Overexpression of γ-Sarcoglycan Induces Severe Muscular Dystrophy

IMPLICATIONS FOR THE REGULATION OF SARCOGLYCAN ASSEMBLY

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Xiaolei Zhu‡‡, Michele Hadhazy‡, Margaret E. Groh‡, Matthew T. Wheeler‡, Robert Wollmann‡ and Elizabeth M. McNally‡‡‡
From the ‡Department of Medicine, Section of Cardiology, and Department of Human Genetics, ¶Department of Molecular Genetics and Cell Biology, and ¶Department of Pathology, The University of Chicago, Chicago, Illinois 60637

The sarcoglycan complex is found normally at the plasma membrane of muscle. Disruption of the sarcoglycan complex, through primary gene mutations in dystrophin or sarcoglycan subunits, produces membrane instability and muscular dystrophy. Restoration of the sarcoglycan complex at the plasma membrane requires reintroduction of the mutant sarcoglycan subunit in a manner that will permit normal assembly of the entire sarcoglycan complex. To study sarcoglycan gene replacement, we introduced transgenes expressing murine γ-sarcoglycan into muscle of normal mice. Mice expressing high levels of γ-sarcoglycan, under the control of the muscle-specific creatine kinase promoter, developed a severe muscular dystrophy with greatly reduced muscle mass and early lethality. Marked γ-sarcoglycan overexpression produced cytoplasmic aggregates that interfered with normal membrane targeting of γ-sarcoglycan. Overexpression of γ-sarcoglycan lead to the up-regulation of α- and β-sarcoglycan. These data suggest that increased γ-sarcoglycan and/or mislocalization of γ-sarcoglycan to the cytoplasm is sufficient to induce muscle damage and provides a new model of muscular dystrophy that highlights the importance of this protein in the assembly, function, and downstream signaling of the sarcoglycan complex. Most importantly, gene dosage and promoter strength should be given serious consideration in replacement gene therapy to ensure safety in human clinical trials.

Muscular dystrophy is a genetically heterogeneous disease, but loss of function mutations in the genes encoding the dystrophin-glycoprotein complex (DGC) contribute significantly to the muscular dystrophy phenotype. Therapy aimed at restoring the missing proteins in muscle has been initiated in humans with these disorders (1). Sarcoglycan is a subcomplex within the DGC, and mutations in α-, β-, γ-, and δ-sarcoglycan genes have been described (2). Most sarcoglycan gene mutations destabilize the entire sarcoglycan complex at the plasma membrane of muscle myofibers and cardiomyocytes (3–7). Expression of dystrophin and sarcoglycans seems to be tightly regulated. Primary mutations in dystrophin lead to drastic reduction of dystrophin-associated proteins including sarcoglycans (8–10).

Replacement of missing DGC components can be achieved with transgenesis or viral-based approaches to evaluate the assembly and function of the DGC. Transgene-mediated expression of dystrophin in the mdx mouse, a mouse model for Duchenne muscular dystrophy (DMD), eliminates dystrophic symptoms (11), and transgenic rescue of dystrophin expression has been used to identify structure-function relationships within the DGC. In these studies, expression or overexpression of dystrophin from the tissue-specific muscle creatine kinase (MCK) promoter was not associated with toxicity (11). However, less is known about sarcoglycan replacement, and unlike dystrophin, the sarcoglycans are transmembrane proteins that assemble within the secretory pathway. Restoration of proper targeting and assembly of the sarcoglycans may involve additional regulatory aspects than those required for expression of cytoplasmic proteins.

Adeno- and adeno-associated viruses carrying normal genes have been successfully used to rescue skeletal muscle defects in mice lacking dystrophin or sarcoglycans (12–15). These studies describe the efficiency of protein expression as well as the immune response generated by the introduction of the transfer viruses and the vectors carrying the gene. The dose response of treatment, including studies of the minimally effective dose and the potential for toxicity related to overexpression, has not yet been explored fully. With the initiation of phase I clinical trials using gene therapy for limb girdle muscular dystrophies (1), it is extremely important to determine the risks and benefits of this potentially very powerful approach.

In this study, we generated transgenic mice expressing different levels of γ-sarcoglycan under the control of the MCK promoter. We found that mice carrying a high copy number of the γ-sarcoglycan gene, concomitant with marked overexpression of γ-sarcoglycan protein, showed profound muscular dystrophy as revealed by extreme muscle wasting and premature death. Histopathology showed muscular dystrophy with variable fiber size, centrally placed nuclei, and increased fibrosis. In addition, fast fibers seem to be preferentially affected. In muscle overexpressing γ-sarcoglycan, we found that γ-sarcoglycan failed to reach the cell membrane and was associated with intracellular aggregates. The remaining sarcoglycan subunits were targeted to the sarcolemma; however, α- and β-sarcoglycans were significantly up-regulated at the muscle membrane. These findings suggest that overexpression of γ-sarcoglycan
and misregulation of α- and β-sarcoglycan can cause muscular dystrophy.

**EXPERIMENTAL PROCEDURES**

*Generation of MCKgsg Transgenic Mice—*Full-length mouse γ-sarcoglycan (nucleotides 152–1027 of GenBank™ accession number AF282801) was obtained using mouse skeletal muscle cDNA as template and amplified with the following primer pairs: gsgF, 5′-AGAAAGCTTGCATGTGGAGCAAGCTAC-3′; gsgR, 5′-TAAGATC- CTCAAAGACAGCGTGCTG-3′. The polymerase chain reaction product was digested with HinIII and BamHI. The digested polymerase chain reaction product was then ligated to the pBluescript II KS (Stratagene, La Jolla, CA) that was treated with HinIII and BamHI. Polyadenylation and termination signals of bovine growth hormone from pcDNA3 (Invitrogen, Carlsbad, CA) were added at the XhoI site. Muscle-specific kinase promoter was amplified with the following primer pairs: MCK-P, 5′-ATCTGAGCCAGCGTGAAGCAAG- GTCT-3′ and MCK-R, 5′-ATAAGCTTGGGGACGCCCCTGTTGCCCC-3′ (16). The resultant 1.35-kb fragment was inserted into the XhoI and HindIII sites. Sequences were verified using cycle sequencing. After digestion with BssHI, the prokaryotic vector was separated from the transgene by sucrose gradient (17). Transgenic mice were generated by microinjection of transgene DNA into the pronucleus of fertilized single-cell BL6/DBA embryos as previously described (18, 19).

*Southern, Northern, and Immunoblot Analysis—*The MCKgsg transgene was detected in transgenic mice by Southern blot analysis of tail DNA with α-32P-labeled DNA probe containing the full-length γ-sarcoglycan coding region (nucleotides 152–1027 of GenBank™ accession number AF282801). genomic DNA was digested with BamHI and HindIII. Signal strength was quantified using ImageQuant software and using PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Fluorescein isothiocyanate- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used. Counterstaining with 4,6-diamidino-2-phenylindole (DAPI) was included in the staining analysis except polyclonal anti-human dystrophin antibody (NovoCastro Laboratories, Newcastle upon Tyne, UK), polyclonal anti-γ- and -δ-sarcoglycan, (6), polyclonal anti-dystrophin antibody (7), and monoclonal anti-myosin heavy chain antibody (Developmental Studies Hybridoma Bank, University of Iowa, IA). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used.

**RESULTS**

*Generation of Transgenic Mice Overexpressing γ-Sarcoglycan—*The MCKgsg transgene was engineered by placing the full-length mouse γ-sarcoglycan cDNA sequence under the control of the 1.35-kb MCK promoter (16) followed by bovine growth hormone termination and polyadenylation signals (Fig. 1A). To assess the potential toxicity of high level γ-sarcoglycan expression, four lines of transgenic mice were selected for this study. Two lines carried low copy numbers of the transgene, and two lines carried high copy numbers of the transgene. Using a full-length γ-sarcoglycan cDNA sequence as a probe, MCKgsg lines 1 and 3 carried approximately three and four copies, respectively, when compared with the endogenous γ-sarcoglycan gene (Fig. 1B) and are considered low copy transgenic mice. MCKgsg lines 2 and 4, considered as high copy transgenic mice, carried ~57 and 36 copies of the γ-sarcoglycan gene, respectively, relative to the endogenous gene (Fig. 1B, asterisk). Northern blot analysis showed the transgenic γ-sarcoglycan mRNA at the predicted size of 1.3 kb (Fig. 1C, arrow). MCKgsg line 2 (high copy) exhibited more mRNA expression than MCKgsg line 1 (low copy), and both showed considerably more mRNA than the endogenous 1.6-kb γ-sarcoglycan mRNA (Fig. 1C, asterisk). The MCK promoter expressed γ-sarcoglycan mRNA in both cardiac and skeletal muscle, although lower levels of mRNA expression were seen in cardiac muscle (Fig. 1C). Immunoblot analysis showed that γ-sarcoglycan protein expression correlated with gene copy number in that high copy number transgenic lines expressed substantially greater amounts of γ-sarcoglycan protein (Fig. 1D, lanes 6 and 9 for MCKgsg lines 2 and 4, respectively). A serial dilution of protein extract from transgenic muscle showed that MCKgsg lines 2 and 4 expressed a 150- and 200-fold increase in the amount of γ-sarcoglycan protein, respectively, when compared with wild.
Marked Overexpression of  \( \gamma \)-Sarcoglycan Produces Severe and Rapidly Progressive, Lethal Muscular Dystrophy—Of the four independent founder lines and their wild type littermate controls, the two high copy MCKsg transgenic mice were found to be significantly smaller than their wild type littermates and the low copy number MCKsg transgenic lines. A representative comparison of a high copy transgenic (MCKsg line 4) and a normal littermate control mouse is shown in Fig. 2, top panel. Mice from MCKsg line 2 and 4 (high copy) were less active and displayed an abnormal gait that was characterized by widened hind limb spacing. We examined skeletal muscles from MCKsg high copy mice, MCKsg low copy mice, and wild type littermate controls. Hind limb from MCKsg line 4 was visibly dystrophic with marked muscle wasting and gross fibrofatty replacement when compared with wild type mice (Fig. 2, top panel, right side). Muscle mass of individual quadriceps in MCKsg lines 2 and 4 (0.07 and 0.05 g, respectively) was significantly lower when compared with two control littermates (0.16 and 0.14 g). Similar findings were observed in fore limbs of high copy MCKsg lines 2 and 4 (data not shown).

The marked muscle wasting was associated with premature death. Two independent high copy founders from MCKsg line 2 and MCKsg line 4 died at day 45 and day 110, respectively. Given the limited survival of the male founder for MCKsg line 2, we attempted to propagate this line through in vitro fertilization but were unsuccessful. The female founder for MCKsg line 4 reproduced once. The progeny from MCKsg line 4 died prematurely and did not reproduce. Thus, neither high copy line was successfully maintained because of the lethal nature of the overexpression of \( \gamma \)-sarcoglycan. We generated an intermediate high copy number line, MCKsg line 5, with a copy number of \( \sim 29 \). These mice also displayed small size and abnormal gait similar to the MCKsg lines 2 and 4. Propagation of MCKsg line 5 was similarly limited.

Histologic analysis of muscles from high copy transgenic mice (MCKsg line 4) using hematoxylin/eosin and Masson trichrome staining showed severe dystrophic changes, including wide variation in fiber size, an inflammatory infiltrate, increased connective tissue, and adipocyte replacement of myofibers (Fig. 2, A and B). Abundant central nuclei were also evident in the dystrophic muscle (Fig. 2A). Muscle from MCKsg line 3, a low copy number line, appeared normal (Fig. 2, C and D) when compared with wild type control muscle (Fig. 2, E and F). The dystrophic phenotype of muscles from high copy transgenic mice was very similar to that seen in a \( \gamma \)-sarcoglycan-null mutant (7) (Fig. 2, G and H).

Overexpression Inhibits Normal Cellular Targeting of \( \gamma \)-Sarcoglycan to the Plasma Membrane—\( \gamma \)-Sarcoglycan is an integral part of the membrane-associated sarcoglycan complex. Because of the similar histologic appearance of high copy MCKsg transgenic muscle and \( \gamma \)-sarcoglycan-null mutant muscle (7), we examined \( \gamma \)-sarcoglycan localization in gastrocnemius muscle from normal mice and MCKsg line 2. Despite a high level of \( \gamma \)-sarcoglycan protein expression, \( \gamma \)-sarcoglycan protein failed to target appropriately to the cell membrane (Fig. 3A). The majority of immunoreactive \( \gamma \)-sarcoglycan protein was detected as punctate staining throughout the cytoplasm of the myofibers and was excluded from nuclei (arrowhead). Similar findings were observed in gastrocnemius muscle from MCKsg line 4 (data not shown). Muscles from low copy MCKsg transgenic mice were similar to wild type control muscle with normal \( \gamma \)-sarcoglycan localization at the plasma membrane (Fig. 3B). Although a small amount of cytoplasmic \( \gamma \)-sarcoglycan protein was seen, this was not toxic to muscle given the normal histology (Figs. 2C and 3B).

Lack of \( \gamma \)-sarcoglycan expression at the cell membrane and increased intracellular \( \gamma \)-sarcoglycan altered the expression of the remaining sarcoglycans in a manner different from loss of function mutations. The genetic loss of \( \gamma \)-sarcoglycan is accompanied by reduced protein expression at the plasma membrane of the residual sarcoglycans despite their normal mRNA levels (4, 7). Because high level \( \gamma \)-sarcoglycan expression similarly results in loss of \( \gamma \)-sarcoglycan at the cell membrane, we expected that reduced levels of the other sarcoglycan proteins...
Disruption of the DGC is responsible for a number of genetically distinct forms of muscular dystrophy. This includes mutations in the dystrophin gene that cause DMD and mutations in the sarcoglycan genes that cause the limb girdle muscular dystrophies (2, 23). Many of these mutations are thought to produce disruption of the DGC by loss of function or loss of protein expression. Because mice null for sarcoglycan genes have been generated (6, 7) and these mice fully recapitulate the membrane instability and other features of human muscular dystrophy, we generated mice expressing γ-sarcoglycan to study replacement approaches for the sarcoglycan gene products.

For these transgenic studies, we used the MCK promoter to drive high level, striated muscle-specific expression. We selected the MCK promoter because it has been used extensively in transgenic gene replacement studies of dystrophin (11). We found a profound, lethal muscle wasting disorder developed in transgenic mice that had high level overexpression of murine γ-sarcoglycan. These mice typically died within 2–3 months of age, were markedly reduced in size, and displayed limited mobility. In the high level γ-sarcoglycan-overexpressing mice, we noted that fast fibers were preferentially affected. This may be consistent with fiber type specificity of the MCK promoter (22) or may be related to observations made in DMD muscle where fast fibers are more sensitive to damage (21). Lower levels of γ-sarcoglycan expression from the same MCK promoter were not associated with muscle damage. The lower levels of expression seen in the hearts of these transgenic animals resulted in near normal γ-sarcoglycan expression and...
no evidence of toxicity or damage (data not shown) and suggests that lethality associated with γ-sarcoglycan overexpression is related to skeletal muscle toxicity. Because cardiomyopathy typically develops later in mice with sarcoglycan gene mutations (6, 7), the premature death in MCKgs g high copy transgenic mice may have limited our ability to detect cardiomyopathy.

The high level of γ-sarcoglycan expression resulting from the MCKgs g transgenes produced several molecular consequences. Overexpression of γ-sarcoglycan resulted in reduced γ-sarcoglycan at the plasma membrane of myofibers. Loss of γ-sarcoglycan expression at the membrane is found in DMD and sarcoglycan-mediated muscular dystrophies. Indeed, a human muscular dystrophy patient carrying the common Δ521-T founder mutation was described who maintained expression of α-, β-, and δ-sarcoglycan and preferentially displayed reduced γ-sarcoglycan (24). Thus, the loss of γ-sarcoglycan at the plasma membrane of myofibers is likely a contributor to the pathogenesis seen in this overexpression model. A second consequence of γ-sarcoglycan overexpression was the accumulation of γ-sarcoglycan immunoreactive aggregates in the cytoplasm of myofibers. Because the sarcoglycan complex is associated with both mechanical and signaling functions (25), mislocalization of γ-sarcoglycan in the cytoplasm could lead to abnormal signaling or alteration of cytoskeletal elements that interact with the γ-sarcoglycan. In its cytoplasmic domain, γ-sarcoglycan interacts with a muscle-specific form of filamin (γ-filamin or filamin 2), and loss of γ-sarcoglycan produces an increased plasma membrane γ-filamin level (26). Once antibodies that recognize murine γ-filamin are available, it will be interesting to determine whether filamin localization is altered in γ-sarcoglycan-overexpressing mice. A third consequence of marked γ-sarcoglycan expression is the increased expression of α- and β-sarcoglycan. Up-regulation of α-sarcoglycan is possi-

FIG. 4. Expression of DGC proteins in γ-sarcoglycan-overexpressing mice. Quadriceps muscle from MCKgs g line 4 (A, C, E, and G) and normal controls (B, D, F, and H) were stained for components of the DGC. Shown in A and B is staining for α-sarcoglycan. C and D represent β-sarcoglycan staining, δ-Sarcoglycan (E and F) and dystrophin (G and H) are also shown. I, Western blot analysis of α-, β-, and δ-sarcoglycans (α-sg, β-sg, and δ-sg), dystrophin (dyst), and myosin in MCKgs g high copy lines (lines 2 and 4), a low copy line (line 1), and a wild type control (wt). Coomassie blue (CB) staining is shown as a loading control.
dystrophin homolog, also can fully rescue the dystrophic phenotype in the mdx mouse (29). In these studies, the same MCK promoter was used but did not result in myofiber toxicity as seen here for γ-sarcoglycan overexpression. This suggests that the skeletal myocyte has different mechanisms for the maintenance of stoichiometric ratios of the different components of the DGC.

These studies highlight the complexity of simple gene replacement strategies and underscore the importance of animal models for the treatment of human disease. Because viral replacement strategies typically lead to high level and often variable levels of expression throughout the transduced tissue, it is likely that some toxicity is occurring in those myofibers that have been transduced at high levels. Moreover, previous viral replacement studies, although critically important in demonstrating that replacement can be effective, have not explored fully the dose response of treatment that includes studies of the minimally effective dose and the potential for toxicity related to overexpression. Because viral replacement gene therapy trials are being initiated in humans (1), thorough testing in animal models is required to document the full range of benefits and the potential risks and toxicity.

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