb-girdle muscular dystrophy 2F, dominant mutations in delta 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 delta sarcoglycans harboring the dominant inherited DCM mutations R71T or R97Q. This study demonstrates that DCM mutant delta sarcoglycan can be stably expressed in adult rat cardiac myocytes, traffic similarly to WT proteins to the plasma membrane, without perturbing assembly of the dystrophin-glycoprotein complex. However, expression of DCM mutant delta 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 delta sarcoglycan R97Q or R71T have increased impermeable dye uptake and undergo contractures at greater frequencies than myocytes expressing normal delta sarcoglycan. Additionally, the R71T mutation creates an ectopic N-linked glycosylation site

that results in aberrant glycosylation of the extracellular domain of delta sarcoglycan. Therefore, appropriate glycosylation of delta sarcoglycan may also be necessary for proper delta sarcoglycan function and overall dystrophin-glycoprotein complex function. These studies demonstrate that DCM mutations in delta sarcoglycan can exert a dominant negative effect on dystrophin-glycoprotein complex function leading to myocardial mechanical instability that may underlie the pathogenesis of delta sarcoglycan associated DCM.

Introduction

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^{103, 160}. The DGC contributes to an important functional linkage between the extracellular matrix (ECM) and cytoskeletal proteins in muscle cells. The DGC attaches and interacts with the cytoskeleton through the binding of dystrophin to f-actin¹⁶¹. The central transmembrane protein dystroglycan completes the link of dystrophin and the DGC to the extracellular matrix, and requires the functional glycosylation of alpha-dystroglycan to serve as a high affinity matrix receptor^{16, 161, 162}. The critical functional roles of the DGC in muscle are still debated, but may include regulation of cell signaling^{44, 51}, muscle force transmission¹⁶³⁻¹⁶⁶, and promoting plasma membrane stability during muscle contraction^{68, 111, 143, 144, 167, 168}. Contained within the DGC is a subcomplex of proteins known as the sarcoglycan complex that includes sarcoglycans and sarcospan¹⁶⁹⁻¹⁷². Delta sarcoglycan

(δ SG) is one of six known transmembrane sarcoglycans (α , β , γ , δ , ϵ , ζ) that are expressed in a tissue specific manner to form the tetrameric sarcoglycan complex^{38, 40, 43, 98, 99, 173, 174}. Delta, beta and gamma sarcoglycan are highly expressed in skeletal and cardiac muscle¹¹⁶ and while alpha-sarcoglycan is the prominent fourth member of the sarcoglycan complex in skeletal muscle, cardiac muscle expresses both alpha and epsilon-sarcoglycan. Alpha and epsilon sarcoglycan proteins are capable of compensating for the loss of each other within the cardiac muscle sarcoglycan complex¹⁰¹.

A critical role of δ SG 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, 2F respectively)¹⁷⁵. Genetic deletion of any of these four sarcoglycans in mice results in a dystrophic muscle phenotype¹⁷⁶. In addition, recessive δ SG mutations are responsible for the cardiac and skeletal muscle phenotypes of the cardiomyopathic BIO 14.6 hamster¹⁷⁷. In addition to progressive skeletal myopathy, patients with sarcoglycan associated LGMD have a high prevalence of cardiomyopathy^{116, 156}. Recessive mutations in δ SG 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 δ SG that result in skeletal muscle dystrophy, dominantly inherited mutations in δ SG have been linked to familial dilated cardiomyopathy¹⁵⁷ (DCM). The dominantly inherited mutations in δ 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 δ SG causing truncation at lysine residue 238 (Δ K238), in some pathological samples, resulted in decrease in sarcoglycan expression at the plasma membrane¹⁵⁷. However, one patient identified with this mutation showed normal expression patterns of the DGC proteins but still developed DCM¹⁵⁷. Transgenic mice expressing the S151A mutation in δ SG that is linked to DCM in the heart¹⁵⁹ show early lethality, loss of the sarcoglycan complex at the plasma membrane in cardiac myocytes, and a sequestration of nuclear proteins in cardiac muscle, 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 (which encodes δ 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 LMGD2F, and there was no family history of heart disease¹⁵³. Despite the controversy raised regarding the link between the S151A mutation and DCM, independent groups have found additional mutations in δ SG in patients with autosomal dominant DCM¹⁵⁸ and more recently polymorphisms in delta sarcoglycan are a risk factor for HCM^{178, 179}. Therefore, significant questions remain about if and how mutations in δ SG cause DCM, and if mutations in δ 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 δ SG exert a dominant effect on DGC function, we expressed wild type δ SG and δ SG containing mutations R97Q or R71T previously linked to DCM^{153, 157, 158} in adult cardiac myocytes to study their direct acute effects on the DGC and function of cardiac muscle cells. Adult cardiac myocytes expressing mutant δ SG R97Q or R71T showed no alterations in the trafficking or localization of the mutant δ SG to the plasma membrane. Additionally, expression of either mutant δ SG R97Q or R71T does not appear to alter expression or assembly of the DGC in rat cardiac myocytes. However using a functional assay previously used to study cardiac myocytes from mice with recessive muscular dystrophy¹⁸⁰, the sarcolemma in cardiac myocytes expressing either mutant δ 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 δ SG R71T show a unique alteration of N-linked glycosylation of δ SG, due to creation of an ectopic consensus N-glycosylation site by the R71T mutation. Together these support the conclusion that DCM-linked mutations δ SG can have a dominant negative effect on cardiac myocyte function similar to dystrophic cardiomyopathy, and the R71T mutation suggests a possible link between post-translational modifications of mutant δ SG and the function of δ SG within cardiac muscle cells.

Methods

Cardiac Myocyte Isolation:

200-gram female Sprague Dawley rats were anaesthetized with 20 mg ketamine and 4 mg xylazine with 400 units heparin to prevent clotting 20 minutes prior to heart removal. The heart was removed and placed on a modified Langendorff perfusion apparatus. Perfusion was performed for 5 minutes at a flow rate of 8 ml/min using Krebs Henselait Buffer (KHB) (118 mmol/L NaCl, 25 mmol/L Hepes, 1.2 mmol/L KH_2PO_4 , 1.2 mmol/L MgSO_4 , 1.0 mmol/L CaCl_2 , 11 mmol/L Glucose). Perfusion proceeded for 5 minutes using KHB without Ca^{2+} at a flow rate of 8 ml/min. 14895 total units of Collagenase type 2 (Worthington Biochem LS004174) was added to the perfusate in 60 ml of perfusate solution. The heart was perfused for 20 minutes using recirculated collagenase-KHB (Ca^{2+} free) solution. After 20 minutes of recirculated perfusion the solution the Ca^{2+} concentration was brought up to 1.5 mmol/L using 4 additions at 225 μl of 100 mmol/L sterile CaCl_2 at 30 second intervals. Recirculating perfusion continued for an additional 20 minutes. The heart was then removed from the perfusion apparatus into a sterile beaker containing 15 ml of Ca^{2+} -collagenase-KHB solution. Aorta, and atrium were removed using scissors and ventricular tissue was minced into 8 small pieces. Ventricular tissue in sterile beaker was manually swirled gently for 5 minutes in a water bath at 37°C. Solution was removed, and discarded. 15 ml of new Ca^{2+} -collagenase-KHB solution was added to ventricular tissue. Gentle swirling was repeated another 5 minutes and solution was again discarded. 15 ml of new Ca^{2+} -collagenase-KHB solution was again added to

ventricular tissue. Gentle swirling, solution removal and new solution addition was performed once more. Remaining ventricular tissue was passed through wide bore sigma-coted (Sigma Aldrich SL2-25) glass pipettes to further liberate ventricular myocytes from tissue. Gentle swirling was repeated for a final 5 minutes-total number of swirling digestions performed was 4. Upon final digestion/swirling the solution was passed through a tissue sieve with #10 mesh screen (Bellco Glass, Inc.). Collected cells and solution were centrifuged at 18g for 3 minutes. Solution was aspirated and isolated cell pellet was resuspended in 10 ml KHB with 1.0 mmol/L Ca^{2+} and 2% bovine serum albumin. Ca^{2+} was increased to 1.75 mmol/L by adding 25 μ l or 100mmol/L CaCl_2 3 times at 5 minute intervals. After final addition of Ca^{2+} and 5 minute incubation the cell suspension was centrifuged at 18g for 3 minutes. Supernatant was aspirated and the cell pellet was suspended in 3 ml MEM (Gibco 11575). Cells were counted using a hemocytometer and suspended at a final concentration of 100,000 cells/ml in MEM with 5% FBS. A total of 6 rats were used for membrane fluorescence experiments. A total of 6 rats were used for stretch assay experiments. A total of 6 rats were used for all western blots. Overall a total of 18 rats were used for these experiments.

Cardiac Myocyte Plating:

Glass cover slips and Bioflex plates (Flex Cell International BF-3001U) were coated with 200 μ l EHS laminin (Sigma Aldrich L2020) at a concentration of 50 μ g/ml or were plated on 100mm culture dishes (Corning 430167) coated with 1 ml EHS laminin at a concentration of 50 μ g/ml. All plates and dishes coated with

laminin were exposed to ultraviolet light for sterilization for 5 minutes and allowed to polymerize for 30 minutes prior to myocyte plating. 20,000 total cells in 200 μ l MEM with 5% FBS were suspended on each cover slip or flex well and allowed to adhere for 2 hours. 1,000,000 total cells in 1 ml MEM with 5% FBS was suspended on each 100 mm plate and allowed to adhere for 2 hours. Media was aspirated from cells adhered to laminin and isolated cardiac myocytes were recovered with 3 ml MEM without FBS on Bioflex plates and glass cover slips and 10 ml MEM without FBS on 100 mm plates.

Cardiac Myocyte Transduction:

Cardiac myocytes were transduced in MEM (Gibco 11575) for 1 hour. MEM was aspirated and replaced with fresh MEM and cells were cultured for 48 hours to express delta sarcoglycan.

Fixed Cardiac Myocyte Immunostaining:

Rat cardiac myocytes were plated on glass cover slips and coated with 200 μ l EHS laminin at a concentration of 50 μ g/ml and transduced with adenoviral vectors. Cardiac myocytes were allowed to express transgenes for 48 hours. Media was aspirated and cardiac myocytes were washed with DPBS (GIBCO 14190). DPBS was aspirated and cardiac myocytes were fixed with 2 ml of 1 mol/L paraformaldehyde (3%) in DPBS for 10 minutes. Paraformaldehyde was aspirated and cardiac myocytes were washed with 5 ml DPBS for 5 minutes 3 times. The final wash of DPBS was aspirated and the cardiac myocytes were blocked for 2 hours with 2 ml block solution (DPBS with 5% BSA and 0.5% Triton-X 100). Block solution was aspirated and cardiac myocytes were

incubated with primary antibodies using 300 μ l/slip of antibodies suspended in block solution for 90 minutes in a humidity chamber to prevent block solution evaporation. Cardiac myocytes were washed with 5 ml DPBS for 5 minutes 3 times. The final wash was aspirated and the cardiac myocytes were incubated with secondary antibodies suspended in block solution using 300 μ l/slip for 1 hour in a humidity chamber to prevent block solution evaporation. Cardiac myocytes were washed with 5 ml DPBS for 5 minutes 3 times. The final wash was aspirated and cover slips were mounted on Superfrost Plus microscope slides (Fisher Scientific 12-550-15) in aqueous permafluor (Lab Vision TA-030-FM). For myc-tagged immunofluorescence primary antibody used was A-14 a rabbit polyclonal anti-myc (Santa Cruz Bioscience) at a titer of 1:100, secondary antibodies used were Cy3 conjugated anti-rabbit IgG (Jackson Immunoresearch) at a titer of 1:200, and DAPI (Sigma-Aldrich) diluted 1:10,000 from a 10 mg/mL stock solution. For delta sarcoglycan immunofluorescence primary antibody used was a goat anti-human delta sarcoglycan (R&D Systems) at a titer of 1:1000, secondary antibodies used were Cy3 conjugated anti-goat IgG (Jackson Immunoresearch) at a titer of 1:200, and DAPI (Sigma-Aldrich) diluted 1:10,000 from a 10 mg/mL stock solution. For glycosylated alpha dystroglycan immunofluorescence primary antibody used was mouse IIH6 anti-alpha dystroglycan (Upstate) at a titer of 1:1000, secondary antibodies used were Cy3 conjugated anti-mouse IgM (Jackson Immunoresearch) at a titer of 1:200, and DAPI (Sigma-Aldrich) diluted 1:10,000 from a 10 mg/mL stock solution. For beta dystroglycan immunofluorescence primary antibody used was mouse anti-beta

dystroglycan (Vector Labs) at a titer of 1:1000, secondary antibodies used were Cy3 conjugated anti-mouse IgG (Jackson ImmunoResearch) at a titer of 1:200, and DAPI (Sigma-Aldrich) diluted 1:10,000 from a 10 mg/mL stock solution.

Live Cell Labeling:

Rat cardiac myocytes were plated on glass cover slips and coated with 200 μ l EHS laminin at a concentration of 50 μ g/ml and transduced with adenoviral vectors. Cardiac myocytes were allowed to express transgenes for 48 hours. Media was aspirated and cardiac myocytes were incubated with primary antibody suspended in MEM (Gibco 11575) for 2 hours at 37°C. Cardiac myocytes were washed with 5 ml MEM for 5 minutes 3 times. After final aspiration of media cardiac myocytes were fixed with 2 ml of 1 mol/L paraformaldehyde (3%) in DPBS for 10 minutes. Paraformaldehyde was aspirated and cardiac myocytes were washed with 5 ml DPBS for 5 minutes 3 times. The final wash of DPBS was aspirated and the cardiac myocytes were blocked for 2 hours with 2 ml block solution (DPBS with 5% BSA and 0.5% Triton-X 100). Block solution was aspirated and the cardiac myocytes were incubated with secondary antibodies suspended in block solution using 300 μ l/slip for 1 hour in a humidity chamber to prevent block solution evaporation. Cardiac myocytes were washed with 5 ml DPBS for 5 minutes 3 times. The final wash was aspirated and cover slips were mounted on Superfrost Plus microscope slides (Fisher Scientific 12-550-15) in aqueous permafluor (Lab Vision TA-030-FM). For myc-tagged immunofluorescence primary antibody used was A-14 a rabbit polyclonal anti-myc (Santa Cruz Bioscience) at a titer of 1:100, secondary antibodies used were

Cy3 conjugated anti-rabbit IgG (Jackson ImmunoResearch) at a titer of 1:200, and DAPI (Sigma-Aldrich) diluted 1:10,000 from a 10 mg/mL stock solution.

Immunofluorescence Microscopy:

All microscope images were taken using an Olympus BX51 Microscope & DP70 Digital Camera System. Baseline for exposure of fluorescence images was established using isolated cardiac myocytes stained without primary antibodies.

Membrane Fluorescence Scoring:

Cardiac myocytes were assessed via random field counts. Five separate experiments were performed using five random field counts each at 200X magnification. Membrane fluorescence was determined as those rod-shaped cells that were also fluorescent above untransduced control cells.

Cell Stretching Assay:

Rat cardiac myocytes were plated on Bioflex plates coated with 200 μ l EHS laminin at a concentration of 50 μ g/ml and transduced with adenoviral vectors. Cardiac myocytes were allowed to express transgenes for 48 hours. Cardiac myocytes were incubated with 50 μ g/ml propidium iodide in culture media immediately prior to stretch protocol. Cardiac myocytes were stretched with an FX4000 Tension System (FlexCell International). Stretch was done using a pressure protocol that imparts a 20% length change across the plate at a frequency of 1 Hz for 24 hours. After completion of stretch protocol cardiac myocytes were undisturbed for 30 minutes. Media and dye solution was aspirated and cardiac myocytes were washed twice 5 ml DPBS (GIBCO 14190). DPBS was aspirated and cardiac myocytes were fixed with 2 ml of 1 mol/L

paraformaldehyde (3%) in DPBS for 10 minutes. Paraformaldehyde was aspirated and cardiac myocytes were washed twice with 5 ml DPBS for 5 minutes. Membranes were cut and mounted on Superfrost Plus microscope slides (Fisher Scientific 12-550-15) in aqueous permafluor (Lab Vision TA-030-FM) for fluorescent microscopy.

Stretch Assay Scoring:

Cardiac myocytes were assessed via random field counts. Five separate experiments were performed using five random field counts each at 200X magnification. Images were taken and a double blind performed. Positive cells are those expressing fluorescence in the nucleus.

Protein Collection:

Rat cardiac myocytes were plated on 100mm culture dishes (Corning 430167) coated with 1 ml EHS laminin at a concentration of 50 $\mu\text{g/ml}$ and transduced with adenoviral vectors. Cardiac myocytes were allowed to express transgenes for 48 hours and were collected with a cell lifter using 500 μl 1% digitonin in DPBS with protease inhibitors (benzamidine 0.6 mmol/L, phenylmethylsulfonyl fluoride 0.4 mmol/L, pepstatin 0.73 $\mu\text{mol/L}$, Aprotinin 2 KIU/ml, Leupeptin 2.34 $\mu\text{mol/L}$).

Cardiac myocytes were vortexed and sonicated for 10 seconds each. Cell debris was spun down at 14,000g for 2 minutes and supernatant/cell lysate was quantified for western blot and glycosidase treatment.

Glycosidase treatment:

30 mg of protein lysate was denatured for either EndoH (New England Biolabs) or PNGaseF (New England Biolabs) treatment in the supplied denaturing buffer

and incubated for 10 minutes at 100°C in a total reaction volume of 80 µl. Protein lysate was then incubated with 2 µl (1000 units) of EndoH with 10 µl of supplied buffer G5 in a total volume of 100 µl or protein lysate was incubated with 2 µl (1000 units) of PNGaseF with 10 µl of supplied buffer G7 and 10 ml of supplied supplement NP-40. Both incubations were performed for 60 minutes at 37°C.

Western Blot:

Protein lysates and glycosidase treated proteins were separated using 3-15% gradient SDS-PAGE gel and transferred to a PVDF membrane at 100 volts for 3 hours. Membranes were blocked using 5% non-fat dairy milk in TBS-T (NaCl 150 mmol/L, Tris pH7.5 50 mmol/L) for 45 minutes. Membranes were incubated with primary antibody in 5% non-fat dairy milk in TBS-T overnight. Membranes were washed for 10 minutes in TBS-T three times and incubated with secondary antibody in 5% non-fat dairy milk in TBS-T for 90 minutes. Membranes were washed for 10 minutes in TBS-T three times and incubated in SuperSignal West Dura Substrate (Thermo Scientific) for 60 seconds. Visualization of western blots was performed using a Fluorochem System (Alpha Innotech). For western blot of myc-tagged proteins primary antibody used was A-14 rabbit polyclonal anti-myc (Santa Cruz Bioscience) at titer of 1:500 and secondary antibody used was HRP conjugated anti-rabbit IgG (Jackson ImmunoResearch) at a titer of 1:1000. For western blot of beta dystroglycan primary antibody used was A-14 rabbit polyclonal anti-myc (Santa Cruz Bioscience) at titer of 1:500 and secondary antibody used was HRP conjugated anti-rabbit IgG (Jackson ImmunoResearch) at a titer of 1:1000. For western blot of beta dystroglycan primary antibody used

was mouse anti-beta dystroglycan (Vector Labs) at a titer of 1:500, secondary antibodies used was HRP-conjugated anti-mouse IgG (Jackson Immunoresearch) at a titer of 1:1000.

Statistics:

For all statistics one-way ANOVA was performed with a Dunnet's post-test, p-value <0.05.

Results

DCM mutant delta sarcoglycans can be expressed in adult cardiac myocytes at similar expression levels to WT delta sarcoglycan.

Adenoviral vectors containing delta sarcoglycan expression constructs were generated using previously described techniques¹⁸¹. The constructed adenoviral vectors use the cytomegalovirus (CMV) promoter to drive expression of wild type (WT) and mutant human δ SG cDNA. A myc tag was engineered on the extracellular C-terminus in order to identify the exogenous protein and study its cellular trafficking and sarcolemma localization (Figure 2-1A). Adult rat cardiac myocytes were isolated and transduced with adenoviral vectors expressing WT or mutant δ SG. Cardiac myocytes were collected at 24, 48, and 72 hours post-transduction. The adenoviral expressed δ SG proteins became first detectable at approximately 18-24 hours post-transduction and continued to increase until expression levels stabilized between 48 and 72 hours post-transduction (Figure 2-1B). Protein expression after 72 hours 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 δ SG protein expression by western blot were obtained in WT, and R97Q and R71T mutant δ SG expressing cardiac myocytes (Figure 2-1B). For wild type δ SG, and δ SG 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 (Figure 2-1C, 2-1D). These data indicate that R97Q and R71T mutant δ SG can be expressed at levels equivalent to WT δ SG and the mutants appear to have normal protein stability when expressed in adult cardiac myocytes.

Adult rat cardiac myocytes expressing R97Q or R71T mutant delta sarcoglycan do not show DGC disruption or loss of cell viability.

Genetic disruption of δ SG or other sarcoglycan members in mice results in loss of expression of the entire sarcoglycan complex at the sarcolemma in muscle cells, and while dystrophin and dystroglycan are still expressed, their expression levels are significantly reduced^{91, 101}, indicating null mutations in δ SG can disrupt DGC assembly in cardiac muscle. In contrast, over-expression of wild type δ SG, δ SG mutant R97Q or R71T using adenoviral vectors has no apparent effect on DGC assembly and expression (Figure 2-2 & 2-3). The expression of beta dystroglycan, an integral membrane bound component of the DGC, is unchanged when mutant δ SG R97Q or R71T is expressed in cardiac myocytes (Figure 2-2A). Expression of glycosylated α DG, an essential DGC component for functional linkage to the extracellular matrix, appears unchanged in cardiac

myocytes expressing δ SG mutants R97Q or R71T (Figure 2-3).

Immunofluorescence staining shows that the expression of α and β sarcoglycan and its localization are also unchanged (Figure 2-3) in cardiac myocytes expressing δ SG mutants R97Q or R71T. Furthermore, the expression of δ SG mutant R97Q, R71T, or wild type δ SG does not appear to directly affect overall adult myocyte viability or cell morphology (Figure 2-2B) at least in quiescent myocytes after 24-48 hour of expression.

Delta sarcoglycan mutants R97Q and R71T properly localize to the plasma membrane

Expression of the δ SG S151A mutation linked to DCM in hearts of transgenic mice reveals that δ SG mutant S151A protein accumulates in the nucleus and does not localize correctly to the plasma membrane¹⁵⁹. Whether this defect was due to the level of overexpression or whether this represents a common mechanism or a property of other δ SG 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 WT 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 δ SG proteins shows no apparent difference in expression or localization of δ SG mutant R97Q or R71T when compared to wild type δ SG expressing cardiac myocytes (Figure 2-2C) 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 δ SG when compared to wild type δ SG, the data does not directly demonstrate that the WT or mutant proteins are properly integrated into the plasma membrane. Delta sarcoglycan is a type-II transmembrane protein that, when inserted into to the membrane, presents the C-terminus to the extracellular milieu. We took advantage of the exposure of the C-terminus myc epitope tag, to develop a live cell staining protocol in unpermeabilized cardiac myocytes to quantify the insertion of the expressed δ SG into the plasma membrane. Using live cell myc antibody staining, both δ SG mutant R97Q and R71T proteins can be labeled at the membrane in unpermeabilized cells (Figure 2-4A) suggesting they are properly inserted into the plasma membrane. To quantify this membrane trafficking, random field counts of cardiac myocytes were scored for detectable membrane fluorescence in live cell labeling assays, at time points when the protein can be first detected at measureable levels. In order to accurately score myocytes, background threshold was established using untransduced myocytes for exposure time and sensitivity settings. All subsequent images were taken using the same exposure time and settings, therefore any myocyte showing fluorescence above untransduced control myocytes was scored as positively labeled at the plasma membrane. Based on this scoring, in adult cardiac myocytes both δ SG mutant R97Q and R71T proteins localize to the plasma membrane with equal efficiency to wild type δ SG (Figure 2-4B). One caveat to this assay is that labeling live cells

with antibodies may cause internalization of the antibody due to endocytosis. To control for this phenomenon cell labeling experiments can be performed at 4° C to decrease endocytosis. Unfortunately the length of time needed to accurately and robustly label live cardiac myocytes is significantly longer than the time that isolated cardiac myocytes can survive at 4° C. To reduce the possible endocytosis of the primary antibody during subsequent labeling steps we immediately fixed the cells after primary antibody incubation. The subsequent secondary antibody labeling uses both fixation and permeabilization and should include any myc antibody that may have been internalized during initial incubation. Importantly, live cell labeling of cardiac myocytes expressing mutant R97Q or R71T is quantitatively and qualitatively similar to wild type expressing cardiac myocytes suggesting the trafficking of delta sarcoglycan to the membrane was unaffected by the DCM mutations R97Q and R71T. Finally, to determine whether the δ SG mutant R97Q and R71T proteins were stable at the plasma membrane, cardiac myocytes were treated with cycloheximide 24 hours after viral transduction and stained for the myc epitope with live cell labeling (Figure 2-5). In the presence of cycloheximide, the sarcolemma incorporated R97Q and R71T δ SG were retained at the plasma membrane at levels equivalent to WT δ SG suggesting they are stably incorporated into the sarcolemma membrane.

Delta sarcoglycan mutant R71T is aberrantly glycosylated

The presence of N-linked glycosylation of δ SG has been demonstrated in skeletal muscle¹¹⁶ and in cultured CHO¹²⁷ and COS-1 cells¹¹⁰. It is unclear what role δ SG glycosylation plays in δ SG protein and overall DGC function in muscle cells or whether δ SG 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 δ SG is glycosylated in isolated rat ventricular myocytes. Delta sarcoglycan mutant R97Q protein appears to have the same glycosylation and expression pattern as wild type δ SG but oddly, δ SG mutant R71T protein shows a higher molecular weight band suggesting altered post-translational modification (Figure 2-6A). Incubating cell lysates with endoglycosidase H (EndoH) or peptide N-glycosidase F (PNGaseF) for one hour was sufficient to restore the migration of δ SG mutant R71T to the same migration pattern of wild type δ SG on Western blots (Figure 2-6B), indicating that the high molecular weight band in cardiac myocytes expressing δ SG mutant R71T represents an alternately N-linked glycosylated form of δ SG. The alternate glycosylation of R71T δ SG does not appear to be cardiac muscle cell specific as the upward shift in MW can be observed when R71T is expressed in other rat muscle cell types including rat smooth muscle cells (A7R5) and rat skeletal muscle myotubes (L6) (Figure 2-7). Interestingly, the occupancy of the 3 putative N-glycosylation sites on WT δ SG appears to be different in smooth muscle cells (Figure 2-7) 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 δ SG. Interestingly, the detection of the expressed protein with anti-human δ SG 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 C-terminus of δ SG in cardiac myocytes. This form of post translation processing does not appear to be different between WT and mutant R97Q or R71T δ SG.

Expression of delta 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 δ SG 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^{180, 182, 183}. This loss of sarcolemma instability in dystrophic cardiac muscle can be demonstrated in isolated cardiac myocytes by subjecting them to mechanical stretching in the presence of cell impermeable dyes^{180, 183, 184}. 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 δ SG mutant R97Q or R71T exerts a dominant negative functional effect on the DGC to cause plasma membrane instability

following cell stretching. Cardiac myocytes expressing mutant δ SG R97Q or R71T exposed to mechanical strain showed a significant increase in impermeable dye uptake and increase in cell contracture leading to rounding of cells (Figure 2-8B). To quantify this, WT and δ SG mutant R97Q and R71T expressing cardiac myocytes were scored in a blinded fashion for cell morphology and dye uptake following mechanical stretch. Delta sarcoglycan mutant R97Q and R71T expressing cardiac myocytes showed a significant decrease in rod-shaped cells, and concomitant increase in round-shaped cells, when compared to wild type δ SG expressing myocytes (Figure 2-9). Nearly all of the round-shaped cells following stretch were propidium iodide positive indicating that sarcolemma rupture likely leads to calcium induced cell contracture. Although there was slight reduction in the total number of cells/cm² after stretch in all groups, the decline in cell density was not statistically different than WT cells. This suggests that R97Q or R71T mutant expression does not significantly alter how tightly the cells are adhered to the laminin substrate, but that expression is sufficient to cause a dominant negative effect of sarcolemma stability in adult cardiac muscle cells comparable to what has been previously observed in dystrophic cardiac cells.

Discussion

Recessive mutations in δ SG that cause limb-girdle muscular dystrophy result in loss of δ SG at the plasma membrane and disrupt assembly and function of the rest of the DGC^{91, 127}. This study investigated the effects of two dominantly

inherited mutations in δ SG, 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 δ SG 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.

Previous work using transgenic mice, demonstrated that cardiac specific over-expression of S151A mutation previously linked to dominantly inherited DCM in resulted in a lethal cardiomyopathy^{157, 159}. 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 of breeding. 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 cardiomyopathy¹⁵³. We chose to focus our attention on other δ SG mutations associated R97Q and R71T using a highly efficient model system of viral mediated gene transfer to adult cardiac

myocytes, that we have previously used to study dominant mutations that lead to hypertrophic cardiomyopathy and other forms of myopathy^{181, 185, 186}. Our data shows human δ SG mutants R97Q or R71T are capable of being expressed at levels comparable to WT δ SG, traffic completely normally in adult cardiac myocytes, and live cell labeling showed they stably insert into the sarcolemma similar to WT proteins without dramatically compromising DGC expression or assembly. While we did observe some intracellular localization of the expressed δ SG, this was not different between WT and mutant proteins indicating that we are likely also detecting a portion of the expressed protein in the secretory pathway of permeabilized adult cardiac myocytes following acute expression. Furthermore, using live-cell labeling, and cycloheximide treatment reveals that mutant δ SG R97Q and R71T are stable at the plasma membrane. The results indicate that the δ SG mutant R97Q and R71T defects do not arise from a deficiency in δ SG localization to the plasma membrane and suggests a potentially dominant negative function of the protein at the sarcolemma underlying its effects to cause DCM.

Delta sarcoglycan protein has been shown to be glycosylated in muscle in vivo and when expressed in vitro^{116, 127}. Although the function of δ SG glycosylation in muscle is unclear it has been established that hypoglycosylation of dystroglycan within DGC is critical to its function as a laminin receptor and results in muscular dystrophy with associated cardiomyopathy^{15, 16, 82}. Surprisingly, when we expressed the δ SG mutant R71T we found an additional higher molecular weight band upon western blotting with both anti human- δ SG

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. Glycosidase treatment to remove N-glycans revealed for the first time that this ectopic glycosylation site in the R71T mutant is highly glycosylated in cardiac muscle cells. The removal of N-glycans by both EndoH and PNGaseF converted all the glycoforms of the WT and δ SG mutant R97Q and R71T to a similar migrating low molecular weight protein. Oddly it was expected that treatment with EndoH would also allow us to reveal any trafficking defects since sensitivity to that particular glycosidase typically indicates a protein is retained in the endoplasmic reticulum. The fact that the WT and mutant proteins appear to be EndoH sensitive is noteworthy, but not completely without explanation. The sarcoglycan complex has previously been found localized to the plasma membrane and is still sensitive to treatment with EndoH⁹¹. Additionally, an epsilon sarcoglycan mutation causing myoclonus dystonia results in an ectopic N-glycosylation site that becomes glycosylated¹²⁶. Epsilon sarcoglycan can localize to the plasma membrane in mouse brain and still retains its sensitivity to EndoH treatment¹⁸⁷. This abnormal post translational processing of 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. However, the occupancy of the glycosylation sites in smooth muscle cells appears to be slightly different than in cardiac muscle cells suggesting a potential role of glycosylation in modifying sarcoglycan function in a tissue specific manner. 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 δ SG may contribute to the functional effects of the R71T mutation in cardiac muscle cells. The ectopic glycosylation and the location of the R97Q mutation suggest that the C-terminal extracellular domains of delta 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 C-terminus in gamma-sarcoglycan can cause limb girdle muscular dystrophy and cardiomyopathy without causing loss of sarcoglycan complex expression¹⁸⁸

Previous studies have shown that loss of α DG glycosylation or dystrophin deficiency causes plasma membrane instability in cardiac myocytes upon mechanical strain^{180, 184}. To test the hypothesis that these dominantly inherited δ SG mutants R97Q and R71T cause a dominant negative effect on overall DGC function, we investigated the effects of mechanical strain upon cardiac myocytes expressing δ SG mutants R97Q and R71T. In these experiments the rat cardiac myocytes are adhered to the deformable substrate using a laminin matrix. Interestingly, untransduced rat cardiac myocytes are overall much less sensitive to mechanical stretching than our previous studies in mouse cells and could withstand much longer mechanical stretching experiments¹⁸⁰. However, when cardiac myocytes expressing δ SG mutant R97Q or R71T are submitted to mechanical strain for 24 hours, there is marked increase membrane permeability (increased dye uptake) and that likely leads to calcium induced cell contracture

(loss of rod-shaped morphology). This does not appear to be a direct effect on cell adhesion in the absence of stretch, given that most of the cells expressing R97Q or R71T remain attached to the substrate equivalently to WT cells. These rounded up cells do not appear to be cells that have died during the experiment, as while most of the rod and round shaped myocytes are quiescent, many of the round shape cells show spontaneous beating behavior (not shown).

Furthermore, some cells die during the cardiac myocyte isolation and exhibit a rounded morphology prior to any experiments, but these cells typically do not adhere well and are easily washed off the laminin substrate. Overall, these results indicate DCM linked δ SG mutations exert a dominant negative functional effect in cardiac myocytes, 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.

The comparable cardiac myocyte phenotypes between mice with hypoglycosylation of α DG and rat cardiac myocytes expressing dominant negative δ SG mutations raises some important new questions about how sarcoglycans may function within the DGC. While, previous studies have suggested sarcoglycans may contribute to the interactions of α DG with the DGC, we did not observe any changes in dystroglycan expression, cleavage of β DG or the amount of glycosylated α DG detected on the cells surface with the IIH6 antibody. Of course it is possible that more subtle protein-protein interactions amongst components of the DGC are altered when mutant sarcoglycans are expressed. However, overall our studies suggest a possible new hypothesis that

the extracellular domains of sarcoglycans contribute directly to the interaction of the cell with matrix either within the DGC or perhaps even in a DGC independent fashion. Recent findings that loss of sarcospan, a component of the sarcoglycan-sarcospan complex appears to have DGC dependent and DGC independent effects on sarcolemma stability in skeletal muscle¹⁸⁹, supports the idea that sarcoglycans may function more than just as an accessory protein in the DGC, but actually play an important prominent direct role in maintaining sarcolemma stability in striated muscle.

Acknowledgements

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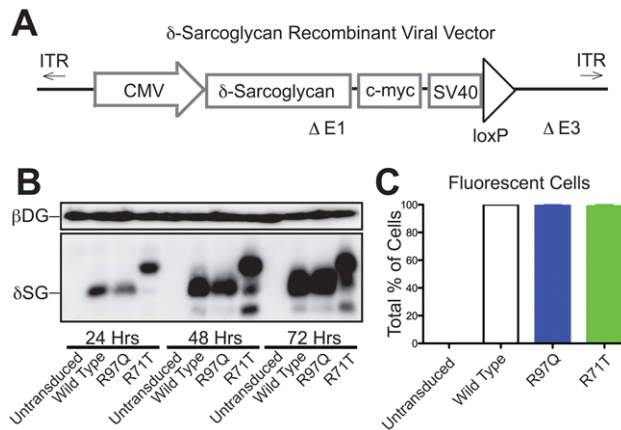


Figure 2-1. Adenoviral vectors driving expression of delta sarcoglycan in cardiac myocytes are highly efficient and comparable across all groups. A, δ SG adenoviral vector design. **B,** Time course of δ SG expression using adenoviral vectors in isolated cardiac myocytes. DCM mutant and wild type δ SG protein expression is detectable as early as 24 hours and increases in expression over time. DCM mutants and wild type protein expression peaks after 48 hours post-transduction and stabilizes thereafter. **C,** Transduction efficiency of DCM mutant and wild type adenoviral vectors. Efficiency of transduction approaches 100 percent for cardiac myocytes and is similar in δ SG mutant and wild type δ SG expressing myocytes. Values are normalized as a percentage of the total population of cells and values are represented as mean \pm S.E.

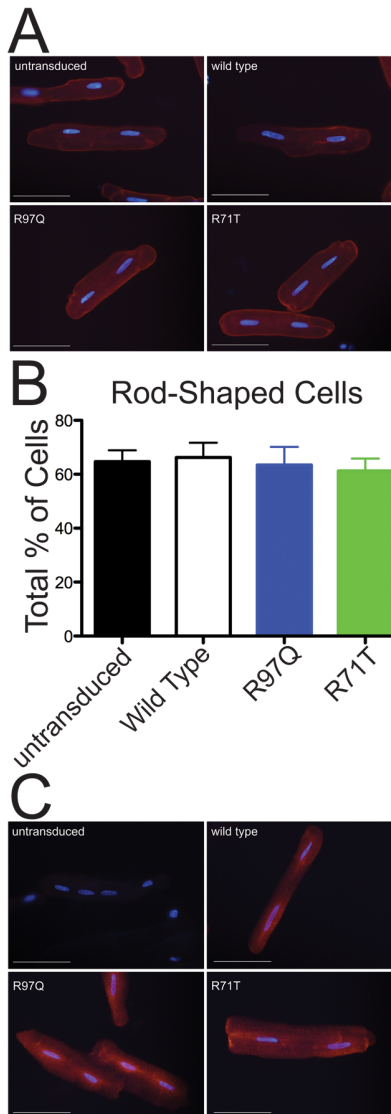


Figure 2-2. Mutant δ SG expression does not affect cardiac myocyte viability or cell morphology and does not alter β DG localization or organization. **A, Immunostaining for β DG. Expressing δ SG mutation R97Q or R71T does not alter expression or localization of membrane bound DGC component β DG. The red channel is beta dystroglycan (anti- β DG), and the blue channel is DAPI stained nuclei. Scale bars are equal to 50 μ m. **B**, Assessment of cardiac myocyte cell morphology. Expressing δ SG using adenoviral vectors does not decrease overall cardiac myocyte viability and does not alter rod morphology. Values are normalized as a percentage of the total population of cells and values are represented as mean \pm S.E. **C**, Immunostaining for δ SG in cardiac myocytes following fixation and permeabilization. The red channel is myc-tagged δ SG (anti-myc) and the blue channel is DAPI stained nuclei. There is no apparent difference in expression levels or localization in wild type or δ SG mutant R97Q or R71T expressing cardiac myocytes. Scale bars are equal to 50 μ m.**

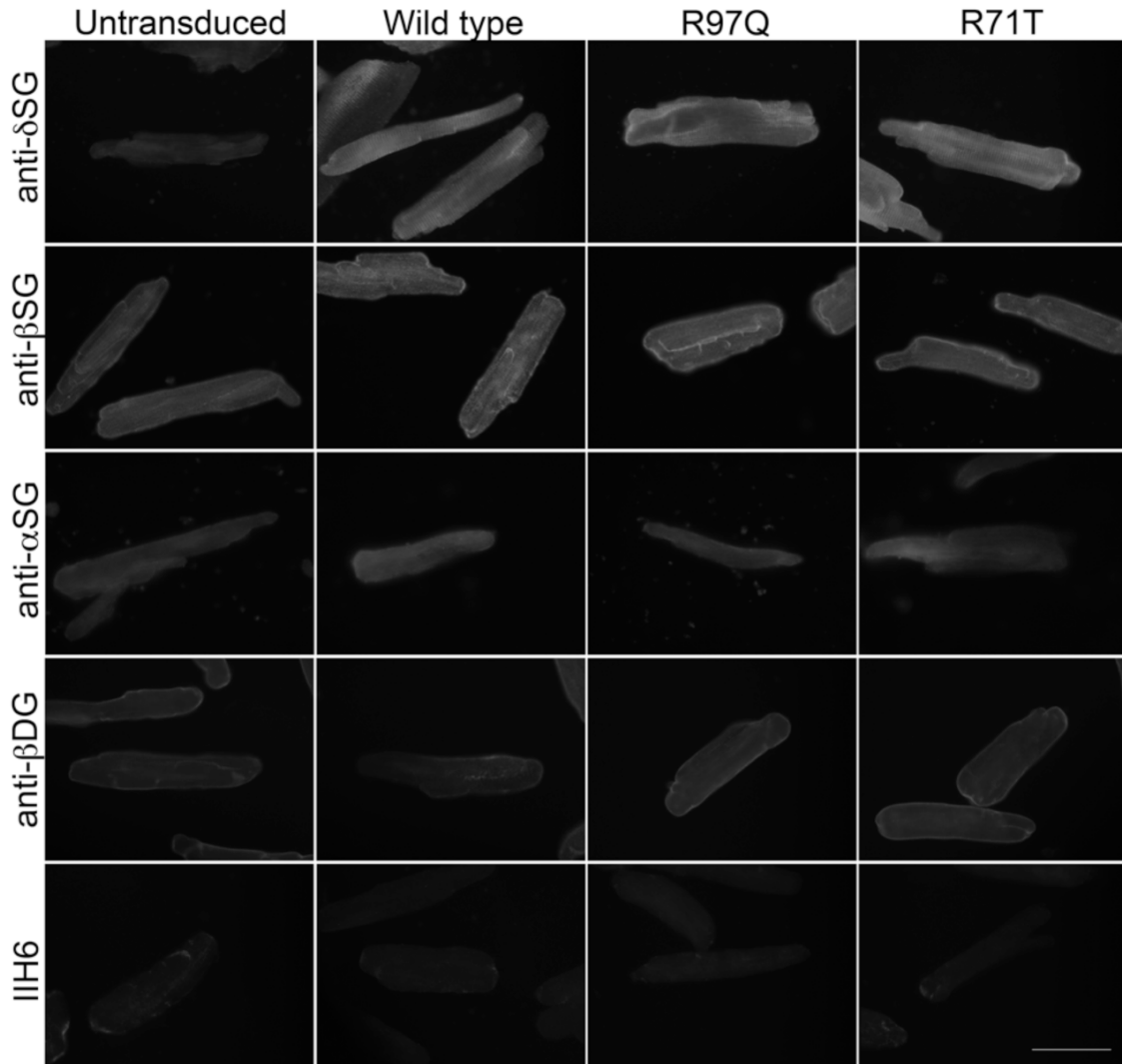


Figure 2-3. Expressing delta sarcoglycan using adenoviral vectors does not alter DGC localization or organization. Immunostaining for δ SG, α SG, β SG, β DG, and glycosylated α DG (IIH6) reveals expression levels for DGC components is unaffected by expression of mutant δ SG protein R97Q or R71T compared to wild type δ SG. DGC localization appears unaffected as well. Scale bar is equal to 50 μ m.

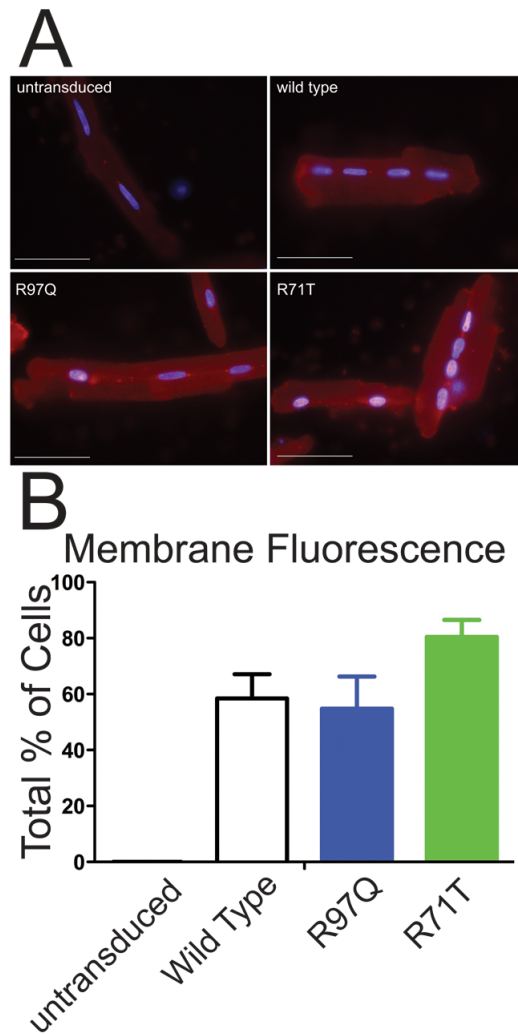


Figure 2-4. DCM mutant δ SG R97Q and R71T localize and insert normally into the plasma membrane. **A**, Live-cell labeling of δ SG. The red channel represents δ SG (anti-myc) and the blue channel is DAPI. Live-cell labeling identifies only δ SG inserted into the plasma membrane. Red levels have been adjusted evenly across all panels to allow visualization of staining above background. Expression levels and membrane labeling are similar in wild type as well as δ SG mutant R97Q and R71T expressing cardiac myocytes. Staining with anti-myc antibody shows labeling only in cells that have been transduced with myc-tagged wild type δ SG or δ SG mutant R97Q or R71T. Scale bars are equal to 50 μ m. **B**, Quantifying cardiac myocytes labeled with δ SG at the plasma membrane. Delta sarcoglycan mutant R97Q and R71T have no significant difference in cardiac myocytes labeled at the plasma membrane when compared to wild type δ SG expressing cardiac myocytes. Values are normalized as a percentage of the total population of cells and values are represented as mean \pm S.E.

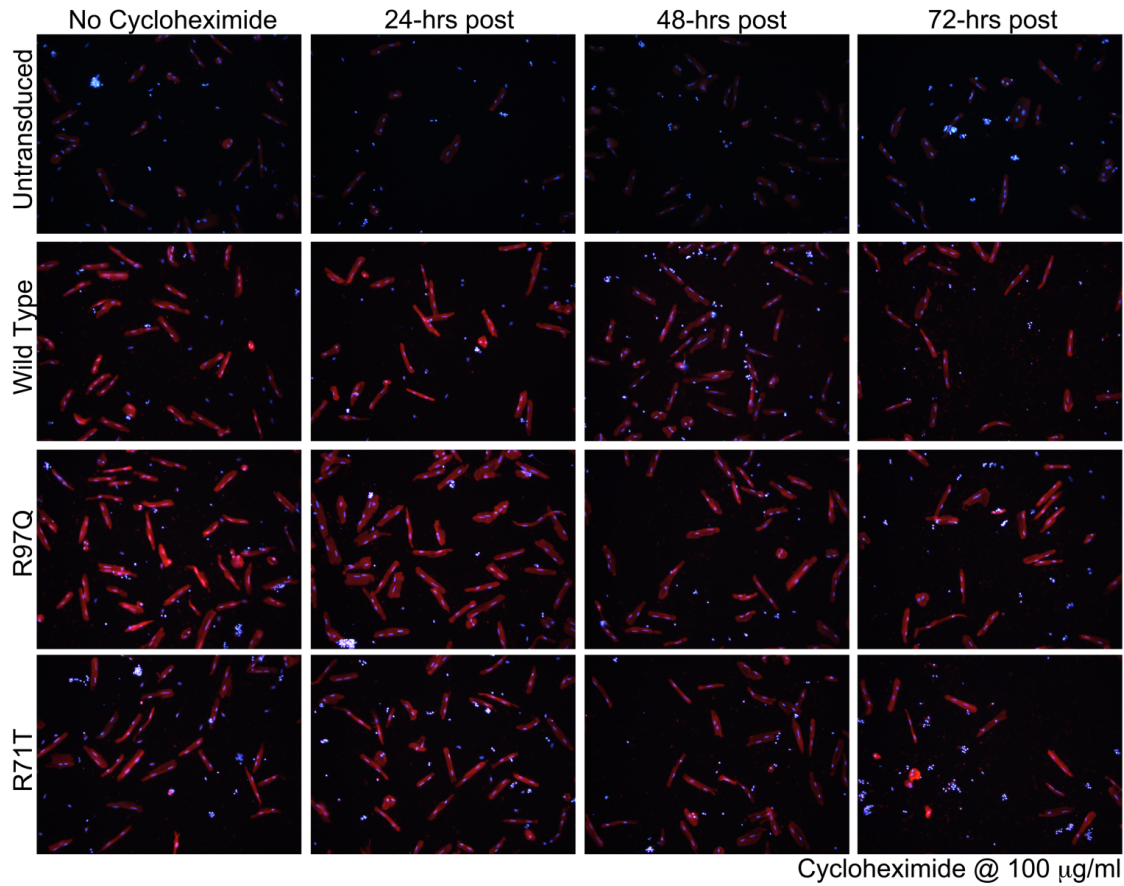


Figure 2-5. Mutant δ SG protein R97Q and R71T are stable at the plasma membrane. Live cell staining and treatment with cycloheximide reveals δ SG protein turnover at the plasma membrane is similar in cardiac myocytes expressing mutant δ SG R97Q and R71T compared to wild type δ SG. All panels have been adjusted equally to better visualize membrane staining. Scale bars are equal to 200 μ m.

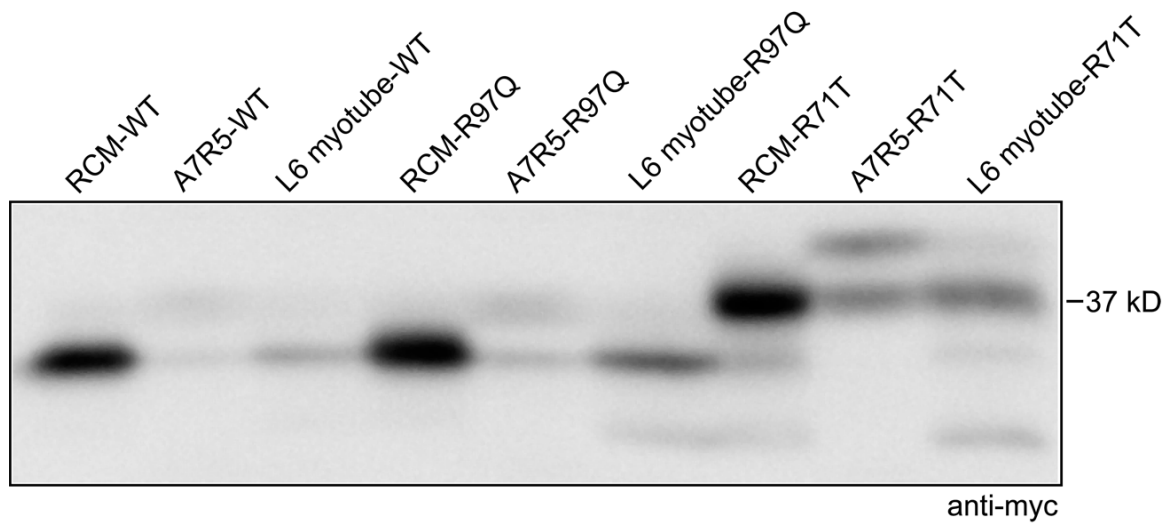


Figure 2-7. Alternative glycosylation of mutant δ SG R71T occurs in other cell culture models of striated muscle and A7R5 cells have increased overall glycosylation. Western blot analysis of whole cell lysates (anti-myc) taken from rat cardiac myocytes, smooth muscle A7R5 cells, and skeletal muscle L6 myotubes. Blotting reveals differential glycosylation of δ SG in rat cardiac myocytes is recapitulated in skeletal muscle L6 myotubes. Additionally, A7R5 cells show multiple bands in both mutant and WT expressing cells and an accompanying molecular weight shift when δ SG mutant R71T is expressed. Similar volumes of lysate were loaded for all groups.

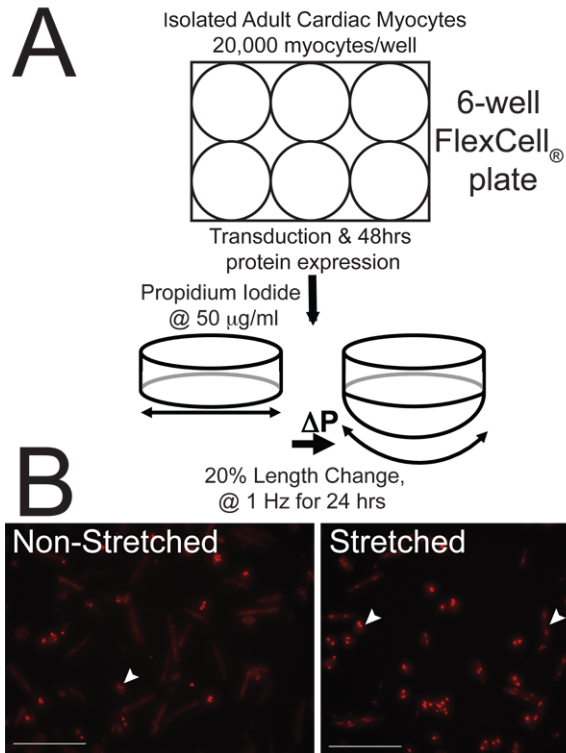


Figure 2-8. Mechanical stretch increases cell contracture and cell impermeable dye uptake in cardiac myocytes. Cells submitted to stretch are more susceptible to dye uptake and cell rounding than cells that are not mechanically strained. **A**, Diagram demonstrating experimental design for subjecting isolated cardiac myocytes to mechanical strain. **B**, Non-stretched and stretched cardiac myocytes. The fields shown are an example of the stretch effect using cardiac myocytes expressing δ SG mutant R71T. Overall fluorescence is lower and there are fewer positively labeled cells in non-stretched fields. Stretching myocytes increases uptake of membrane impermeable propidium iodide and causes an increases in total number of round-shaped cells. White arrows show examples of positively labeled cells. Scale bars are equal to 200 μ m.

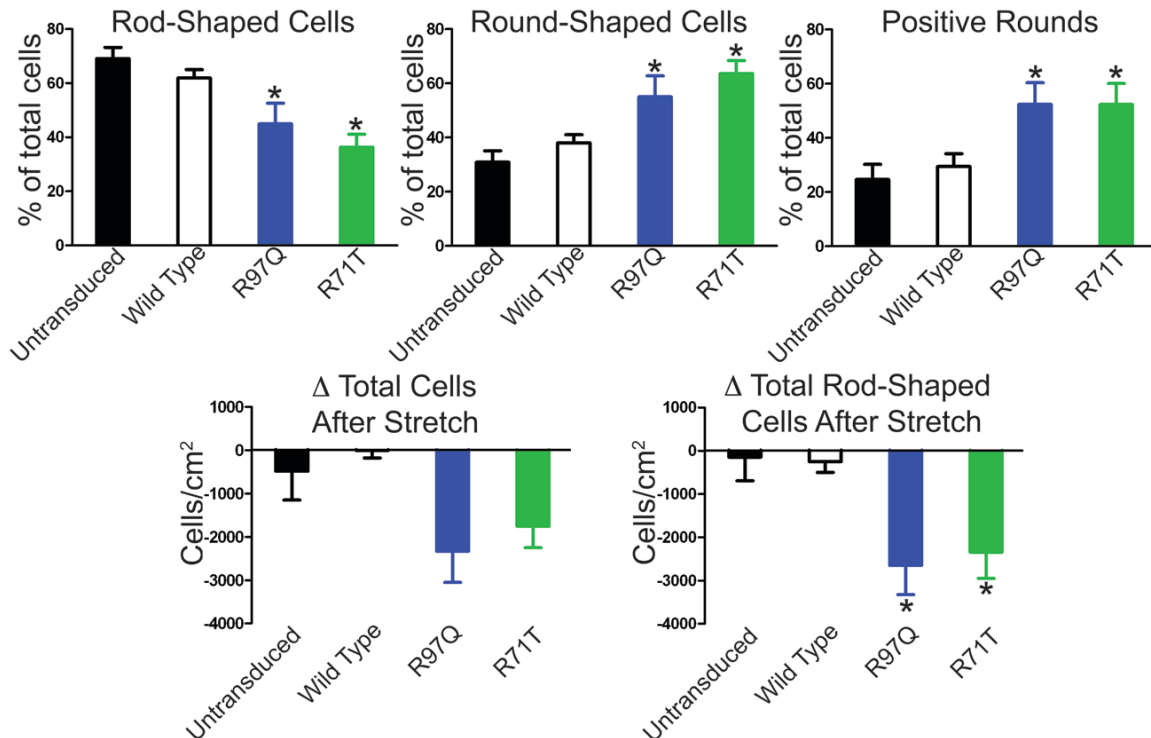


Figure 2-9. Cardiac myocytes expressing DCM mutants δ SG R97Q and R71T are more susceptible to sarcolemmal damage due to mechanical strain compared to cardiac myocytes expressing wild type δ SG. Scoring cardiac myocytes following a 24-hour protocol of mechanical strain. The top graphs are normalized as a percentage of the cell population within each experiment. The top-left graph shows the overall percentage of cells retaining the normal rod-shaped morphology. The top-middle graph shows the overall percentage of cells that are abnormally round-shaped. The top-right graph shows the number of positively labeled round-shaped cardiac myocytes. Cardiac myocytes expressing δ SG mutant R97Q or R71T have a statistically significant decrease in normal rod-shaped cells and a significant increase in abnormal round-shaped cells. Additionally, cardiac myocytes expressing δ SG mutant R97Q or R71T have a statistically significant increase in the number of positively labeled round cells. Positive labeled cells are those cells defined as having fluorescence above background. The bottom graphs show the change in number of cardiac myocytes counted before and after mechanical strain. The bottom-left graph shows the change in total number of cardiac myocytes. The bottom-right graph shows the change in normal rod-shaped cardiac myocytes. Cardiac myocyte populations expressing δ SG mutant R97Q or R71T do not show a statistically significant difference in overall total cells when exposed to mechanical strain. Cardiac myocyte populations expressing δ SG mutant R97Q and R71T do show a statistically significant decrease in rod-shaped cells. Delta was expressed by subtracting the number of cells counted without stretch from the number of cells counted after stretch (Δ =myocytes after stretch-unstretched myocytes).

Chapter 3

Glycosylation of Delta Sarcoglycan's Extracellular Domain is Necessary to Maintain Binding to Laminin During Mechanical Events in Cardiac Myocytes

Abstract

Delta sarcoglycan is an integral component of the dystrophin-glycoprotein complex and necessary for sarcoglycan complex assembly and function. Mutations in the extracellular domain of delta sarcoglycan are responsible for both limb-girdle muscular dystrophy 2F and are linked to development of dilated cardiomyopathy. The extracellular domain of delta sarcoglycan also undergoes a number of post-translational modifications including three putative sites for N-linked glycosylation at threonine residues 60 and 108, as well as serine residue 284. The purpose of this study was to investigate the role these residues play in delta sarcoglycan function. Using isolated rat cardiac myocytes as a model these data demonstrate that N-linked glycosylation of delta sarcoglycan only occurs at residues 108 and 284. Additionally, residue 108 is N-glycosylated on all delta sarcoglycan proteins whereas residue 284 appears to be occupied on only one quarter of all delta sarcoglycan proteins. The loss of glycosylation at residue 108 or 284 also does not affect delta sarcoglycan localization to the plasma membrane. Furthermore loss of glycosylation at residue 108 causes decreased myocyte adhesion to laminin following mechanical strain. Together these data suggest that fully glycosylated delta sarcoglycan may play a supporting role in

stabilizing the attachment of the dystrophin-glycoprotein complex to laminin during mechanical events.

Introduction

Delta sarcoglycan (δ SG) is member of the tetrameric sarcoglycan complex expressed highly at the lateral sarcolemma in striated muscle transcribed by the SGCD gene in humans on chromosome 5. The δ SG protein was first identified as a 35-kD glycoprotein associated with the dystrophin-glycoprotein complex (DGC) almost 20 years ago⁴¹. That same year it was determined that an autosomal recessive mutation in the SGCD gene was responsible for limb-girdle muscular dystrophy type 2F (LGMD2F)¹⁵⁶. Not long after the initial discovery of δ SG it was identified as the gene product deficient in the BIO 14.6 Syrian hamster¹⁷⁷, a widely used animal model of dilated cardiomyopathy (DCM). Later candidate gene studies revealed that dominantly inherited mutations in the SGCD gene were linked to familial DCM¹⁵⁷. Since the initial discovery of δ SG many more mutations have been identified linked to both LGMD2F^{112, 153-155} and DCM¹⁵⁸. The presence of multiple mutations that cause varying severe muscle diseases suggests that the δ SG protein plays an integral role in muscle function. Despite this, δ SG protein function remains largely a mystery. It is hypothesized that the sarcoglycans stabilize the DGC at the sarcolemma^{97, 128, 134, 142, 190, 191}, but how the sarcoglycan complex performs this action is not completely clear.

Mutations in δ SG that cause LGMD2F also destabilize the sarcoglycan complex^{94, 112, 192} making it difficult to establish a possible functional role for the

singular protein δ SG beyond the overall function of the sarcoglycan complex. This is likely due to δ SG being an obligate half of the δ/β sarcoglycan complex core. The disruption of the δ/β sarcoglycan core causes complete sarcoglycan complex loss and a subsequent dystrophic phenotype^{110, 128, 129}. The δ/β sarcoglycan core is not only the first necessary step in sarcoglycan complex assembly but also appears to interact with dystrophin through both δ and β SG cytosolic tails¹¹⁰.

Previous work in our lab suggests that DCM linked δ SG mutations R97Q and R71T act as dominant negative proteins when integrated into the DGC and sarcolemma (Chapter 2). These two δ SG mutants functionally disrupt plasma membrane stability during mechanical strain. The dominant negative action of these two mutants suggests that δ SG has a functional role that can be disrupted without also disturbing the sarcoglycan complex. Additionally, the mutation at residue 71 from an arginine to a threonine creates an ectopic consensus site for N-linked glycosylation. Despite the addition of this ectopic glycan the mutant δ SG localizes to the plasma membrane and integrates with normal efficiency. Previous data has suggested that residues 57-92 in δ SG are necessary for proper association with β SG¹¹⁰. The mutation of residue 71 and ectopic glycosylation would normally suggest that this mutation has a role in destabilizing the δ SG and β SG interaction. However, expression of δ SG mutant R71T does not have an apparent change in β SG expression or localization to the plasma membrane (Chapter 2). Expressing δ SG mutant R71T and retaining an intact sarcoglycan complex naturally leads to questions about the role that glycan

residues play in δ SG protein function and how mutations in the extracellular domain of δ SG alter this function.

The extracellular domain of δ SG comprises over 80 percent of the amino acids that make up the δ SG protein. Additionally, due to glycosylation it likely also makes up the bulk of δ SG's mass. Sequence analysis identifies three consensus sites for N-linked glycosylation at residues 60-62 (NFT), 108-110 (NVT), and 284-286 (NTS). These putative sites have not been experimentally verified, and more importantly the role of N-linked glycosylation in δ SG protein function is still unknown. Prevailing thought concerning sarcoglycan glycosyl residues is that they are responsible and necessary for proper protein folding and processing through the secretory pathway¹⁹³⁻¹⁹⁶. However, three pieces of evidence exist that suggest N-linked glycosylation of sarcoglycans may play a more active role in δ SG and sarcoglycan complex function. 1) Ectopic glycosylation at residue 71 in mutant δ SG R71T is processed and localizes normally to the sarcolemma in cardiac myocytes (Chapter 2). 2) Glycosylation of DGC protein α DG is responsible for binding to the extracellular matrix through laminin^{16, 81-87}. 3) Gain-of-glycosylation mutations in epsilon sarcoglycan (ϵ SG) can cause myoclonus dystonia¹²⁶

This study investigated the functional role δ SG glycan residues play in sarcolemma stabilization and extracellular matrix interactions. Adenoviral vectors were designed mutating the asparagine to glutamine residues in each of the three putative glycosylation sites in δ SG. Isolated rat cardiac myocytes expressing glycan mutant demonstrate that glycosylation does not occur on

asparagine residue 60. Asparagine residue 108, is glycosylated on all δ SG proteins in cardiac myocytes. Finally, asparagine residue 284 is glycosylated on approximately 23 percent of δ SG proteins in cardiac myocytes. Additionally, ablation of glycan residues at either 108 or 284 does not inhibit δ SG protein localization to the plasma membrane. Using a functional stretch assay reveals that cardiac myocytes expressing glycan mutant N108Q have decreased cell adhesion to laminin after mechanical strain. These findings demonstrate that glycan residues on δ SG are not necessary for protein processing through the secretory pathway. Additionally, the data presented here suggests that δ SG glycosylation may support DGC adhesion to laminin especially during mechanical events.

Methods

Recombinant Adenovirus Generation:

Human delta sarcoglycan full-length cDNA was obtained from Open Biosystems (MHS1011-7509692) Point mutations creating glycosylation deficient δ SG were engineered using a QuickChange II site directed mutagenesis kit (Agilent 200523). Adenovirus was generated as previously described (Chapter 2).

Cardiac Myocyte Isolation:

Anaesthetization (20mg ketamine/4mg xylazine, 400 units heparin) was administered via intraperitoneal injection on 200 gram Sprague Dawley female rats (Charles River Labs). 20 minutes after anaesthetization rats received bilateral thoracic incision and the heart was removed and placed in ice-cold

Krebs Henselait Buffer (KHB) (118 mmol/L NaCl, 25 mmol/L Hepes, 1.2 mmol/L KH_2PO_4 , 1.2 mmol/L MgSO_4 , 1.0 mmol/L CaCl_2 , 11 mmol/L Glucose). Excess lung, pulmonary vessels, thymus, and fat were removed prior to attaching to a sterile canula and transferring to a modified Langendorff perfusion apparatus. Retrograde perfusion was performed through the aorta using 37° KHB for 5 minutes at approximate 10 ml/min. Temperature was maintained by recirculating water bath in an outer chamber. Perfusion is changed to KHB without Ca^{2+} for another 5 minutes at a flow rate of approximately 10 ml/min. Type 2 Collagenase (Worthington Biochem LS004174) was added to perfusate at a concentration of 230 u/ml in a total of 80 ml solution. Perfusion proceeded for 25 minutes with recirculation of Ca^{2+} -free KHB solution with collagenase. Ca^{2+} concentration is increased to 1.5 mmol/L using 225 μl of 100 mmol/L sterile CaCl_2 added 4 times at 30-second intervals. Perfusion and recirculation continued for an additional 25 minutes. After perfusion the heart is removed from the Langendorff into 20 ml of collagenase- Ca^{2+} -KHB solution in a sterile beaker. Ventricular tissue is removed and minced into 8-12 small pieces. Ventricular tissue underwent gentle manual swirling for 5 minutes at 37°C in a water bath. Solution was removed and discarded. 20 ml of new collagenase- Ca^{2+} -KHB solution was added and swirling was repeated for an additional 5 minutes. Solution was removed and discarded. 20 ml of new collagenase- Ca^{2+} -KHB solution was added and ventricular tissue was passed through wide-bore pipettes coated with sigma-cote (Sigma Aldrich SL2-25). Swirling was repeated again for an additional 5 minutes. Solution was removed and discarded. 20 ml of new collagenase- Ca^{2+} -KHB solution was added

and ventricular tissue was passed through wide-bore pipettes coated with sigmacote (Sigma Aldrich SL2-25). Swirling was repeated again for an additional 5 minutes bringing the total number of incubations to 4. After the final swirling and incubation remaining tissue was passed through a 60-mesh tissue screen. Collected myocytes were centrifuged at 300 rpm for 3 minutes. Collagenase- Ca^{2+} -KHB solution was aspirated and pelleted cardiac myocytes were resuspended in 10 ml stop solution (2% bovine serum albumin in KHB). Calcium was increased to 1.75 mmol/L by adding 5, 10, 15, 20, and 25 μl 100 mmol/L CaCl_2 at 5-minute intervals. Cardiac myocytes were centrifuged at 300 rpm for 3 minutes. Stop solution was aspirated and pelleted cardiac myocytes were resuspended in 5 ml minimum essential media (MEM) (Gibco 11575). Cells were totaled and counted using a hemocytometer and suspended at a final concentration of 100,000 cells/ml in MEM with 5% FBS. A total of 4 rats were used for stretch assay experiments. A total of 4 rats were used for membrane fluorescence and live-cell staining experiments. A total of 3 rats were used for western blots. Overall a total of 11 rats were used for experiments in this cohort.

Cardiac Myocyte Plating:

Flexcell plates were coated with 300 μl EHS laminin (Sigma Aldrich L2020) at a concentration of 50 $\mu\text{g}/\text{ml}$. Glass cover slips were coated with 100 μl EHS laminin at a concentration of 50 $\mu\text{g}/\text{ml}$. 100mm culture dishes (Corning 430167) were coated with 1 ml EHS laminin at a concentration of 50 $\mu\text{g}/\text{ml}$. All plates and coverslips covered with laminin were sterilized prior to plating myocytes using ultraviolet light for 5 minutes and allowed to polymerize at least 30 minutes prior

to plating. 30,000 cardiac myocytes were suspended on each bioflex plate and allowed to adhere for 2 hours. 10,000 cardiac myocytes were suspended on each glass cover slip and allowed to adhere for 2 hours. 1,000,000 cardiac myocytes were suspended on each 100 mm plate and allowed to adhere for 2 hours. Following the 2-hour incubation media was aspirated from cardiac myocytes adhered to laminin and replated in MEM without FBS.

Cardiac Myocyte Transduction:

Cardiac myocytes were transduced in MEM for at least 1 hour. MEM was aspirated and replaced with fresh MEM and cardiac myocytes were allowed to express transduced proteins for 48 hours.

Fixed Cardiac Myocyte Immunostaining:

After being allowed to express transduced proteins media was aspirated from cardiac myocytes and replaced with Dulbecco's phosphate buffered saline (DPBS) (Gibco 14190). DPBS was aspirated and cardiac myocytes were fixed with 500 μ l of 1 mol/L paraformaldehyde in phosphate buffered saline (PBS) for 15 minutes. Paraformaldehyde was aspirated and fixed cardiac myocytes were washed with 500 μ l DPBS for 5 minutes a total of 3 times. Cardiac myocytes were blocked for 2 hours with 500 μ l block solution (DPBS with 5% BSA and 0.5% triton-X 100) to permeabilize myocytes. Block solution was aspirated and cardiac myocytes were incubated in primary antibody using 100 μ l/slip antibody suspended in block solution for 90 minutes in a humidity chamber. Primary antibody was aspirated and cardiac myocytes were washed with 500 μ l DPBS for 5 minutes a total of 3 times. DPBS was aspirated and cardiac myocytes were

incubated with secondary antibody using 100 μ l/slip using antibody suspended in block solution for 1 hour in a humidity chamber. Secondary antibody was aspirated and cardiac myocytes were washed using 500 μ l DPBS for 5 minutes a total of 3 times. DPBS was aspirated and cover slips were mounted in aqueous permafluor (Lab Vision TA-030-FM) on Superfrost Plus microscope slides (Fisher Scientific 12-550-15). For labeling myc tags primary antibody used was 9B11 mouse monoclonal anti-myc (Cell Signaling 2276S) at a titer of 1:50, secondary antibodies used were Cy3 conjugated anti-mouse IgG (Jackson Immunoresearch) at a titer of 1:500 and DAPI (Sigma-Aldrich) diluted 1:10,000 from a 10 mg/ml stock.

Live Cell Labeling:

After being allowed to express transduced proteins media was aspirated from cardiac myocytes and replaced with primary antibody in 100 μ l MEM (Gibco 11575) at 37°C for 2 hours. Media and antibody were aspirated and cardiac myocytes were washed with 500 μ l MEM for 5 minutes a total of 3 times. Cardiac myocytes were washed once with 500 μ l DPBS (Gibco 14190) and then fixed in 500 μ l 1 mol/L paraformaldehyde for 15 minutes. Paraformaldehyde was aspirated cardiac myocytes washed with 500 μ l DBS for 5 minutes a total of 3 times. DPBS was aspirated and myocytes were blocked using 500 μ l block solution (5% BSA and 0.5% triton-X 100 in DPBS) for a total of 2 hours. Block solution was aspirated and replaced with 100 μ l secondary antibody suspended in block solution for 1 hour in a humidity chamber. Secondary antibody solution was aspirated and cardiac myocytes were washed with 500 μ l DPBS for 5

minutes a total of 3 times. DPBS was aspirated and cover slips were mounted in aqueous permafluor (Lab Vision TA-030-FM) on Superfrost Plus microscope slides (Fisher Scientific 12-550-15). For labeling myc tags primary antibody used was 9B11 mouse monoclonal anti-myc (Cell Signaling 2276S) at a titer of 1:50, secondary antibodies used were Cy3 conjugated anti-mouse IgG (Jackson ImmunoResearch) at a titer of 1:500 and DAPI (Sigma-Aldrich) diluted 1:10,000 from a 10 mg/ml stock.

Immunofluorescence Microscopy:

Images were taken using a Leica SP8 confocal microscope with direct detection. Fluorescence baseline was established using myocytes stained without primary antibody.

Membrane Fluorescence Scoring:

Membrane fluorescence was scored using random field counts. Four experiments were performed generating five random field counts for each group at 200X magnification. Membrane fluorescence was determined as those rod-shaped cells that were fluorescent above untransduced control cell threshold. For evaluating membrane localization, pixel density was evaluated selecting regions of interest using Leica LAS-AF-Lite software. Regions evaluated were total cell fluorescence, peripheral fluorescence, intercalated disc fluorescence, and lateral membrane fluorescence. Pixel density was used to normalize for variance in the size of areas selected. 4 random myocytes from each sample group were used to score regions of interests

Cell Stretch Assay:

Isolated myocytes were incubated with 2 $\mu\text{g/ml}$ 7-aminoactinomycin D (7-AAD) (Molecular Probes A1310) in MEM (Gibco 11575) immediately prior to mechanical stretch. Myocytes were stretched on Bioflex plates via an FX4000 Tension System (FlexCell International). Stretch was performed using a strain protocol imparting 20% length change across the membrane at 1 Hz for 24 continuous hours. After mechanical strain myocytes were incubated for 30 minutes undisturbed. Media was aspirated and cardiac myocytes were washed with DPBS (Gibco 14190) twice for 5 minutes each. DPBS was aspirated and myocytes were fixed using 1 mol/L paraformaldehyde in DPBS for 15 minutes. Paraformaldehyde was aspirated and myocytes were washed with DPBS for 5 minutes 2 total times. Myocytes and membranes were mounted in aqueous permafluor (Lab Vision TA-030-FM) on SuperFrost Plus microscope slides (Fisher Scientific 12-550-15) for fluorescent microscopy.

Cell Stretch Assay Scoring:

Myocytes were counted via random fields. In total 3 separate rat isolations were performed. Each experiment generated 15 fields per sample group. Of the 15 fields generated for each group 5 fields were assessed at random. Images were taken and myocyte morphology was scored for all cells in a field. Positive-labeled cells were evaluated as having staining in the nucleus above background.

Protein Collection:

After being allowed to express transduced proteins media was aspirated from cardiac myocytes and collected using a cell lifter in 0.5% SDS in DPBS with protease inhibitors (leupeptin 2.34 $\mu\text{mol/L}$, aprotinin 2 KIU/ml pepstatin 0.73

$\mu\text{mol/L}$, phenylmethylsulfonyl fluoride 0.4 mmol/L, benzamidine 0.6 mmol/L).

Lysate and collected myocytes were vortexed, sonicated, and centrifuged at 14,000g for 2 minutes to pellet cell debris and supernatant was stored at -80°C for future use.

Western Blots:

All samples were run on a 3-15% SDS-PAGE gradient gel and wet-transferred to PVDF membranes at 100 volts for 3 hours. Membranes were blocked with 5% non-fat dairy milk in TBS-T (NaCl 150 mmol/L, Tris pH 007.5 50 mmol/L) for 1 hour. Following block, membranes were incubated using primary antibody in 5% non-fat dairy milk in TBS-T overnight. Membranes were washed using TBS-T for 10 minutes 3 total times. Membranes were incubated using secondary antibody in 5% non-fat dairy milk in TBS-T for 2 hours. Membranes were washed using TBS-T for 10 minutes 3 total times and incubated in SuperSignal West Dura Substrate (Thermo Scientific) for 1 minute. Membranes were visualized on a Fluorochem System (Alpha Innotech). For labeling myc tags primary antibody used was 9B11 mouse monoclonal anti-myc (Cell Signaling 2276S) at a titer of 1:500, secondary antibodies used were HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch) at a titer of 1:1000. For labeling beta-dystroglycan primary antibody used was a mouse monoclonal anti- βDG (AbCam) at a titer of 1:500, secondary antibodies used were HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch) at a titer of 1:1000. For labeling beta-sarcoglycan primary antibody used was a mouse monoclonal anti- βSG (AbCam) at a titer of 1:500, secondary antibodies used were HRP-conjugated anti-mouse IgG

(Jackson ImmunoResearch) at a titer of 1:1000. For labeling alpha-sarcoglycan primary antibody used was a mouse monoclonal anti- α SG (University of Iowa Developmental Studies Hybridoma Bank IVD (1)A9) at a titer of 1:500, secondary antibodies used were HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch) at a titer of 1:1000.

Statistics:

One-way analysis of variance (ANOVA) was performed with Dunnet's post-test. p-value<0.05

Results

Recombinant Adenoviruses Driving Expression of Glycosylation Deficient Delta Sarcoglycan is Highly Efficient

Glycosylation deficient delta sarcoglycan adenovirus was engineered using previously described techniques¹⁸¹. Vectors used in adenovirus use the cytomegalovirus (CMV) promoter to drive expression. Delta sarcoglycan was engineered with a C-terminus myc tag and glycosylation consensus sites were mutated altering the asparagine residues into glutamine residues (Figure 3-1A) using a quick-change site directed mutagenesis kit (Agilent). Rat cardiac myocytes were isolated and transduced at varying titers to determine optimal transduction efficiency (data not shown). Cardiac myocytes were also isolated, transduced and collected at 24, 48, and 72 hours post-transduction to determine optimal time for expression (data not shown). In cardiac myocytes expressing wild type δ SG two distinct bands are present – the lower highly expressed band

and an upper band of relatively low expression. In cardiac myocytes expressing N60Q mutant δ SG there appears to be no change in expression compared to wild type δ SG indicating that N-linked glycosylation does not occur at the consensus sequence from residues 60-62 (Figure 3-1B). In cardiac myocytes expressing N108Q mutant δ SG both bands are present but shifted downward indicating these bands run at a lower molecular weight than wild type δ SG. In cardiac myocytes expressing N284Q mutant δ SG the bottom band is still present but the upper band is lost entirely (Figure 3-1B). The one band remaining in lysates from N284Q expressing cardiac myocytes runs at an apparently similar level to the lower band of wild type delta sarcoglycan expressing cardiac myocytes. This suggests that the reduction to one band in the mutant N284Q expressing cardiac myocytes is due to ablation of glycosylation at residue 284 and is not due to some form of proteolytic cleavage. Additionally, the downward shift of both bands in cardiac myocytes expressing mutant N108Q appears to have a magnitude of around 5-6 kD which is similar to the molecular weight shifts observed in other model systems ablating specific sites of N-linked glycans¹⁹⁷,¹⁹⁸, given there may be differences in complexity and charge of the N-linked glycans. The upper band in wild type expressing cardiac myocytes representing δ SG that has been glycosylated on both residue 108 and residue 284 is approximately 23 percent of the total δ SG protein indicating that glycosylation on residue 284 is significantly less efficient than residue 108 (Figure 3-1C). When site 108 is mutated causing loss of N-linked glycosylation at this residue the

upper band representing δ SG glycosylated at residue 284 comprises almost half of all δ SG proteins.

Adult Rat Cardiac Myocytes Expressing Glycosylation Deficient Delta

Sarcoglycan has no Adverse Effects on DGC Expression or Cell Viability

Previous studies have demonstrated that δ SG is an integral component in sarcoglycan complex assembly. The sarcoglycans are necessarily expressed in a 1:1:1:1 ratio during assembly^{116, 127} and the first step in sarcoglycan assembly is the association of δ SG with β SG^{110, 128, 129}. Destabilization of the sarcoglycan complex via loss of δ SG N-glycans could disrupt assembly of the sarcoglycan complex or dystroglycans at the plasma membrane and could alter DGC component stoichiometry. Scoring cardiac myocytes expressing glycan mutant δ SG shows that loss of glycosylation at residue 108 or 284 has no apparent change in cell morphology indicating that overall cell viability is not affected by expression of δ SG glycan mutants (Figure 3-2A). Additionally, expression of δ SG glycan mutant N108Q or N284Q in cardiac myocytes does not affect apparent expression levels of the dystroglycans or sarcoglycan complex (Figure 3-2B).

Glycosyl Residues on Delta Sarcoglycan are not Necessary for Sarcoglycan Complex Assembly or Localization to the Sarcolemma

It has been suggested that many of the mutations in δ SG that cause loss of the sarcoglycan complex may be due to trafficking defects that could be rescued using pharmaceutical chaperones through the secretory pathway or inhibition of endoplasmic-reticulum-associated protein degradation¹⁹³⁻¹⁹⁶. DCM-

linked δ SG mutant S151A in mice have a trafficking defect causing foci and sequestration of the sarcoglycan complex in the nucleus of cardiac myocytes¹⁵⁹ suggesting that pathogenesis from some mutations in δ SG may be caused by mislocalization away from the plasma membrane. Whether N-linked glycosylation is necessary for δ SG processing through the secretory pathway and localization to the sarcolemma is not clear. Immunofluorescent staining shows that expression of δ SG glycan mutant N108Q and N284Q is similar to wild type δ SG (Figure 3-3). Taking advantage of the extracellular C-terminus myc tag, live-cell staining allows monitoring of δ SG protein arrival at the plasma membrane. Myocytes expressing glycan mutant δ SG N108Q or N284Q showed marked antibody staining in live cell configuration indicating the δ SG protein mutants localized to the sarcolemma similar to wild type δ SG expressing cardiac myocytes (Figure 3-4). To quantify this trafficking, we scored populations of live cell labeled cardiac myocytes revealing that glycan deficient mutant δ SG proteins N108Q and N284Q are detectable at the plasma membrane at comparable levels to wild type delta sarcoglycan (Figure 3-5A). High resolution imaging using confocal microscopy showed that expressed wild type appears to localize predominantly to either end of cardiac myocytes in locations that previously formed intercalated disks in the intact heart (Figure 3-4). To quantify this distribution and targeting of delta sarcoglycan in normal and mutant cells, we analyzed cardiac myocytes by quantifying the lateral membrane and end membrane fluorescence in cardiac myocytes. There is not a statistically significant difference in the distribution of mutant N108Q or N284Q at the plasma

membrane compared to wild type δ SG (Figure 3-5B), indicating that mutant N108Q or N248Q appear to spatially localize to similar locations in cardiac myocytes as compared to WT.

Loss of glycosylation at residue 108 causes myocyte detachment during mechanical strain

The function of δ SG and the sarcoglycans have not been well established. Previous work in our lab suggests that δ SG may play a functional role in stabilizing the sarcolemma during mechanical events (Chapter 2). The mechanism for this stabilization remains a mystery but could work through the interaction of the DGC to laminin. Additionally the cytosolic tail of δ SG has been shown to bind dystrophin in vitro¹¹⁰. Mutations in δ SG may somehow affect this interaction with dystrophin. Whether δ SG glycan residues are necessary for δ SG protein interactions has not been established. Adhesion of cardiac myocytes to laminin is unaffected when δ SG glycan mutant N108Q or N284Q are expressed (Figure 3-6). Oddly when cardiac myocytes are stretched for 24 hours cardiac myocytes expressing δ SG glycan mutant N108Q show increased detachment from laminin compared to wild type δ SG expressing cardiac myocytes (Figure 3-6). Furthermore, the expression of δ SG glycan mutant N108Q or N284Q does not alter sarcolemmal integrity or cell morphology when submitted to mechanical strain (Figure 3-7).

Discussion

The δ SG protein appears critical for function of striated muscle. Until very recently the only functional understanding of δ SG was the role it played in proper assembly and function of the sarcoglycan complex in total. Recent data from our lab has suggested that the δ SG protein may play an active functional role in maintaining sarcolemmal stability during mechanical events (Chapter 2). The mechanism to this function is still unclear but any and all of three hypotheses is likely. 1) Delta sarcoglycan binds directly to some ligand in the extracellular matrix. 2) Delta sarcoglycan binds directly to dystrophin and/or utrophin within the cytosol. 3) Delta sarcoglycan binds directly to another member of the DGC acting similar to an anchor within the sarcolemma. How δ SG may perform any and all of these actions is unclear. The N-linked glycans are obvious targets of investigation for δ SG protein function given the role glycosylation plays in DGC adhesion to laminin via glycosylated α DG. Additionally, our previous work has suggested that adding ectopic glycan residues does not inhibit δ SG trafficking to the plasma membrane as would be expected. Furthermore, it has been shown in ϵ SG that a gain-of-glycosylation mutation can cause myoclonus dystonia¹²⁶ suggesting that N-glycans on the sarcoglycans may be more than simple chaperones through the secretory pathway. This study investigated whether N-linked glycans in the extracellular domain are necessary for δ SG function. These results show that δ SG does undergo N-linked glycosylation at residues 108 and 284 in cardiac myocytes.^{199, 200} Furthermore the glycan at residue 108 plays a role in DGC binding to laminin during mechanical events. The mechanism of this binding remains a mystery but suggests strongly that either δ SG directly binds

laminin in some capacity or that δ SG is necessary for a stable linkage of laminin to α DG. The absence of glycosylation at the putative residue 60 can be explained due to its close proximity to the transmembrane domain of delta sarcoglycan (residues 36-56, Figure 3-1A). Amino acids as far away as 12 residues from transmembrane domains are not readily accessible to the oligosaccharyltransferase that imparts sugar residues to proteins within the ER^{199, 200} inhibiting glycosylation of consensus sequence sites that fall within this 12 residue threshold. Labeling δ SG at the plasma membrane reveals that δ SG proteins localize predominantly to regions at the plasma membrane corresponding to the intercalated discs in the intact myocardium. Loss of glycosylation at either residue 108 or 284 did not significantly alter the localization of δ SG to the plasma membrane nor its spatial distribution to membrane compartments in cardiac myocytes. The enrichment at the intercalated discs suggests that δ SG may be involved somehow in the ability of cardiac tissue to act as a functional syncytium. Although expressing glycosylation deficient δ SG did not alter the preferred localization to the intercalated discs it is possible that loss of these glycan residues could also alter the function of δ SG at this location of the plasma membrane.

There are a number of regions within δ SG proposed as critical for sarcoglycan complex assembly or for trafficking to the plasma membrane. Truncation of δ SG prior to the cysteine-rich motif is sufficient to cause a decrease of the sarcoglycan complex at the plasma membrane^{110, 154, 156}. Additionally in COS-1 cells it was established that mutating the asparagine at residue 108 to a

lysine reduces δ SG at the plasma membrane¹¹⁰. This suggests a possible role for glycosylation at residue 108 in membrane trafficking, but the change from an asparagine to a lysine may alter protein structure and/or interactions sufficient to explain the decrease in δ SG at the plasma membrane. The alteration of the asparagine at residue 108 to a glutamine is a much less drastic change in overall protein properties but still ablates glycosylation at residue 108. These results show that δ SG glycan mutant N108Q actually does localize to the plasma membrane in cardiac myocytes suggesting that N-linked glycosylation at residue 108 has some other role in δ SG protein function. It is possible, that N-linked glycosylation of δ SG has tissue specific roles. This would explain the discrepancy between our findings in cardiac myocytes and previous data obtained in COS-1 cells. Furthermore, δ SG mutant R71T exhibits a gain-of-glycosylation and appears to cause DCM but not skeletal muscle dystrophy¹⁵⁸ supporting the hypothesis that δ SG function is slightly altered from skeletal muscle to cardiac muscle.

δ SG glycan mutant N284Q also localizes normally to the plasma membrane suggesting that neither glycan residue at 108 or 284 alone is responsible for δ SG processing through the secretory pathway. Unfortunately generation of δ SG glycan mutant N108Q/N284Q proved difficult preventing investigation into loss of all the N-linked glycosylation within the extracellular domain of δ SG. It may be that either glycan residue is capable of covering for loss of glycosylation at the other site. These results showing residue 284 is glycosylated on only 23 percent of δ SG proteins suggest that glycosylation at 284

may only play a supporting role for δ SG protein when processing through the secretory pathway is somehow disrupted. Another possible explanation is that δ SG plays more than one functional role within cardiac myocytes and each glycan residue plays a different function. There is some evidence to suggest that δ SG can form a tricomplex distinct from the normal tetrameric sarcoglycan subcomplex^{110, 128, 132}. Additionally δ SG has been shown to localize to the sarcoplasmic reticulum²⁰¹ and associate with 16-kD vacuolar ATPase²⁰². Taken together these results indicate that δ SG may undergo alternate processing depending on the final destination or interactions of δ SG within the cell. It is likely that the vast majority of δ SG proteins are located at the lateral sarcolemma as a part of the tetrameric sarcoglycan complex. Targeting of δ SG to the sarcoplasmic reticulum or formation of a trimeric sarcoglycan complex may be accomplished via differential glycosylation at residues 108 and 284. This could explain why residue 284 is not occupied on all δ SG proteins.

It is attractive to hypothesize that alternative glycosylation on residue 284 is responsible for differential protein trafficking or function, however there are also other explanations that have support from the literature and are related to the mechanistic nature of glycosylation. Residue 284 is only 5 amino acid residues removed from the C-terminal end of δ SG. It has been shown that N-linked glycosylation consensus sequences within 60 residues of the C-terminus are less efficiently glycosylated than consensus sequences greater than 60 residues proximal to the C-terminus^{199, 203} suggesting this may be the mechanism for partial glycosylation of residue 284. Additionally, N-linked consensus

sequences containing threonine at the third residue of the consensus site can be glycosylated up to 40 times more efficiently than consensus sequences containing serine at the third residue^{204, 205}. Together the presence of serine at residue 286 and the consensus sequence close proximity to the C-terminus are the likely explanation for inefficient glycosylation on residue 284.

Although ablating the N-glycan residue at 284 did not disturb cell adhesion to laminin similar to losing the N-glycan at residue 108, residue 284 may still be involved somehow in cell adhesion to the basal lamina. We only tested cell adhesion via DGC to laminin, but it is possible that adhesion to another ECM protein such as collagen would be affected by the loss of N-glycan on δ SG residue 284. Furthermore, the functional role of each glycan residue could be tissue specific. Losing N-glycosylation at residue 284 may be more pathogenic in skeletal muscle or smooth muscle, whereas the N-glycan at residue 108 is more important for protein interactions within cardiac myocytes and the myocardium. Our previous data suggested that overall glycosylation of the δ SG protein is slightly altered in smooth muscle compared to cardiac myocytes and L6 myotubes (Chapter 2), supporting the idea that the functional role of δ SG glycosylation is different for each tissue type. It is also entirely possible that δ SG glycosylation is not relevant in DGC binding to laminin or the basal lamina, but that integrating δ SG mutant N108Q into the DGC is disruptive to overall DGC stability.

These results are the first to suggest that glycosylation is relevant for δ SG protein function beyond simple protein processing through the secretory

pathway. How loss of δ SG N-glycan at residue 108 disrupts laminin adhesion is still unclear. Determining whether δ SG directly binds laminin would go a long way in explaining both the function of δ SG as well as the function of the sarcoglycan complex. Finally, it would be useful to explore whether N-glycan residues on δ SG have a role in binding other ECM proteins.

Acknowledgements

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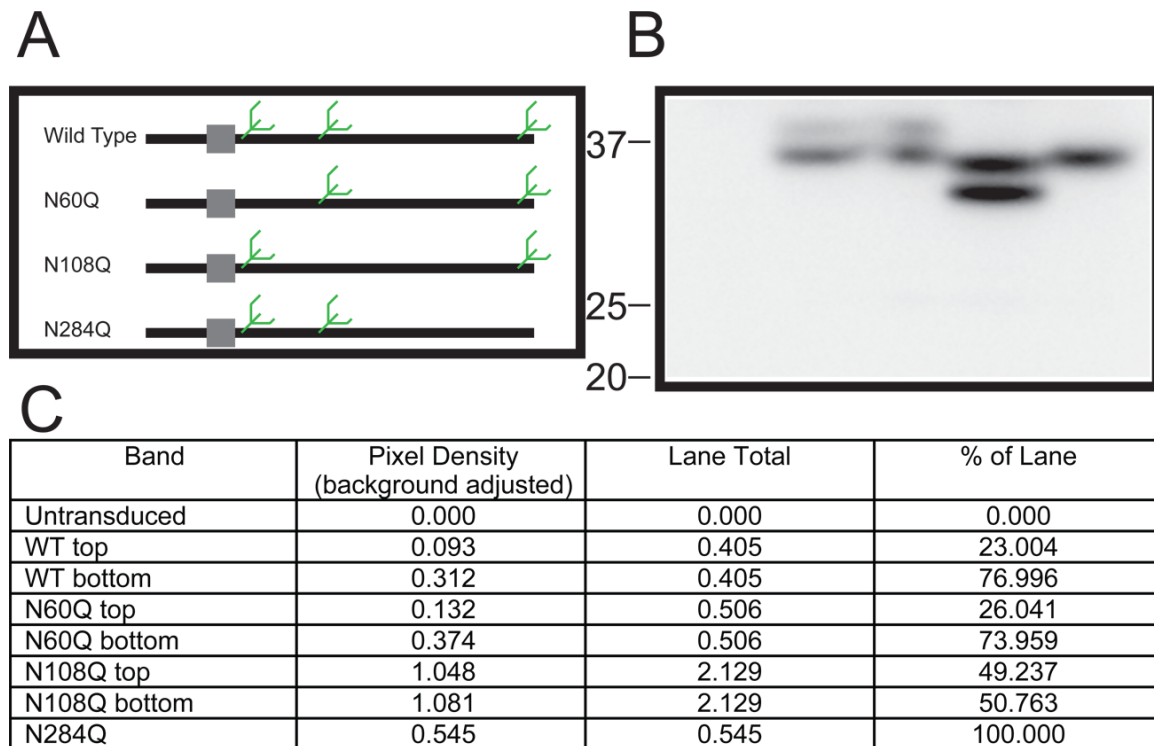


Figure 3-1. Glycosylation deficient adenoviral vectors expressed in cardiac myocytes reveals that delta sarcoglycan undergoes N-linked glycosylation at residues 108 and 284 in cardiac myocytes. (A) The four δ SG constructs used. The top construct is wild type δ SG with all three putative N-linked glycosylation sites intact. Mutant constructs each have one of the three putative glycosylation sites ablated. Light gray box represents the transmembrane domain, green tridents are putative N-glycan residues (B) δ SG mutant protein expression compared to wild type δ SG in cardiac myocytes. (C) Relative expression levels of δ SG protein show glycosylation occupancy at the N284 site on approximately 23 percent of δ SG proteins.

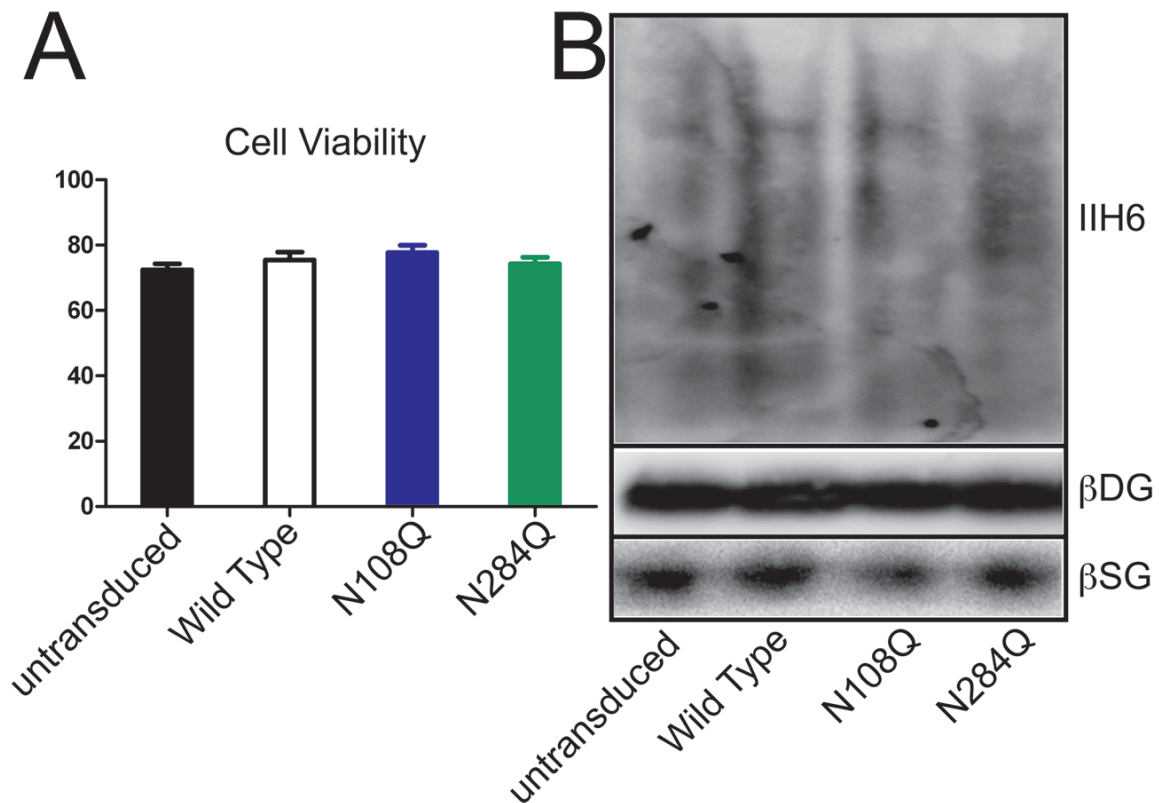


Figure 3-2. Loss of glycosylation in delta sarcoglycan at residue 108 or 284 in cardiac myocytes does not alter cell viability or DGC expression. (A) Expressing glycan mutant δ SG proteins does not alter cardiac myocytes morphology or cell viability. Cell viability was assessed by scoring normal rod-shaped cells. (B) Total expression of glycosylated alpha-dystroglycan (IIH6), beta-dystroglycan (β DG), and beta-sarcoglycan (β SG). Values are presented as a percentage of the total cell population and represented as mean \pm S.E. $P < 0.05$.

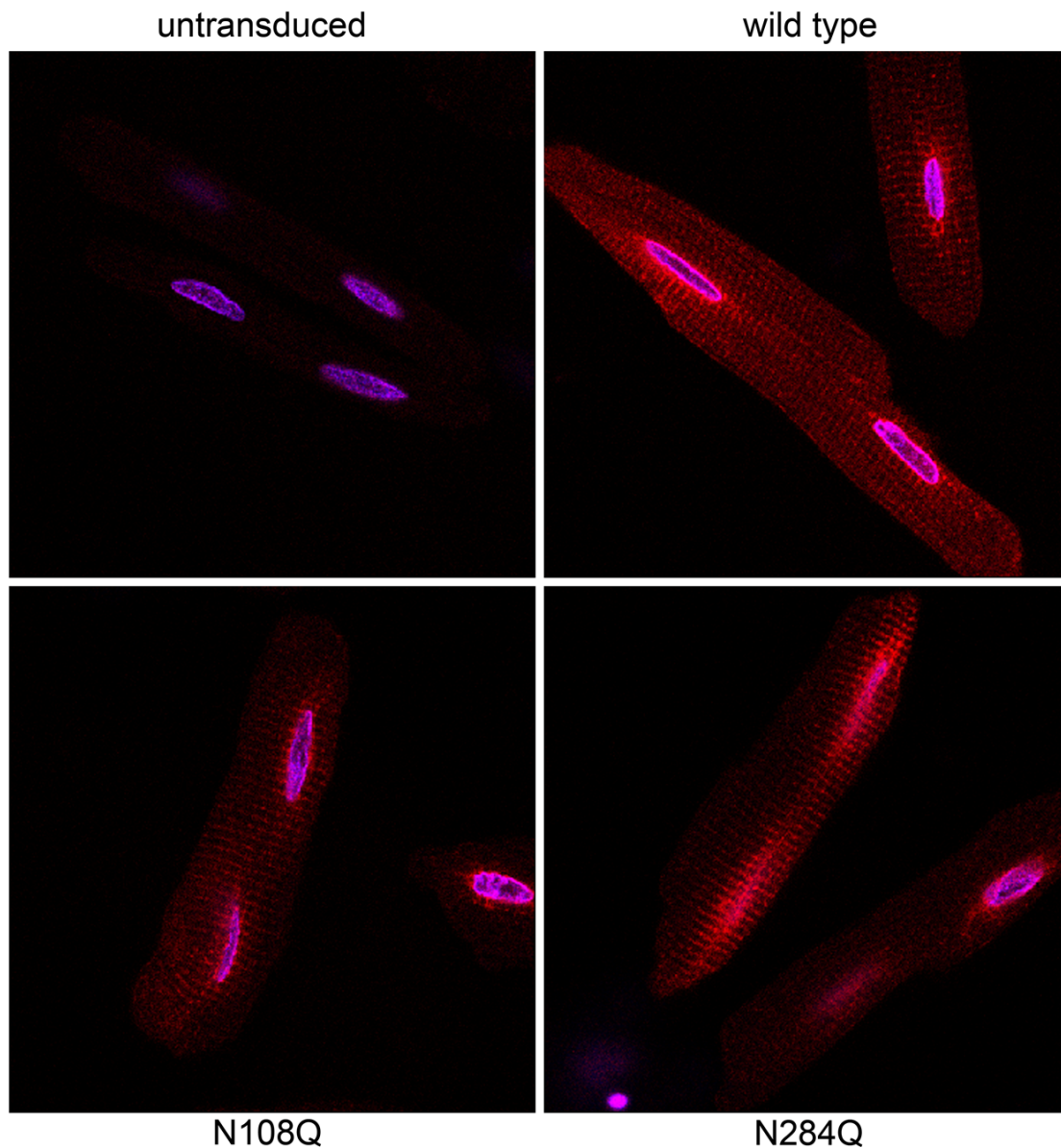


Figure 3-3. Loss of glycosylation in delta sarcoglycan at residue 108 or 284 in cardiac myocytes does not alter expression or localization of the delta sarcoglycan protein. Immunofluorescent staining of cardiac myocytes expressing wild type and glycan mutant δ SG proteins. Cells were permeabilized and fixed. Red-anti myc, blue-DAPI. The red channel has been enhanced to visualize untransduced cardiac myocytes. DAPI staining is slightly detected in the red-channel.

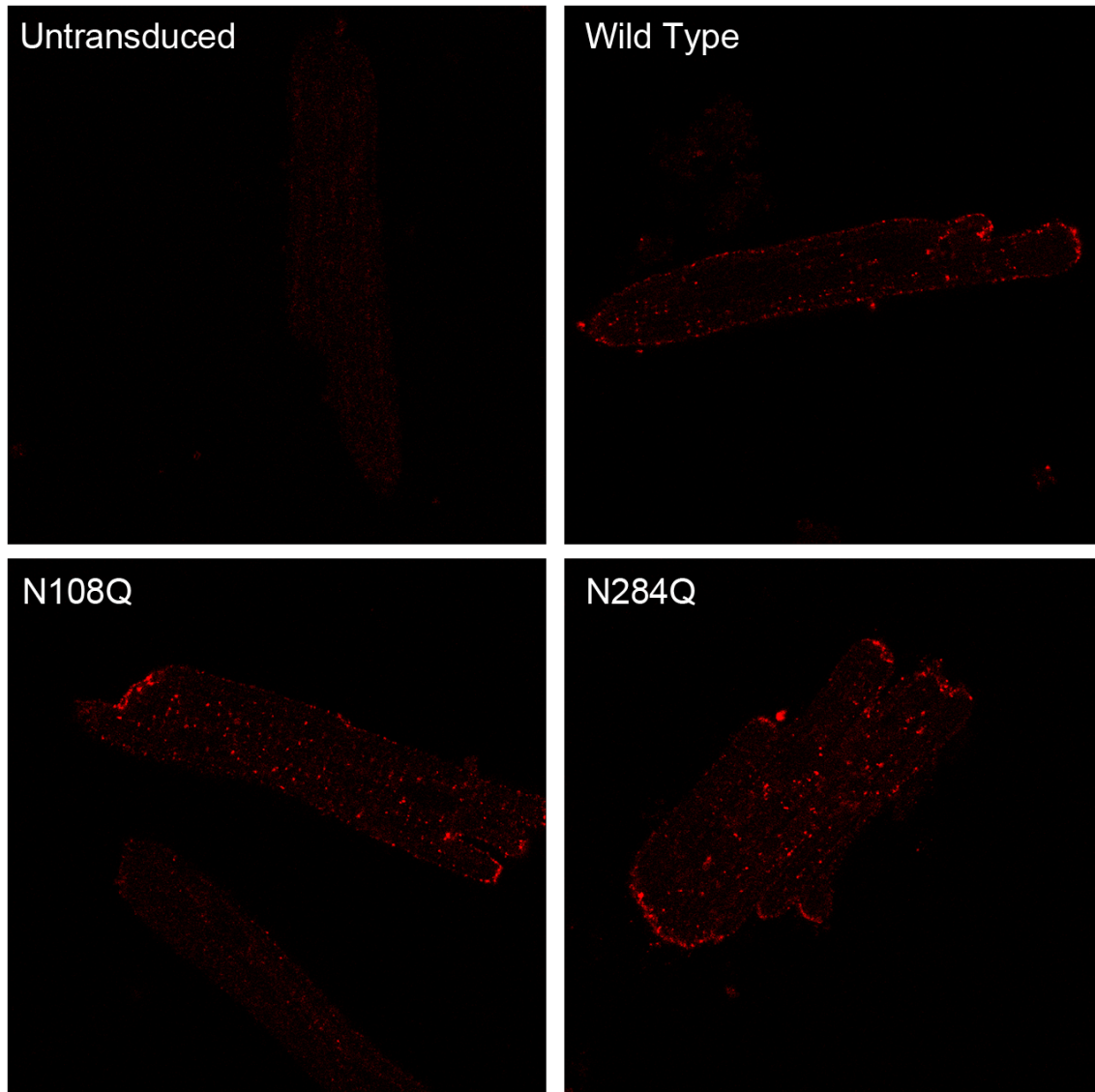


Figure 3-4. N-glycan deficient mutant delta sarcoglycan N108Q and N284Q localize and insert to the plasma membrane with equal efficiency as wild type delta sarcoglycan. Live-cell labeling of cardiac myocytes expressing wild-type and glycan mutant δ SG proteins. Initial labeling with primary antibodies is performed in media using no permeabilization or fixation to identify only δ SG proteins exposing the C-terminus myc tag on the extracellular side of the sarcolemma. The red channel has been enhanced to visualize untransduced cardiac myocytes.

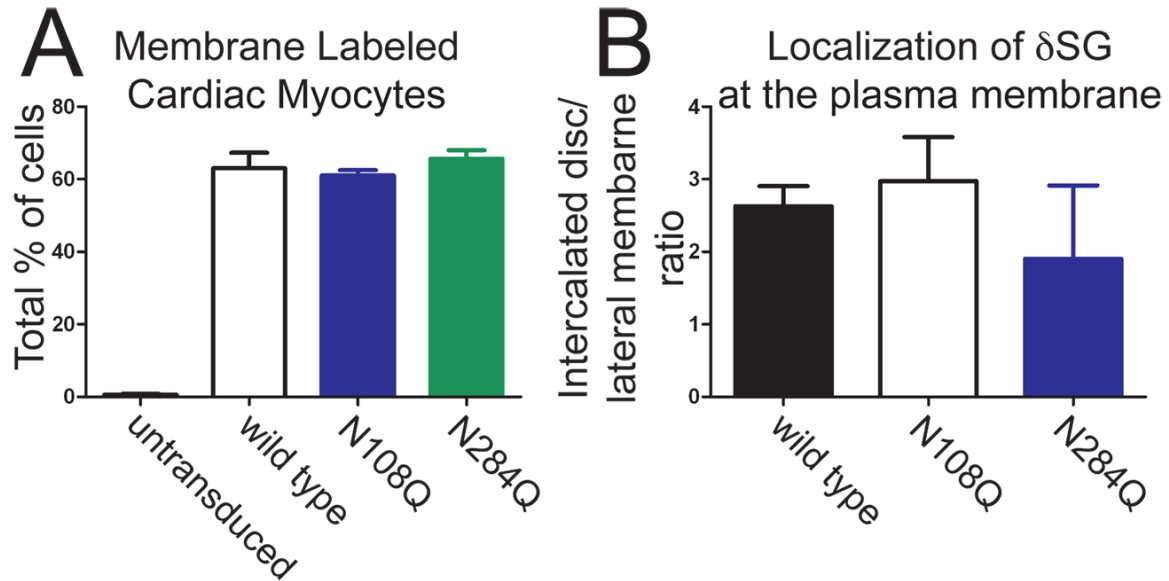


Figure 3-5. Loss of N-linked glycosylation does not alter delta sarcoglycan membrane localization or enrichment at the intercalated discs. (A) Scoring of delta sarcoglycan immunofluorescence at the plasma membrane. Glycosylation deficient mutants are labeled at the plasma membrane at comparable levels to wild type δ SG (n=4 rats). **(B)** Protein localization at the plasma membrane. Delta sarcoglycan predominantly localizes to the intercalated discs and loss of glycosylation at residue 108 or 284 does not alter this natural enrichment. Values are represented as mean \pm S.E., n= 4 myocytes P<0.05.

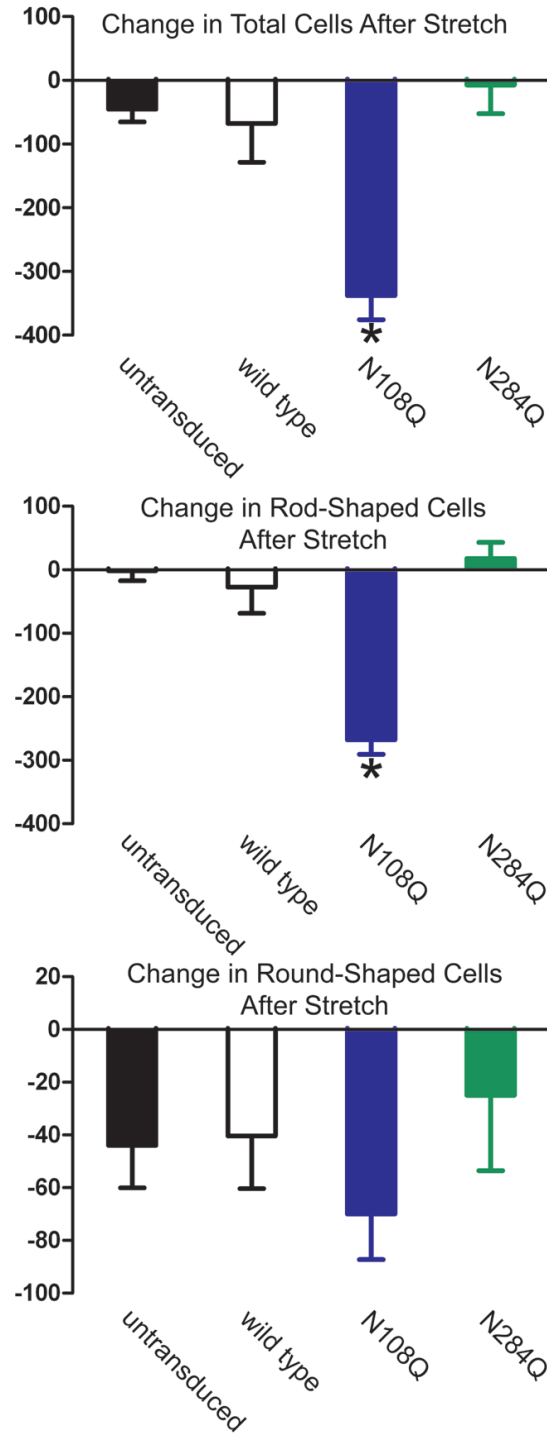


Figure 3-6. Loss of N-linked glycosylation on delta sarcoglycan residue 108 causes myocyte detachment from laminin after mechanical strain. Cardiac myocytes adhered before and after stretch. **(top)** The difference in total cells (rods and rounds). **(center)** The difference in rod-shaped cells. **(bottom)** The difference in round-shaped cells. Change is calculated as the difference in cells counted after stretch and cells counted without stretch (stretch – no stretch = change). Values are represented as mean \pm S.E. P<0.05. n=3 rats

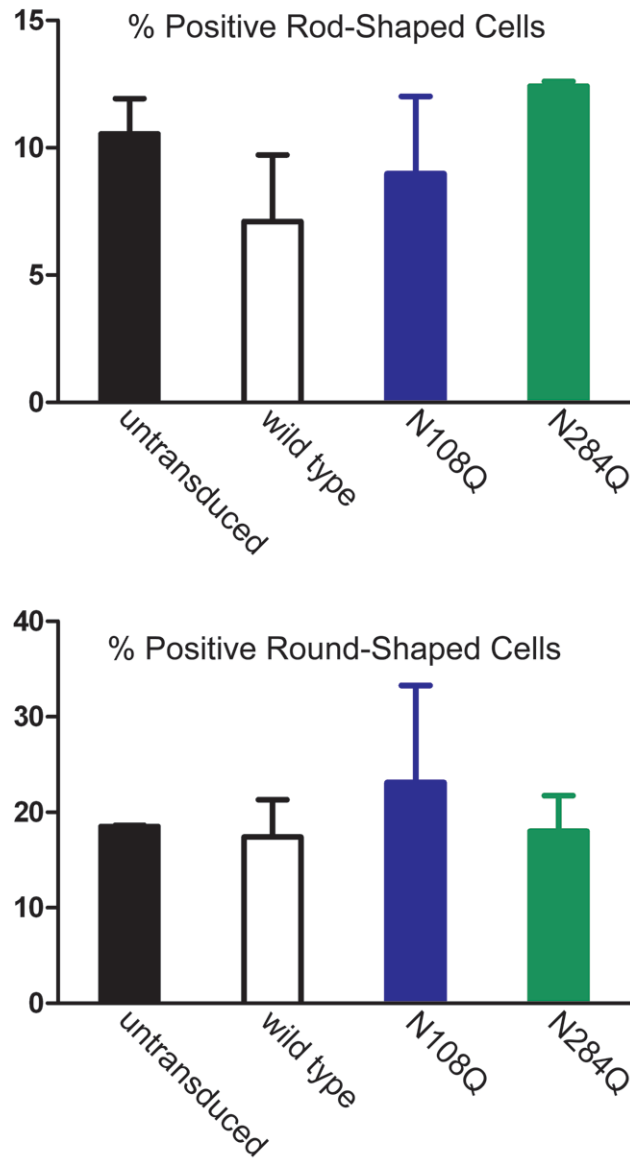


Figure 3-7. Expression of glycosylation deficient delta sarcoglycan mutant N108Q or N284Q in cardiac myocytes does not affect sarcolemmal stability. Fluorescently labeled cardiac myocytes after mechanical stretch. (**top**) The percentage of positively labeled rod-shaped cells. (**bottom**) The percentage of positively labeled round-shaped cells. Values are presented as a percentage of the total cell population and represented as mean \pm S.E. $P < 0.05$. $n = 3$

Chapter 4

Conclusions and Future Directions

Summary of Thesis Work

Mutations within the genes encoding protein members of the DGC not only cause devastating skeletal muscle dystrophy, but also frequently lead to development of fatal cardiomyopathy^{37, 38, 40, 73, 74, 156}. The presentation and development of cardiomyopathy in dystrophy-associated mutations of the DGC are dependent upon the member of the DGC affected (for reference see Table 1.1). DGC mutations may cause functional deficits and development of cardiomyopathy through disruption of either of two distinct connections. The first obligate connection is within the intracellular space where dystrophin functionally links the DGC to the cytoskeleton via F-actin²⁶⁻²⁸. The second obligate connection is on the extracellular face where glycosylated α DG binds to the basal lamina via laminin¹²⁻¹⁶. Defects within the DGC disrupting either or both of these links cause sarcolemmal instability allowing large extracellular molecules and ions such as Ca^{2+} to pass across the sarcolemma and into the cell when myocytes are mechanically strained^{63, 65-68} (Figure 1). The unregulated passage of extracellular ions likely causes a host of cellular dysfunctions especially due to the many signaling mechanisms involving Ca^{2+} . Although glycosylated α DG and dystrophin are two key members of the DGC establishing these connections mutations in additional members of the DGC, notably the sarcoglycans, cause limb-girdle muscular dystrophy with concomitant sarcolemmal instability⁶⁵.

Mutations within the extracellular domain of the δ SG protein have also been linked to development of dilated cardiomyopathy^{157, 158}. This suggests that δ SG function is critical to DGC function beyond simply assembly of the overall complex. Mutations in the extracellular domain of δ SG may affect one or both of the two obligate connections of the DGC stabilizing the sarcolemma in cardiac tissue. The critical nature of the δ SG protein and its post-translational modifications in maintaining DGC stability was the basis for the experiments performed in chapter 2 and 3. These two chapters explored the physiological outcome when δ SG mutants were expressed in isolated cardiac myocytes. It is not entirely clear in cardiac muscle whether the sarcolemma is simply more permeable when the DGC is disrupted or whether mechanical action is what causes membrane permeability. Overall, the experiments performed in this thesis were designed to determine the mechanism by which mutations in the protein δ SG exert a dominant negative effect on cardiac myocyte function possibly leading to dilated cardiomyopathy. The experiments described in chapters 2 and 3 and summarized hereafter support the overall hypothesis that dominant mutations in the extracellular domain of delta sarcoglycan alter protein structure or post-translational modifications directly causing DGC dysfunction and leading to disrupted mechanical stability of cardiac myocytes.

Chapter 2 described the functional and biochemical consequences of expressing DCM-linked δ SG mutations in cardiac myocytes. Expression of mutant δ SG R97Q or R71T is sufficient to cause sarcolemma instability during mechanical strain. Although previous work has suggested that DCM-linked δ SG

mutants may primarily owe disease pathology to trafficking defects¹⁵⁹, δ SG mutant R97Q and R71T both localize and are stable at the sarcolemma. This suggests that, unlike LGMD2F causing recessive mutations, δ SG mutants R97Q and R71T exert dominant negative effects on overall DGC function. Interestingly, when protein lysates from cardiac myocytes expressing δ SG mutant R71T were run on western blot it revealed a strikingly different pattern of post-translational modification compared to wild type δ SG. Treatments using endoglycosidases reveal the additional bands from mutant R71T to be alternatively glycosylated forms of δ SG. The change in amino acid at residue 71 from an asparagine to a threonine forms a new consensus site for N-linked glycosylation. Whether the ectopic glycosylation at residue 71 is responsible for sarcolemma instability is unclear and led to investigation of the role glycan residues on δ SG may play in δ SG and overall DGC function.

To further investigate the role of glycan residues on δ SG in protein function we generated adenovirus expressing mutants lacking the putative sites of N-linked glycosylation in δ SG. Chapter 3 described the functional consequences of disrupting N-linked glycosylation on δ SG. In ventricular muscle δ SG appears to be N-glycosylated at only two of the putative sites in the extracellular domain; asparagine residues 108 and 284. Mutating δ SG to ablate N-glycosylation at either of these two residues does not impact δ SG protein localization and integration into the plasma membrane. Additionally expression of glycosylated alpha dystroglycan, beta dystroglycan, and beta sarcoglycan is unchanged when expressing N-glycan mutant δ SG. These results indicate that

N-glycosylation of δ SG is not responsible for protein trafficking through the secretory pathway in cardiac myocytes or assembly of the sarcoglycan complex as previously hypothesized from ectopic expression in non-muscle cell types. Exposing cardiac myocytes expressing N-glycan mutant δ SG to mechanical stretch revealed no obvious sarcolemma instability. This was contrary to chapter 2 data showing that aberrantly glycosylated δ SG mutant R71T expression is sufficient to cause plasma membrane instability during mechanical strain. Interestingly, cardiac myocytes expressing δ SG lacking glycosylation at glutamine residue 108 have increased detachment from laminin after mechanical stretch. Our results indicate that post-translational glycosylation on δ SG may be necessary for DGC function at the plasma membrane during mechanical events in a way that more dramatically impacts overall cell adhesion. Importantly these results are the first to suggest that δ SG may have a functional role in matrix adhesion during mechanical strain.

The most likely explanation for the discrepancy between phenotypes concerning ectopic glycosylation caused by mutant R71T and glycosylation deficiency caused by mutant N108Q relates to the relative location of each mutation. In COS-1 cells the residues from 57-92 are necessary for association and binding with β SG. Mutation of the arginine to threonine at residue 71 and subsequent ectopic glycosylation at residue 69 probably disturbs this interaction leading to disruption of sarcoglycan complex function. N-linked glycans that are sialylated also have a negative charge and it's also possible that electrostatic interactions of the glycan near the membrane somehow impacts delta

sarcoglycan protein structure, nearby membrane proteins or the membrane itself. Conversely the loss of glycosylation at residue 108 falls outside of the domain proposed for interaction with β SG and may be exposed more directly to the extracellular matrix. This may explain why the N108Q δ SG mutation affects myocardial cell adhesion in isolated cardiac myocytes.

It is difficult to infer much more from the spatial location of each mutation within δ SG. The reason for this is that a number of disease causing polymorphisms within δ SG are spatially in close proximity to one another and yet can present with vastly different phenotypes, some with muscular dystrophy and some with dilated cardiomyopathy (for reference see figure 1-5). For example, the R71T mutation discovered by Tsubata et al. and examined in chapter 2 lies within the β SG interaction domain of δ SG¹¹⁰ and is linked to DCM¹⁵⁷. The LGMD2F causing G76C mutation¹¹² resides only 5 residues from the R71T mutation and is also within the domain necessary for interaction with β SG. These two mutations are extremely close to each other and both reside in the same domain necessary for a specific protein interaction and yet cause two distinct phenotypes. One appears to result in a null protein mutation (G76C), and based on my thesis data R71T would be predicted to have normal protein expression in patients but have dominant negative effects on δ SG function. Furthermore, the domain between residues 93 and 262 contains 6 verified mutations causing either DCM or LGMD2F. This domain has not been implicated in any specific protein-protein interactions of δ SG protein but is apparently essential for some aspects of protein function. It is possible that the domain from residues 93-262 is

responsible for interaction with one of the other sarcoglycans, dystroglycans, or other extracellular matrix ligands which could help explain why mutations in that region are pathogenic and cause DGC dysfunction but does not elucidate why mutations in this domain can cause disparate phenotypes.

One possible explanation for phenotypic dissimilarity of mutations in δ SG relates to truncation of the protein as opposed to point mutants. The cysteine-rich motif in δ SG has been implicated in proper localization to the plasma membrane of the sarcoglycan complex¹¹⁰. Two of the five known mutations causing LGMD2F are caused by truncation of δ SG prior to the cysteine-rich motif and furthermore one other LGMD2F causing mutation is located immediately proximal to this motif. This suggests that disruption of the cysteine-rich motif may be a contributing factor to complete loss of protein stability, resulting in a null mutation, loss of delta sarcoglycan expression and development of LGMD2F as opposed to only DCM.

Delta sarcoglycan is not the only protein within the DGC that exhibits dissimilarity in phenotypes as a consequence of disease causing polymorphisms. Although Becker Muscular Dystrophy (BMD) is caused by mutations in the same dystrophin gene that causes Duchenne muscular dystrophy, patients with BMD have a remarkably heterogeneous presentation. Skeletal myopathy in BMD is normally not as severe as that observed in DMD^{206, 207}. However, despite mild or no skeletal muscle weakness patients can present with severe cardiomyopathy^{208, 209}.

It is also possible that the disparate phenotypic outcomes of different mutations within δ SG relate more to differences in the physiology of cardiac muscle and skeletal muscle. Skeletal muscle is typically only recruited and activated in situations where it is needed to produce force. Skeletal muscles that are more active such as the muscles of the shoulders and those muscles used during ambulation in the hips and torso are far more affected by limb-girdle muscular dystrophy. In general in DMD/BMD patients and patients with other forms of muscular dystrophy such as sarcoglycanopathy or dystroglycanopathy, cardiac disease is more slowly progressive than skeletal disease. However, cardiac disease becomes highly penetrant in older individuals even in the presence of very mild skeletal muscle dysfunction or even as the first sign of disease individuals. In many cases heart disease can deteriorate to a level requiring cardiac transplant, even though the patient retains a high degree of skeletal muscle function supporting normal ambulation. One possibility is that the DCM linked δ SG mutants R71T, R97Q, S151A, or Δ K238 may have fairly mild dysfunction leading to low or incomplete penetrance of DCM-linked δ SG mutations such that skeletal muscle disease does not reach clinical significance but cardiac disease appears later in the patients lifespan as DCM. This may be similar to disease causing mutations in FKRP (a dystroglycanopathy) that first presented as cardiac disease in human patients with complete absence of skeletal muscle symptoms²¹⁰⁻²¹². Indeed, work by Bauer et al. concluded that the S151A mutation in δ SG previously linked to DCM may be a rare non-disease causing polymorphism¹⁵³, but does not account for the possibility that the

S151A mutation may simply have reduced penetrance with respect to DCM. Also of note is the fact that sub-physiological expression of the sarcoglycans in skeletal muscle from *mdx* mice appears to be mildly compensatory to dystrophic pathology²¹³ suggesting that even low levels of functional sarcoglycans can help prevent development of skeletal muscle myopathy. It is entirely probable that low penetrance of DCM-linked mutations of δ SG coupled with expression of one functional copy of the δ SG protein is sufficient to prevent skeletal muscle disease and may help explain the tissue specific effects of DCM-linked mutations in δ SG

Future Directions

Mechano-Protection of the Sarcolemma by Delta Sarcoglycan in Cardiac Myocytes

In the dystrophin glycoprotein complex the sarcoglycans exist as a tetrameric subcomplex^{37, 40, 41, 88}. Despite identification of the sarcoglycans in striated muscle almost 20 years ago^{37, 40, 41, 102, 105} very little is known about their function. The DGC performs the critical function at the lateral sarcolemma of mechanical protection during stretch and contraction^{59-63, 66}. When the sarcoglycan complex is lost due to mutation or knockdown the sarcolemma becomes increasingly permeable to extracellular molecules and ions^{91, 97, 141-144}. This originally led to the hypothesis that the sarcoglycan complex functionally stabilizes the DGC at the plasma membrane. The identification of DCM-linked mutations in δ SG that do not cause apparent skeletal myopathy and do not result in complete loss of the sarcoglycan at the plasma membrane were some of the

first evidence to suggest that δ SG may have a functional role outside of DGC assembly and/or stabilization¹⁵⁷. The mechanism of these DCM-linked δ SG mutations was unclear but subsequent study using transgenic mice overexpressing the S151A δ SG mutation showed a trafficking defect concomitant with a loss of the sarcoglycan complex at the plasma membrane and disruption of nuclear membrane proteins¹⁵⁹. The results in chapter 2 challenge this conclusion regarding the mechanism of action for DC mutants. There are three important limitations to the conclusions in chapter 2. 1) Identifying mutant δ SG R97Q and R71T interactions with other sarcoglycans or members of the DGC proved difficult due to the limited amount of protein produced in the isolated cardiac myocyte model, 2) isolated cardiac myocytes are only stable in culture for a matter of days disallowing investigation into long-term expression of mutant δ SG R97Q or R71T, and 3) sarcolemmal instability in isolated cardiac myocytes may not necessarily be recapitulated in vivo where cells are in 3-dimensional contact with the matrix. Because δ SG mutant R97Q and R71T do not show a trafficking defect nor subsequent disruption of nuclear proteins similar to S151A it is reasonable to hypothesize that production of transgenic mice expressing these two mutants would not have the high lethality observed in transgenic mice overexpressing δ SG mutant S151A.

Transgenic mice expressing δ SG mutant R97Q and R71T would overcome the limitations of protein quantity and allow investigation into chronic expression of mutant δ SG proteins in vivo. Co-immunoprecipitation experiments using pulldown of myc-tagged transgenic proteins would allow probing for

sarcoglycans, dystrophin, and dystroglycans proving finally that mutant δ SG R97Q and R71T not only localize and insert into the plasma membrane but also associate and integrate tightly with the DGC. It would be predicted that δ SG mutants fully integrate into the DGC in vivo at the sarcolemma and pull down the remaining members of the DGC.

Transgenic mice would also allow evaluation of sarcolemmal integrity in vivo by injecting mice with Evan's blue dye and stimulating the heart either pharmacologically with isoproterenol or via exercise using a treadmill. Given the sarcolemmal instability observed in mutant δ SG expressing cardiac myocytes outlined in chapter 2 it should follow that sarcolemmal integrity is compromised in vivo causing an increase in Evan's blue dye uptake that is likely exacerbated by cardiac stimulation. It should be noted that knock-in mice could be used rather than transgenic mice. Although generation of knock-in mice is significantly more time consuming and technically difficult a heterozygous knockin mouse for either R71T or R97Q would be the most accurate genetic representation of patients with δ SG linked DCM. There is also an alternative to generating transgenic or knock-in mice if given a more limited timeframe. Mutant δ SG R97Q or R71T could be engineered into AAV and injected into δ SG knockout mice or the BIO 14.6 hamster as previously performed by Holt et al²¹⁴. AAV serotype 9 would be selected for its superior global cardiac gene transfer in rodents²¹⁵.

There has been some evidence that calls into question whether dominantly inherited δ SG dilated cardiomyopathy actually exists¹⁵³. A study of a large consanguineous family containing both an A131P δ SG mutation causing

LGMD2F and DCM-linked S151A δ SG mutation showed no signs of development of cardiomyopathy even in the presence of heterozygous LGMD2F causing mutations. The study concluded that the S151A mutation doesn't actually cause dominantly inherited DCM and questioned the role of other dominantly inherited δ SG mutations in DCM pathology. There are crucial experiments that may silence lingering criticism about whether δ SG mutants R97Q or R71T cause DCM despite the conclusions by Bauer et al. The δ SG knockout mouse is a well-characterized model of human LGMD2F. Hearts could be isolated from δ SG knockout mice and transduced using adenoviral vectors expressing δ SG mutant R97Q or R71T. Sarcolemmal integrity could easily be tested using modified stretch protocols similar to chapters 2 and 3. Delta sarcoglycan knockout mice could also be crossed to transgenic or knock-in mice expressing δ SG mutant R97Q or R71T. Expression of mutant δ SG R97Q or R71T in δ SG deficient cardiac tissue would be predicted to not rescue the compromised membrane integrity in δ SG deficient animals.

Despite the presence of sarcolemmal instability in cardiac myocytes expressing mutant δ SG R97Q or R71T and a subsequent contracture of myocytes, the mechanism of this dysfunction is lacking. Previous studies have suggested that perturbation of the link between glycosylated alpha dystroglycan and the extracellular matrix are responsible for plasma membrane fragility¹⁸⁰. Calcium influx through fragile membranes has been hypothesized as the initiating event for contracture and development of a dystrophic phenotype in both skeletal and cardiac muscle. It would further the understanding of dominant negative δ SG

mutations to identify whether the phenotypes of sarcolemmal instability observed in chapter 2 are indeed caused by Ca^{2+} influx.

A previous study showed that inhibiting calcium influx through TRPC3 channels in dystrophic skeletal muscle dramatically improved disease pathology²¹⁶. One of the models in this study was a cross of the δ SG knockout mouse to a transgenic mouse overexpressing a dominant negative TRPC3 channel inhibitor using the human skeletal actin promoter. An adenovirus expressing the same dominant negative TRPC6 (dnTRPC6) using the cytomegalovirus promoter could easily be generated. Isolated cardiac myocytes from transgenic mice expressing δ SG mutant R97Q or R71T transduced with adenovirus expressing dnTRPC6 allows investigation into the role of Ca^{2+} influx as an initiating event for cell contracture in vitro. For in vivo studies AAV9 expressing dnTRPC6 could be injected into transgenic mice pups expressing R97Q and R71T and hearts from these mice could be examined for dystrophic pathology and Evan's blue dye uptake.

Identifying how δ SG mutants R97Q or R71T cause DGC dysfunction and ultimately dystrophic cardiomyopathy would further understanding of the mechanism of DGC disruption causing sarcolemmal instability. Previous studies have shown the N-terminal domain of alpha dystroglycan is cleaved and shed into human serum implicating this as a potential contributing factor to DGC dysfunction due to reduction of alpha dystroglycan at the plasma membrane acting as a high affinity laminin receptor²¹⁷. Collecting serum from transgenic or knockin mice expressing δ SG mutant R97Q or R71T and probing for the N-

terminal of alpha dystroglycan could reveal whether the mechanism of sarcolemmal fragility is via a secondary alpha dystroglycanopathy. Shedding of alpha dystroglycan into the serum of R97Q or R71T transgenic or knockin mice would support the hypothesis that the sarcoglycan complex has a role in stabilizing the dystroglycans. Some studies have also identified beta dystroglycan cleavage via a matrix metalloprotease (MMP)^{135, 136}. This has been proposed as a mechanism for DGC destabilization due to the fact that beta dystroglycan cleavage occurs in dystrophies where the sarcoglycan complex is absent. However, because mutant δ SG R97Q and R71T are predicted to integrate and form a fully mature sarcoglycan complex it would not be hypothesized that beta dystroglycan would undergo proteolytic cleavage by this MMP. If it were established in transgenic mice expressing mutant δ SG R97Q or R71T that sarcoglycan trafficking and assembly is unperturbed yet beta dystroglycan still undergoes proteolytic cleavage it can be assumed that the sarcoglycan complex is not responsible for spatially inhibiting access to the cleavage site on beta dystroglycan, but is instead involved in some other mechanism of proteolytic cleavage inhibition.

Delta Sarcoglycan function in Extracellular Matrix Adhesion

The data presented in chapter 3 suggests that in addition to having a role in mechano-protection δ SG may also play a role in adhesion to the extracellular matrix. These are not the first results to implicate the sarcoglycans in cell matrix adhesion^{218, 219}. Previous work by Yoshida et al discovered that pulldown of

alpha sarcoglycan or dystrophin in L6 myotubes includes co-immunoprecipitation of $\alpha_5\beta_1$ integrin and focal adhesion proteins vinculin, talin, and paxillin. Integrin α_5 is a receptor for fibronectin and is upregulated in dystrophic muscle. Anti-sense oligomers directed against alpha and gamma sarcoglycan cause reduction in focal adhesion proteins and a significant reduction in cell adhesion. Additionally, adhesion of L6 myotubes to collagen or fibronectin results in tyrosine phosphorylation on alpha and gamma sarcoglycan. The authors concluded that there exists a system of bidirectional signaling between the sarcoglycan complex and the integrins during cell adhesion. Notably, the authors did not examine δ SG using their model. Due to the necessity of the sarcoglycan complex to be expressed as a unit it is likely that pulldown of δ SG would also co-immunoprecipitate $\alpha_5\beta_1$ integrin and focal adhesion proteins.

Delta sarcoglycan mutant N108Q described in chapter 3 revealed that glycosylation on residue 108 appears to play a role in myocyte adhesion to laminin. These results are distinct from hypoglycosylated alpha dystroglycan cells that show no apparent defect in cell adhesion¹⁸⁰. Two critical experiments can be performed to determine if ablation of N-glycosylation at residue 108 in δ SG affects the $\alpha_5\beta_1$ integrin adhesion system. The first is using fibronectin as a matrix substrate for culturing and stretching cardiac myocytes. Fibronectin is bound by α_5 integrins but not alpha dystroglycan so if the contribution of δ SG to ECM adhesion is not laminin specific it would be expected that we would obtain similar results using fibronectin as the results reported in chapter 3. The second crucial experiment is pulling down wild type and mutant δ SG N108Q and

examining for co-immunoprecipitation with $\alpha_5\beta_1$ integrin and other focal adhesion proteins. It would be expected that if myocyte adhesion is reduced through the $\alpha_5\beta_1$ integrin system that pulldown of glycan deficient δ SG mutant N108Q would show reduced or no co-immunoprecipitation with focal adhesion proteins compared to wild type δ SG. It should be noted that attempting to perform this experiment in isolated cardiac myocytes could prove difficult due to limitations in total protein making co-immunoprecipitation experiments troublesome. To overcome this it would be necessary to use the adenoviral vectors described in chapter 3 in a different cellular model such as COS or CHO cells. Neither of these cell lines model cardiac tissue however, so it would be more relevant to use either HL-1 cells or a transgenic mouse expressing glycan mutant δ SG N108Q.

In addition to bidirectional signaling between the integrins and sarcoglycans it has been suggested that upregulation of $\alpha_7\beta_1$ integrin can compensate for loss of the sarcoglycan complex in dystrophic muscle. This is supported by data showing upregulation of α_7 integrin in the muscle of δ SG and γ SG knockout mice²¹⁸. Additionally, mice knocked out for both α_7 integrin and gamma sarcoglycan showed marked increase in dystrophy and a subsequent decrease in sarcolemmal stability²¹⁸. A separate study identified that transgenic overexpression of α_7 integrin reduces cardiomyopathy in dystrophic mice²²⁰. Together these data suggest that the integrin system of cell adhesion works in parallel with the DGC to maintain sarcolemmal integrity and cell adhesion to the extracellular matrix. The data in chapter 3 suggesting myocyte adhesion to

laminin is decreased in cardiac myocytes expressing δ SG mutant N108Q does not address whether this adhesion defect is mediated through dystroglycan or the integrins. Our lab has generated a transgenic mouse overexpressing the glycosyltransferase LARGE driven by the MCK promoter. This results in hyperglycosylation of alpha dystroglycan and increased laminin binding. Cardiac myocytes isolated from this transgenic model and transduced using the adenoviral vectors described in chapter 3 can be stretched and observed for myocyte detachment. If decreased cell adhesion from expression of N-glycan mutant δ SG is mediated through the integrins it would be predicted that overexpression of LARGE would partially or fully rescue this phenotype. Conversely, if rescue does not occur we can perform the same test in transgenic mice overexpressing α_7 integrin²²⁰. If decreased cell adhesion from expression of δ SG mutant N108Q is mediated through the DGC it would be predicted that overexpression of α_7 integrin would partially or fully rescue this phenotype.

Data in chapter 3 reveals an adhesion defect but it lacks an explanation. Glycosylation residues on delta sarcoglycan may destabilize the sarcoglycan complex resulting in dissociation from the sarcolemma. To further investigate the role of δ SG in myocyte attachment to laminin through alpha dystroglycan it would be useful to examine protein-protein interactions within the DGC that may be disturbed when δ SG mutant N108Q is expressed. Previous studies have performed co-immunoprecipitation using increasingly stringent conditions to identify which interactions are the weakest among the sarcoglycans and dystroglycans^{128, 221}. These experiments can be performed in transgenic mice

overexpressing δ SG N108Q to identify whether loss of glycosylation at δ SG residue 108 causes decreased interaction of δ SG with any of the other members of the DGC.

As described in the previous section cleavage of alpha dystroglycan or beta dystroglycan has been proposed as a mechanism for DGC dysfunction. The decrease in adhesion when δ SG N108Q is expressed may be due to an increased cleavage of either of these two proteins. Collecting the media from stretched cardiac myocytes expressing δ SG N108Q and analyzing for either alpha dystroglycan or beta dystroglycan released into the media would help explain decreased adhesion to laminin after mechanical strain especially if this phenotype is mediated via DGC rather than integrin adhesion to laminin.

Conclusions

The results described in this dissertation reveal that δ SG contributes directly to sarcolemmal stability during mechanical events. Importantly this work demonstrates the DCM-linked mutations in δ SG have a dominant negative effect on overall DGC function. Importantly it also demonstrates that the N-glycan at residue 108 in δ SG contributes to protein function in a manner that appears distinct from δ SG DCM mutations and other DGC mutations. These are the first data to reveal a functional role for N-linked glycosylation of δ SG beyond simple sarcoglycan assembly or expression. Additionally these data reveal a functional role for δ SG in cell adhesion that has not previously been proposed.

Prior to this study investigation into the functional role of δ SG has proved difficult due to the fact that many mutations in δ SG behave as nulls and destabilize the entire sarcoglycan complex. With the exception of a few signaling mechanisms individual function of any sarcoglycan has not been identified. Given the nature of myocyte detachment from laminin in cardiac myocytes expressing δ SG mutant N108Q it is intriguing to propose that δ SG may bind directly to laminin or other matrix ligands. Mutations in δ SG that alter its extracellular domain structure or post-translational modification likely exert their effects through dystroglycans but may also influence other matrix receptors or directly contribute to matrix binding. Elucidating these alternative mechanisms may reveal important new functions for δ SG that are DGC independent and these new mechanisms may be important for therapeutic intervention. In the future treatments targeted to rescuing or enhancing δ SG function at the sarcolemma in cardiac tissue may help prevent the gradual development of dilated cardiomyopathy.

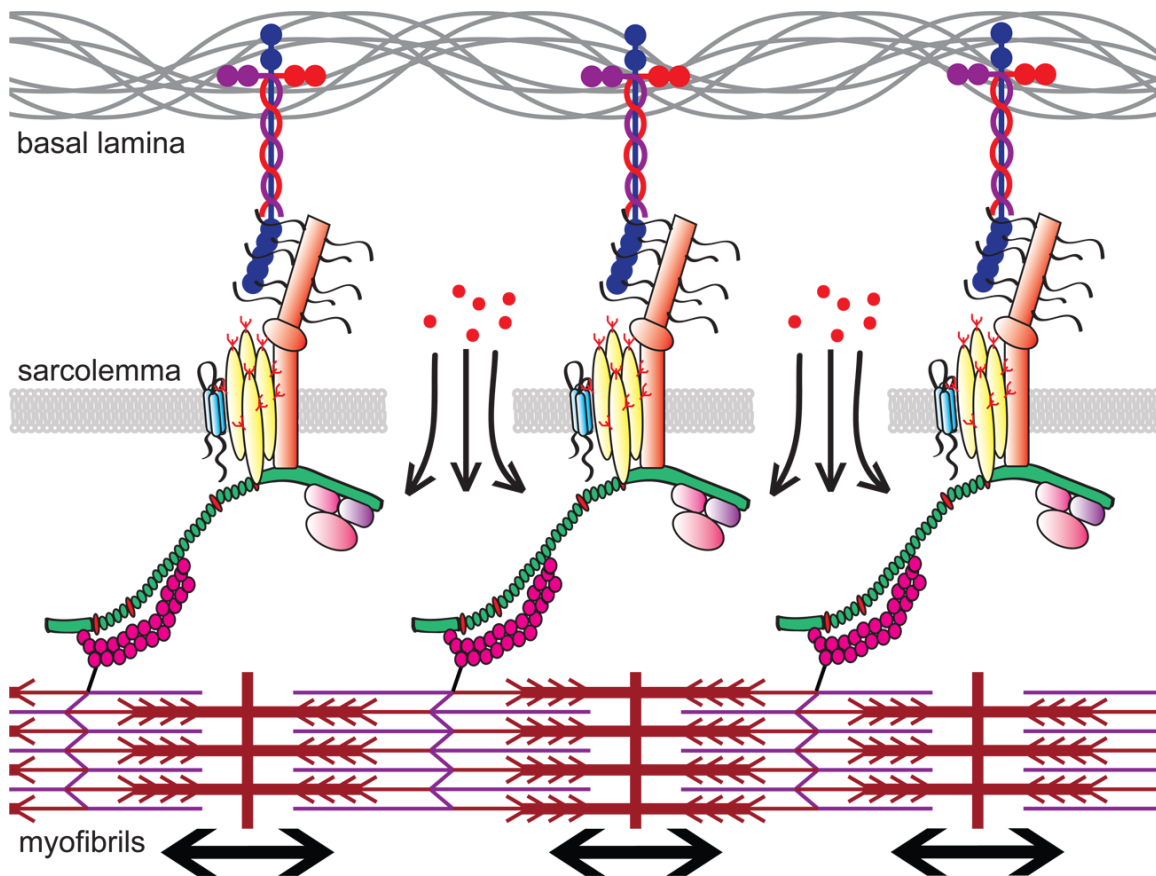


Figure 4-1. Sarcolemma integrity is disrupted in mutations of the DGC proteins and exacerbated by mechanical strain. The DGC forms a functional linkage between the cytoskeleton in striated muscle and basal lamina. Disruption of the DGC causes micro-tears in the sarcolemma during mechanical cycles of stretch or contraction allowing unregulated passage of extracellular molecules and ions into the cytosol.

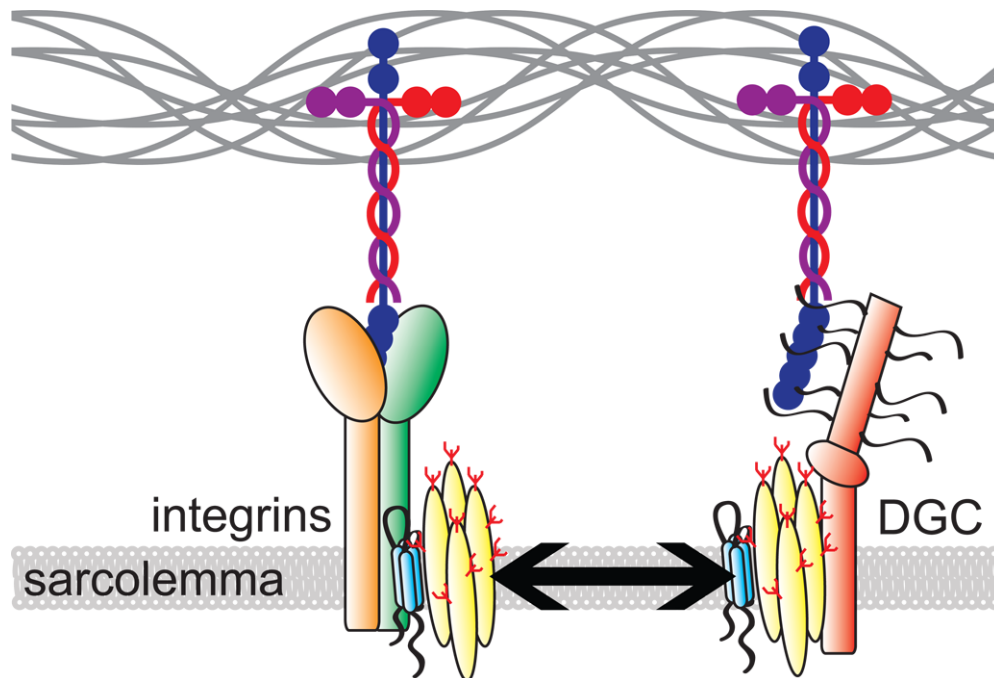


Figure 4-2. Delta sarcoglycan may mediate cell adhesion to laminin through the integrins or DGC. Bidirectional signaling and association with the integrins suggests that the sarcoglycan complex supports cell adhesion to laminin through multiple systems. Glycosylation on residue 108 in delta sarcoglycan may confer a specific protein interaction aiding one or both of these systems during mechanical strain.

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