

Molecular Recognition by LARGE Is Essential for Expression of Functional Dystroglycan

Motoi Kanagawa,^{1,5} Fumiaki Saito,^{1,5} Stefan Kunz,²
Takako Yoshida-Moriguchi,¹ Rita Barresi,¹
Yvonne M. Kobayashi,¹ John Muschler,³
Jan P. Dumanski,⁴ Daniel E. Michele,¹
Michael B.A. Oldstone,² and Kevin P. Campbell^{1,*}

¹Howard Hughes Medical Institute
Department of Physiology and Biophysics
and Department of Neurology
The University of Iowa Roy J. and Lucille A. Carver
College of Medicine
Iowa City, Iowa 52242

²The Scripps Research Institute
Division of Virology
Department of Neuropharmacology
La Jolla, California 92037

³California Pacific Medical Center
Research Institute
San Francisco, California 94115

⁴Uppsala University
Department of Genetics and Pathology
Uppsala 751 85
Sweden

Summary

Reduced ligand binding activity of α -dystroglycan is associated with muscle and central nervous system pathogenesis in a growing number of muscular dystrophies. Posttranslational processing of α -dystroglycan is generally accepted to be critical for the expression of functional dystroglycan. Here we show that both the N-terminal domain and a portion of the mucin-like domain of α -dystroglycan are essential for high-affinity laminin-receptor function. Posttranslational modification of α -dystroglycan by glycosyltransferase, LARGE, occurs within the mucin-like domain, but the N-terminal domain interacts with LARGE, defining an intracellular enzyme-substrate recognition motif necessary to initiate functional glycosylation. Gene replacement in dystroglycan-deficient muscle demonstrates that the dystroglycan C-terminal domain is sufficient only for dystrophin-glycoprotein complex assembly, but to prevent muscle degeneration the expression of a functional dystroglycan through LARGE recognition and glycosylation is required. Therefore, molecular recognition of dystroglycan by LARGE is a key determinant in the biosynthetic pathway to produce mature and functional dystroglycan.

Introduction

Dystroglycan (DG) is a central component of the dystrophin-glycoprotein complex (DGC). Deficiency or genetic disruption of proteins within the DGC or its extracellular matrix (ECM) ligands leads to muscular dystrophy,

indicating that the integrity of this complex is crucial for muscle cell viability (Cohn and Campbell, 2000; Burton and Davies, 2002; Tomé, 1999). DG is encoded by a single gene and cleaved into α - and β -DG by posttranslational processing (Ibraghimov-Beskrovnaya et al., 1992). α -DG is a highly glycosylated extracellular peripheral membrane protein that binds to several ECM proteins. In turn, the transmembrane protein β -DG anchors α -DG at the extracellular surface of the plasma membrane and the cytoplasmic domain of β -DG interacts with dystrophin, a cytoplasmic protein that binds to the actin cytoskeleton. Thus, DG plays a central role in the DGC to stabilize the plasma membrane by acting as an axis through which the ECM is tightly linked to the cytoskeleton. Furthermore, evidence suggests that the interaction of DG with ECM proteins is crucial for basement membrane assembly (Williamson et al., 1997; Henry and Campbell, 1998) and morphogenesis of certain organs such as kidney, brain, and peripheral nerve (Durbeej et al., 1998; Moore et al., 2002; Saito et al., 2003).

α -DG is comprised of three distinct domains: the N-terminal, the mucin-like, and the C-terminal (Figure 1A). The N- and C-terminal domains are globular and are joined by a central mucin-like domain that is highly glycosylated by O-linked oligosaccharides (Brancaccio et al., 1995, 1997). Sugar chain moieties comprise up to two-thirds of the molecular mass of α -DG (Ibraghimov-Beskrovnaya et al., 1992; Ervasti and Campbell, 1993). Certain sugar chain structures of α -DG play an important role in its binding to ligand proteins (Yamada et al., 1996; Chiba et al., 1997). Thus, the mucin-like domain is believed to mediate these interactions. Several ECM components, such as laminin-1, laminin-2, agrin, neu-rexin, and perlecan, were shown to serve as ligands of α -DG (Ibraghimov-Beskrovnaya et al., 1992; Sunada et al., 1994; Bowe et al., 1994; Sugita et al., 2001; Peng et al., 1998). Each of these ligands has laminin G (LG)-like domains that mediate their interaction with α -DG (Hohenester et al., 1999). However, the functional domains of α -DG have not been characterized systematically, and to date, the nature and location of the laminin binding site in α -DG remains elusive.

Mutations in known or putative glycosyltransferase genes have been identified in patients with congenital muscular dystrophy: *fukutin* in Fukuyama congenital muscular dystrophy (FCMD); *fukutin-related protein (FKRP)* in congenital muscular dystrophy 1C (MDC1C); *POMGnT1* in muscle-eye-brain disease (MEB); *POMT1* in Walker-Warburg syndrome (WWS); and *LARGE* in congenital muscular dystrophy 1D (MDC1D), as well as in the myodystrophy (*Large^{myd}*) mouse, a rodent model of MDC1D (Michele and Campbell, 2003; Muntoni et al., 2004; Grewal et al., 2001; Longman et al., 2003; Manya et al., 2004). In these disorders, α -DG is hypoglycosylated and its laminin binding activity is greatly reduced (Michele et al., 2002; Hayashi et al., 2001; Kano et al., 2002). These findings indicate that the posttranslational glycosylation processing of DG is essential for its biological activity as an ECM receptor and is correlated with

*Correspondence: kevin-campbell@uiowa.edu

⁵These authors contributed equally to this work.

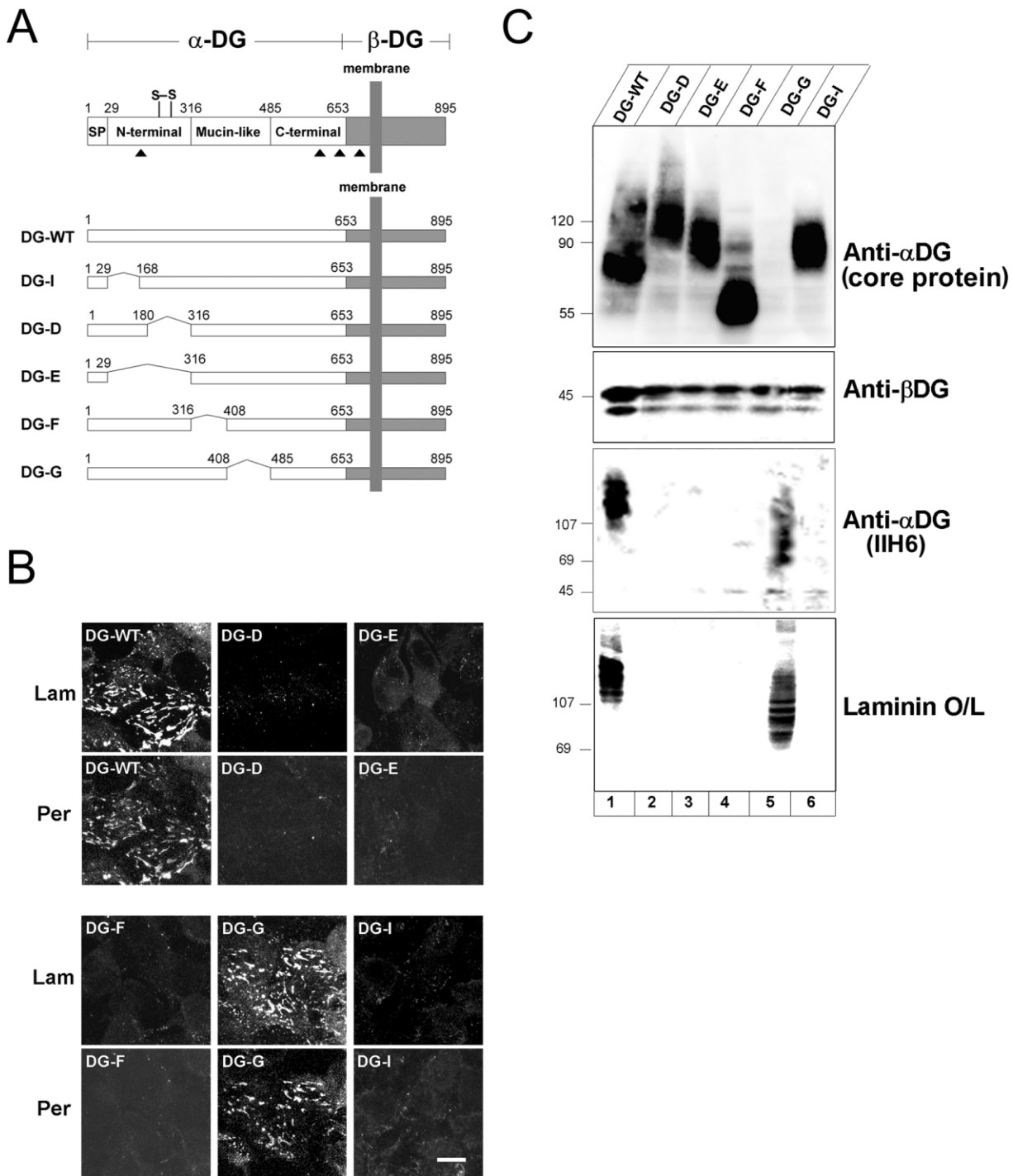


Figure 1. Analysis of α -DG Functional Domains

(A) Schematic representation of DG deletion mutant proteins. α -DG is composed of a signal peptide (SP, amino acids 1–29), an N-terminal domain (amino acids 30–316), a mucin-like domain (amino acids 317–485), and a C-terminal domain (amino acids 486–653). The disulfide bond is shown by S-S and potential N-glycosylation sites are indicated by arrowheads. The hatched box represents β -DG.

(B) The N-terminal domain of α -DG is necessary for cell surface laminin and perlecan organization. Laminin-1 (Lam) and perlecan (Per) form clusters of plaque-like morphology on the surface of cells expressing DG-WT and DG-G. The scale bar represents 10 μ m.

(C) Both the N-terminal domain and the first half of the mucin-like domain (amino acids 30–408) of α -DG are necessary for laminin binding. Western blotting and laminin domain overlay (O/L) assays show that laminin-1 binds to DG-WT and DG-G.

muscle and central nervous system pathology (Michele et al., 2002). Recently, we have found that the overexpression of LARGE, which contains two structurally and

perhaps functionally distinct glycosyltransferase-like domains (Grewal et al., 2001; Peyrard et al., 1999), can bypass defects of α -DG laminin receptor function in

WWS, MEB, and FCMD patients' cells (Barresi et al., 2004), suggesting a novel and flexible pathway for expression of functional DG.

In order to better understand the posttranslational processing pathway for expression of functional DG, we characterized the functional domains of DG *in vitro* and *in vivo*. We first demonstrate that in addition to the first half of the mucin-like domain, the N-terminal domain of α -DG is essential for the production of functional α -DG as a laminin receptor. We provide evidence of a molecular interaction between LARGE and the N-terminal domain of α -DG, and these data indicate that LARGE uses this N-terminal domain to recognize DG as a substrate. Furthermore, we show evidence that the N-terminal domain of DG is cleaved by convertase-like activity, but the cleavage does not affect DG ligand binding activity. Therefore, the data indicate that with regard to ligand binding, the N-terminal domain of α -DG is necessary only for intracellular formation of an enzyme-substrate intermediate between DG and LARGE, while the C-terminal domain of α - and/or β -DG mediates the assembly of the DGC at the sarcolemma. Significantly, using viral gene transfer to skeletal muscle-specific DG null mice, we provide *in vivo* evidence that the disruption of the DG-laminin linkage, through the absence of the critical glycosylation or LARGE recognition, is central to muscle cell degeneration in muscular dystrophy.

Results

Posttranslational Modification Regulates Laminin Receptor Function of DG

To examine the nature of the α -DG-laminin interactions, we generated a set of adenovirus (AdV) constructs with deletions in the N-terminal and/or the mucin-like domain of α -DG (Figure 1A). These deletion constructs, when expressed in embryonic stem (ES) cells, can properly target α - and β -DG proteins to the cell surface membrane (Kunz et al., 2001). First, we performed a laminin-1 and perlecan clustering assay on DG null (DG^{-/-}) ES cells after infection with each AdV construct to define the DG domains necessary for DG-mediated basement membrane assembly. Laminin-1 and perlecan formed clusters, which consolidated into plaque-like morphologies on the surface of ES cells expressing DG-WT or DG-G. No clusters were observed on the cells expressing DG-I, DG-D, DG-E, or DG-F (Figure 1B). Next, the laminin binding domain was characterized by blot overlay assay. Because antibodies against α -DG do not recognize all of the deletion mutants, the protein amount was adjusted by immunoreactivity against β -DG (Figure 1C). Laminin-1 bound to DG-WT and DG-G (Figure 1C). The monoclonal antibody IIH6, whose epitope is sensitive to the glycosylation status of α -DG (Ervasti and Campbell, 1993), also detected only DG-WT and DG-G. Unexpectedly, the results demonstrated that in addition to the N-terminal half of the mucin-like domain, the whole N-terminal domain of α -DG is necessary for laminin and IIH6 binding as well as laminin-1 and perlecan organization on the cell surface.

To further investigate the interaction between α -DG and laminin, we generated full-length and truncated α -DG molecules as IgG Fc fusion proteins (DGFc) by

transfecting TSA201 cells. The majority of the expressed full-length fusion protein (DGFc5) was secreted as a 100–120 kDa protein into the culture media although some was detected in the cell lysate. We observed, by blot-overlay assay, that laminin-1 bound to the secreted DGFc5, but not to DGFc5 in the cell lysate (Figure 2A). Interestingly, the species of DGFc5 that binds to laminin migrates slower than that stained by Coomassie blue (CB). Antibody FPD, against the core protein of α -DG, identified broader bands than those stained by CB (Figure 2B). Laminin and IIH6 reacted to the slow migrating species of the FPD-reactive bands (data not shown). This broad appearance of the fusion protein suggests extensive posttranslational modification, as is the case with native α -DG in skeletal muscle (Ervasti and Campbell, 1991). Enzymatic deglycosylation slightly decreased the molecular mass of DGFc5 and did not affect laminin binding activity (Figure 2B). However, chemical deglycosylation greatly reduced the molecular mass and abolished laminin binding activity. These results are consistent with previous observations that the sugar chain structure of α -DG is necessary for the interaction with laminin (Ervasti and Campbell, 1993). It is evident that only a small population in the DGFc5 preparations possess the specific carbohydrate modification required for ligand binding activity. Taken together, these data indicate that strict posttranslational glycosylation is necessary for the interaction between α -DG and laminin.

To confirm that DGFc5 conserves the ligand binding properties of native α -DG, we demonstrated that DGFc5, when compared to native α -DG, has similar laminin binding affinity (Figure 2C), can quantitatively compete laminin binding to native α -DG (data not shown), and shows similar ligand selectivity by nonlabeled ligand competition assays (Figure 2D).

LARGE-Dependent Functional Modification of α -DG

We recently demonstrated that forced expression of LARGE produced glycan-rich α -DG species and restored its function as a laminin receptor in FCMD, MEB, and WWS patients' cells (Berresi et al., 2004). We investigated the molecular mechanism for LARGE-dependent functional modification of DG using DGFc fusion proteins. Several deletion constructs of DGFc proteins were generated and expressed in TSA201 cells (Figure 3A). Ligand overlay assays on these fusion proteins further confirmed our previous results in ES cells: both the N-terminal domain and the first half of the mucin-like domain are essential for DG function as an ECM-receptor (Figure S1 on the *Cell* website). Furthermore, adenoviral expression of LARGE with each DGFc fusion protein increased reactivity to IIH6 and laminin-1 binding in the secreted forms of DGFc3 and DGFc5, but not in DGFc2 and DGFc6 (Figure 3B). However, in cell lysates, regardless of LARGE overexpression, we did not observe significant reactivity of any DGFc proteins to laminin-1 or IIH6 even when detection sensitivity was increased and/or the fusion protein was loaded in equal amounts compared to the secreted DGFc protein experiments. A solid phase laminin-1 binding assay on secreted DGFc5 demonstrated that LARGE modification quantitatively increased the laminin-1 binding activity (Figure 3C). Al-

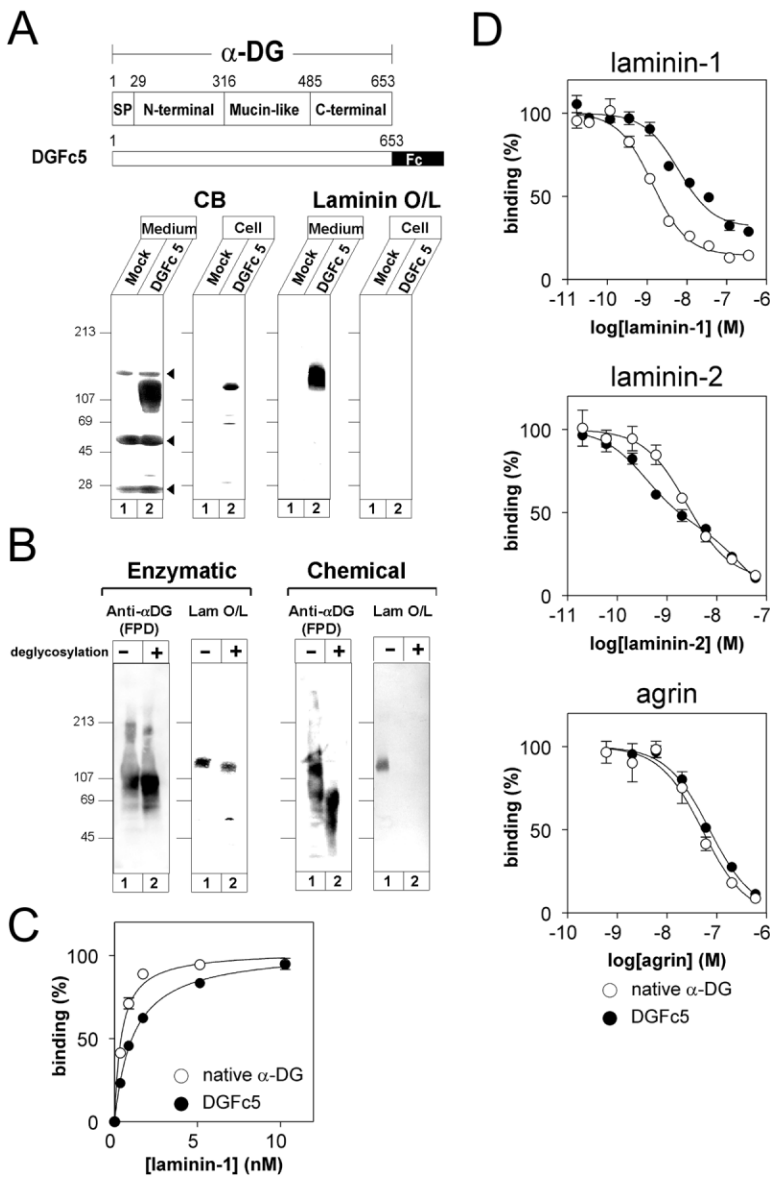


Figure 2. Expression of Full-Length α -DG-IgG Fc Fusion Protein (DGFc5) and Its Interaction with Laminin

DGFc5 was purified from the conditioned medium or cell lysate. Arrowheads indicate contaminated immunoglobulin light chain, heavy chain, and heavy chain dimer. O/L, overlay. (A) Laminin-1 binds to DGFc5 in conditioned medium but does not bind to that in cell lysate. A population of DGFc5 to which laminin-1 binds migrates more slowly than that stained by CB. Lane 1, mock transfection; lane 2, transfection with DGFc5. A schematic representation of DGFc5 is shown at the top of panel (A). Black boxes indicate the Fc portion.

(B) Glycosylation is essential for laminin binding activity of DGFc5. DGFc5 was deglycosylated either enzymatically or chemically. By chemical deglycosylation, the molecular mass of DGFc5 is greatly reduced, and laminin binding is abolished. Lane 1, untreated sample; lane 2, treated sample for deglycosylation.

(C) Binding of laminin-1 to DGFc5 (closed circle) and native α -DG (open circle). By nonlinear regression analysis, the dissociation constants of DGFc5 and native α -DG are estimated to be 1.1 ± 0.1 and 0.4 ± 0.1 nM, respectively.

(D) DGFc5 represents similar ligand selectivity to native α -DG. Competitive binding of biotinylated laminin-1 to native α -DG (open circle) or DGFc5 (closed circle) was measured in the presence of nonlabeled laminin-1, laminin-2, and agrin. All nonlabeled ligands compete the binding of biotinylated laminin-1 to both native α -DG and DGFc5.

though DGFc6 contains the entire mucin-like domain, we did not see any functional modification of DGFc6 even after the LARGE overexpression, suggesting that the N-terminal domain is required for the functional modification by either LARGE or other intrinsic glycosyltransferases. Notably, the secreted form of DGFc5 and DGFc6 migrated with similar molecular weight on SDS-PAGE, and the majority of DGFc2 prepared from conditioned medium seemed to be degraded. These findings led us to hypothesize that the N-terminal domain was proteolytically processed. We found that culturing TSA201 cells expressing DGFc2 in the presence of decanoyl-Arg-Val-Lys-Arg-CMK (CMK), an inhibitor for pro-protein convertases (endoprotease family), prevented degradation of DGFc2. Accordingly, the secreted DGFc2 migrated at the same molecular size (approximately 65 kDa) as DGFc2 prepared from cell lysate. Neither preparation showed any detectable laminin binding activity, with or without LARGE coexpression (data not shown). These data also support previous findings that essential glycosylation indeed occurs in the mucin-like domain.

A molecular weight shift following protease inhibition was also found in secreted DGFc3 and DGFc5, but not in DGFc6 (data not shown). CMK treatment caused secreted DGFc5 to migrate around 150 kDa and become reactive to an antibody to an N-terminal peptide of α -DG (rabbit 73) (Figure 4A). Without the CMK treatment, no significant reactivity to rabbit 73 was obtained with DGFc5 prepared from cell culture media. On the other hand, the DGFc5 preparations from cell lysate showed reactivity against rabbit 73 regardless of CMK treatment (Figure 4A). These data suggest that a convertase-like activity processes the N-terminal domain of α -DG as it is secreted from cells or in the extracellular environment. N-terminal amino acid sequence analyses of DGFc5 from CMK-treated cells and full-length DGFc5 cleaved by furin *in vitro* revealed that a convertase-like activity cleaves α -DG between Arg³¹² and Gln³¹³ (Figure S2 on the Cell website).

Our evidence of proteolytic processing of the N-terminal domain of secreted DG excluded the possibility that the ternary structure of the N-terminal domain is

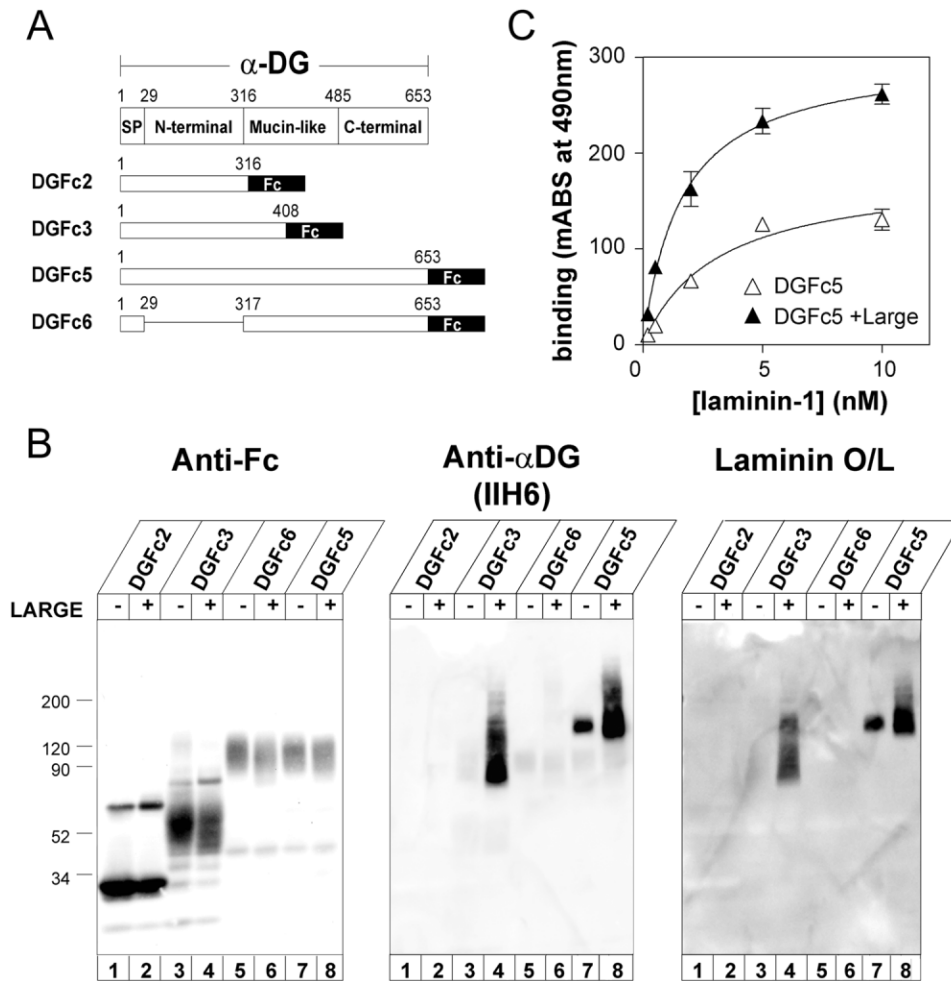


Figure 3. LARGE-Dependent Glycosylation Requires the N-Terminal Half of α-DG

(A) Schematic representation of deletion mutants of DGFc proteins.

(B) The N-terminal domain and the first half of the mucin-like domain of α-DG are necessary for LARGE-dependent glycosylation. Western blotting and a laminin overlay (O/L) assay shows that DGFc3 and DGFc5 are functionally glycosylated by LARGE. Neither the N-terminal nor the mucin-like domain itself is sufficient for the functional glycosylation by LARGE and/or intrinsic glycosyltransferases.

(C) LARGE-dependent glycosylation of DGFc5 increases laminin binding activity. By nonlinear regression analysis, the dissociation constants of DGFc5 (open triangles) and LARGE-modified DGFc5 (closed triangles) to laminin-1 are estimated to be 3.1 ± 1.1 and 1.6 ± 0.1 nM, respectively.

directly required for laminin binding to the mucin-like domain. Since DGFc5 isolated from cells contains the N-terminal domain, we hypothesized that the N-terminal domain may be required intracellularly for transient recognition by glycosyltransferases to form an enzyme-substrate intermediate. To test this hypothesis, we examined molecular interactions between LARGE and DGFc proteins. Protein A affinity beads coupled to DGFc proteins were incubated with LARGE-overexpressed TSA201 cell lysates. Bound materials were eluted with Laemmli sample buffer (LSB) and analyzed by Western blotting using an anti-LARGE antibody (Figure 4B). We found that LARGE interacted with DGFc2 (lane 2) and DGFc5 prepared from cell lysate (lane 3). Importantly, although the secreted form of DGFc5 did not bind LARGE (lane 4), inhibition of proteolytic cleavage with CMK successfully reconstituted the DGFc5-LARGE complex (lane 5). Mature glycosylation does not seem to affect the interaction between α-DG and LARGE. Together, these data suggest that the N-terminal domain

of α-DG is recognized by LARGE to form the enzyme-substrate intermediate, which is necessary for the subsequent glycosylation on the first half of the mucin-like domain. The N-terminal domain is then proteolytically cleaved after the glycosylation occurs.

In Vivo Evidence for Significance of DG-LARGE Interaction

In order to determine the significance of the molecular recognition of the N-terminal domain by LARGE, and the functional glycosylation of α-DG in vivo, we expressed deletion mutants of DG in skeletal muscle of MCK-DG null mice in which the endogenous DG gene is selectively ablated in striated muscle. MCK-DG null mice show concomitant loss of sarcoglycans and sarcospan and develop muscular dystrophy at 4–5 weeks of age (Cohn et al., 2002). Five weeks after viral gene transfer, more than 80% of the muscle fibers were transduced and converted to DG positive in each of the injected mice (Figure 5). Interestingly, each of the expressed

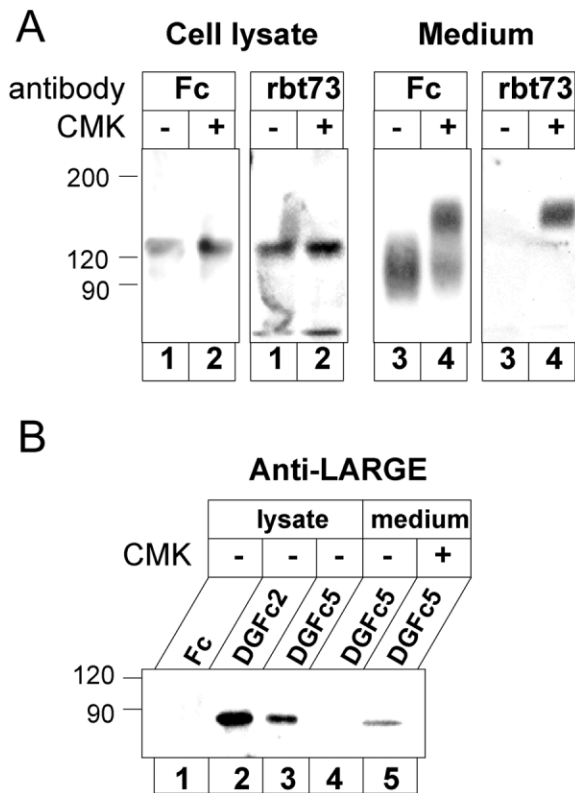


Figure 4. Mechanism for Glycosylation of α -DG by LARGE
(A) Proteolytical processing of the N-terminal domain of α -DG. Western blotting analysis with antibodies to the Fc or the N-terminal sequence of α -DG (rbt73) reveal that the secreted form of DGFC5 lacks its N-terminal domain. DGFC5 in cell lysates contains its N-terminal domain. Treatment with a convertase inhibitor, CMK, prevents proteolytic processing of the N-terminal domain of the secreted form of DGFC5. (B) Molecular interaction between α -DG and LARGE. The following DGFC-protein A beads were prepared and then incubated with LARGE-expressing TSA201 cell lysate: Fc (cell lysate), DGFC2 (cell lysate), DGFC5 (cell lysate), DGFC5 (medium), and DGFC5 (CMK-treated, medium). Western blotting with anti-LARGE antibody demonstrates that LARGE recognizes the N-terminal domain of α -DG to form an enzyme-substrate intermediate complex.

DG proteins (DG-WT, DG-I, DG-D, DG-E, DG-F, DG-G) restored the DGC assembly at the sarcolemma including sarcoglycans and sarcospan. The expression of a shorter DG-H protein lacking the entire N-terminal and mucin-like domains of α -DG also restored the DGC assembly. In Figure 5, the expression profile is represented by DG-E and DG-H. Dystrophin and α 1-syntrophin, which are preserved in MCK-DG null mice, were also normally expressed in these muscles. These results provide novel evidence that the C-terminal domain of α -DG and/or β -DG mediate the stable assembly of the DGC at the sarcolemma.

Surprisingly, hematoxylin-eosin staining revealed that expression of DG-I, DG-D, DG-E, DG-F, or DG-H exacerbated the dystrophic phenotype of the mutant mice in spite of full restoration of the DGC (Figure 6). The pathological phenotype of the MCK-DG null mouse includes numerous regenerating fibers in skeletal muscle with no evidence of tissue fibrosis and/or fatty infiltration (Cohn

et al., 2002). However, after expression of DG-D, DG-E, DG-F, or DG-H, fibrosis and fatty infiltration were commonly observed (Figure 6A). In contrast, expression of DG-WT or DG-G, proteins that bind to laminin, completely rescued the mouse from dystrophic pathology (Figure 6A). Quantitative morphometric analyses were performed by recording the percentage of fibers with centrally located nuclei and fiber diameter variability, which reflect ongoing muscle regeneration and are both increased in muscular dystrophy. MCK-DG null mice injected with saline (Mock) or expressing DG-I, DG-D, DG-E, DG-F, or DG-H had a significantly higher percentage of fibers with central nuclei and fiber diameter variability than control Cre-negative mice. The expression of DG-WT or DG-G in mutant mice prevented the increase in central nucleation and fiber diameter variability (Figures 6B and 6C). Taken together, these results provide in vivo evidence that the DGC assembly mediated by β -DG with the C-terminal domain of α -DG is not sufficient and that the α -DG-laminin interaction is crucial to prevent muscle cell degeneration. These results demonstrate that the molecular recognition of α -DG by LARGE through the N-terminal domain of α -DG is essential for the subsequent functional glycosylation that underlies the pathology of several forms of muscular dystrophy.

Discussion

In the present study, we dissected the functional domains of DG necessary for ECM protein organization, ligand interactions, DGC assembly, and recognition by glycosyltransferases. We provide strong evidence, summarized below and in Figure 7, for a direct role of the ECM ligand binding domain of α -DG in the pathogenesis of muscular dystrophy and a unique processing pathway for expression of functional DG. The N-terminal domain of α -DG serves as an intracellular substrate recognition site for LARGE, which initiates subsequent functional glycosylation of α -DG. The glycosylation, which is essential for ligand binding and cell surface laminin/perlecan organization, takes place within the first half of mucin-like domain. β -DG with the C-terminal domain of α -DG mediates the DGC assembly. This assembly is not sufficient to prevent muscular dystrophy. Rather, the α -DG-laminin linkage is necessary to prevent muscle cell degeneration. These findings demonstrate in vitro and in vivo that molecular recognition of DG by LARGE is a key determinant of DG functional expression.

Mechanism of Posttranslational Modification of α -DG for ECM Binding

DG-mediated formation of laminin clusters on the cell surface is thought to be critical for embryonic basement membrane assembly (Williamson et al., 1997; Henry and Campbell, 1998). We observed laminin clustering on the surface of DG-WT- or DG-G-expressing ES cells (Figure 1). In addition, only these two proteins show laminin binding activity, indicating that the N-terminal domain and the N-terminal half of the mucin-like domain (amino acids 30–408) are required for functional expression of DG as a laminin receptor. This observation is consistent with the "DG-facilitated laminin network model," which

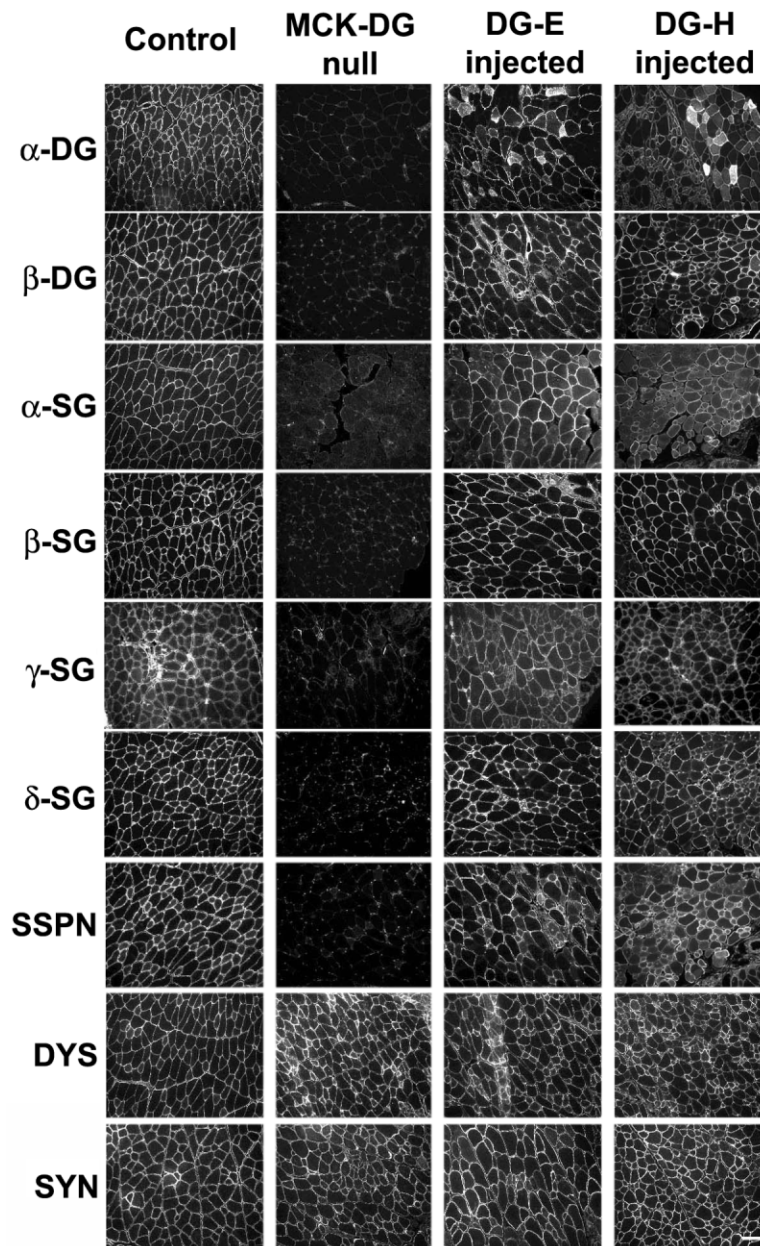


Figure 5. Restoration of the DGC in Skeletal Muscle of MCK-DG Null Mice Expressing DG Deletion Mutant Proteins

The C-terminal domain of α -DG and/or β -DG is responsible for the DGC assembly. The DGC assembly was examined by immunofluorescence analysis after expression of deletion mutants of DG in vivo. The figure shows DG-E- and DG-H-injected skeletal muscle, which represent all constructs tested. All the DGC components are restored at the sarcolemma after gene transfer. Equivalent results were obtained by expression of DG-WT, DG-I, DG-D, DG-F, or DG-G. SG, sarcoglycan; SSPN, sarcospan; DYS, dystrophin; and SYN, syntrophin.

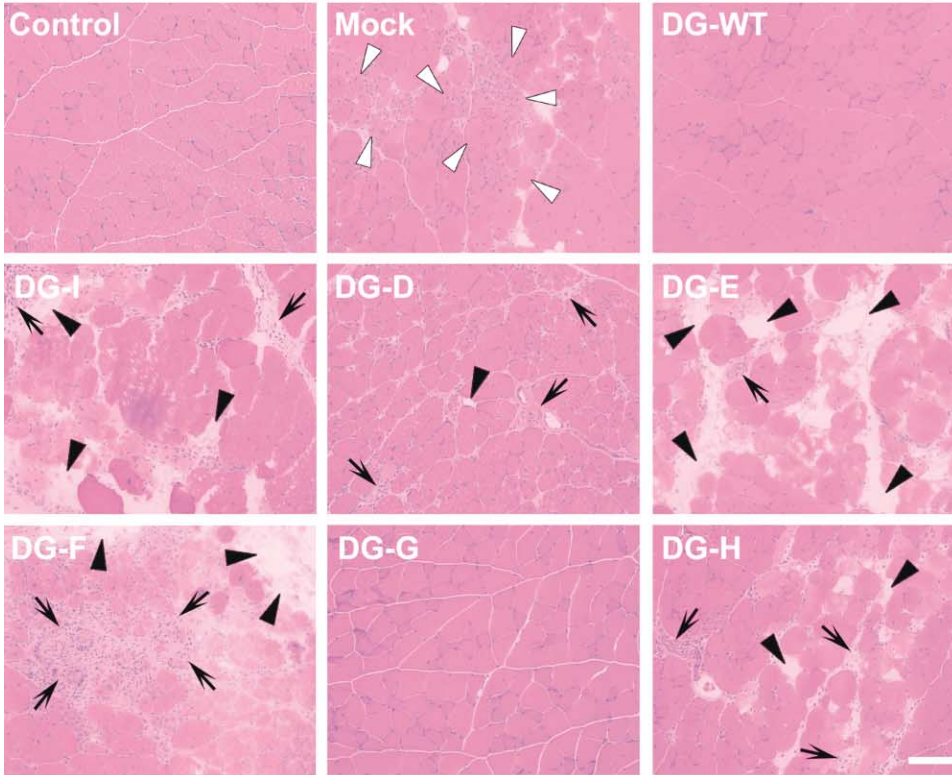
proposes that both laminin self-association and DG are necessary for the formation of a laminin network on the cell surface (Colognato et al., 1999). Myotubes differentiated from WWS and FCMD patients fail to form laminin clusters (Barresi et al., 2004). After LARGE expression in these cells, restoration of laminin organization is observed, suggesting that DG function is also required for laminin organization in skeletal muscle cells.

Previous studies suggest that α -DG in skeletal muscle is extensively glycosylated, thus contributing to the broad appearance of α -DG on Western blot (Ervasti and Campbell, 1991). Consistent with this notion, we observed a broad appearance of DGFc5 secreted into culture media (Figures 2 and 3). The population of DGFc5 stained by CB may represent immature DG in terms of functional posttranslational modification. In contrast,

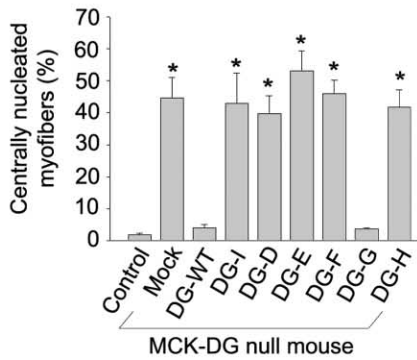
the population of DGFc5 with a higher molecular weight and little CB staining may be a mature form of α -DG because both laminin and IIH6 bind only to this portion. In this context, DGFc5 isolated from cells seems to be immature. Players for DG glycosylation appear, or are hypothesized, to locate in the Golgi apparatus (Esapa et al., 2003). These observations together suggest that DGFc5 matures intracellularly in the Golgi apparatus and is secreted immediately so that there is very little detected in the cell lysate by laminin overlay assay. However, full maturation, at least in TSA201 cells, is not essential for secretion of α -DG into the extracellular environment.

Recently, we found that overexpression of LARGE in cells from WWS, MEB, and FCMD patients induced the synthesis of an α -DG species enriched in glycans with

A



B



C

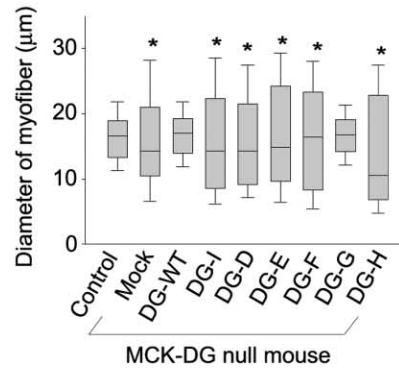


Figure 6. Histological Analysis of MCK-DG Null Muscles after Expression of DG Deletion Mutant Proteins

Laminin binding activity is essential for maintaining muscle cell integrity.

(A) The pathological phenotype after expression of deletion mutants of DG in vivo was examined by hematoxylin-eosin staining. Numerous regenerating fibers with central nuclei are seen in MCK-DG null mice with mock injection (open arrowhead). In the muscles expressing DG-I, DG-D, DG-E, DG-F, or DG-H, fibrous connective tissue is increased (arrow), and fatty infiltration is detected (closed arrowhead). In sharp contrast, the muscles expressing DG-WT and DG-G are fully rescued from dystrophic pathology.

(B) The percentage of myofibers with centrally located nuclei in the MCK-DG null mice expressing DG-WT and DG-G was not statistically different from that in the control mice.

(C) The variability of myofiber diameter in the MCK-DG null mice expressing DG-WT and DG-G was decreased and not statistically different from that of the control mice. The bar in the box represents the mean diameter. The boxes indicate the 25 and 75 percentile values, and the error bars represent the 10 and 90 percentile values.

high affinity for extracellular ligands, leading to restoration of α -DG as a functional laminin receptor (Barresi et al., 2004). These findings indicate that a LARGE-dependent posttranslational pathway can be a target for a

viable therapeutic strategy for glycosyltransferase-deficient muscular dystrophies. In the present report, we observed a direct increase of laminin binding activity in DGFC5 after LARGE expression (Figure 3). Laminin

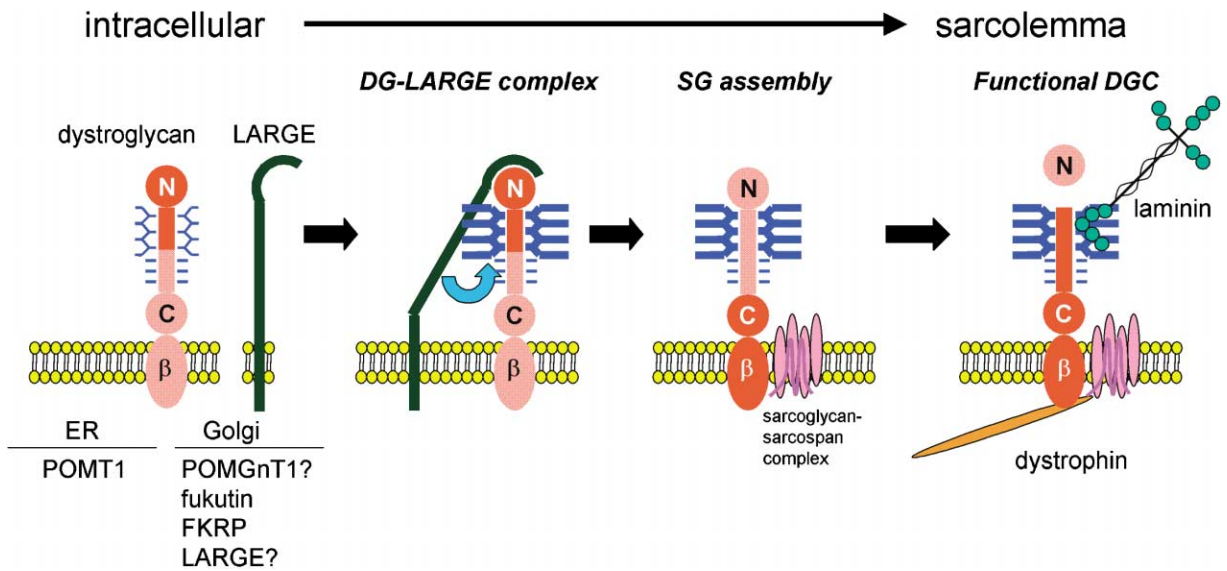


Figure 7. Multistep Molecular Pathway for the Functional Expression of DG

Distinct domains are responsible for each step of posttranslational modification of DG. Important domains in each step are represented in dark red. The N-terminal domain and the first half of the mucin-like domain are necessary for functional glycosylation. The N-terminal domain is recognized by LARGE. LARGE-dependent modification takes place within the first half of the mucin-like domain. The C-terminal domain and/or β -DG is responsible for the sarcoglycan (SG) assembly. All steps are necessary for the functional expression of DG to maintain muscle cell integrity and basement membrane assembly.

overlays indicated that the N-terminal domain is a prerequisite for LARGE-dependent functional glycosylation on the mucin-like domain of α -DG (Figure 3) as is the case with intrinsic glycosyltransferases in ES and TSA201 cells (Figure 1; see also Figure S1 available on the *Cell* website at <http://www.cell.com/cgi/content/full/117/7/953/DC1/>). The involvement of the N-terminal domain in the functional expression of α -DG was of particular interest. This observation first raised the possibility that, in addition to the sugar chain moieties, the core protein structure in the N-terminal domain is also important in the interaction with laminin. At first, we speculated that the N-terminal domain acts as a regulator by providing a tertiary structure to promote accessibility of the receptor sugar chains in the mucin-like domain. In addition, the core protein structure might provide a distinct binding domain, which directly interacts with its ligands in cooperation with the mucin-like domain. However, surprisingly, the N-terminal sequence analysis revealed that mature α -DG lacks the N-terminal domain (Figure 4; Figure S2 on the *Cell* website). On the other hand, Western blotting analysis with rabbit 73 showed that intracellular DGFC5 contains the N-terminal domain (Figure 4). Therefore, we proposed the alternative hypothesis that the N-terminal domain may be required for intracellular substrate recognition by essential enzymes that glycosylate α -DG and formation of a transient enzyme-substrate intermediate. Indeed, we demonstrated an interaction between the N-terminal domain of α -DG and LARGE (Figure 4). This LARGE-DG intermediate may be critical to the specificity for α -DG functional glycosylation because we observed specific increases in glycosylation of α -DG in cells and tissues overexpressing LARGE, and the high level of LARGE expression did not cause muscle pathology in wild-type skeletal muscle

(Barresi et al., 2004). This seems to be a unique mechanism in terms of substrate recognition. That is, LARGE recognizes amino acid sequences located in a different part of the protein from the acceptor region for the glycoconjugate. It is also suggested that the intrinsic glycosyltransferases involved in α -DG modification in TSA201 cells also recognize the N-terminal domain. Therefore, we conclude that the N-terminal domain of α -DG determines specificity for glycosylation on the mucin-like domain.

We demonstrated that the N-terminal domain is proteolytically processed by intrinsic convertase-like activity after the functional glycosylation of α -DG. Because cellular DGFC5 contains the N-terminal domain, the processing seems to occur in the extracellular space or immediately preceding secretion. The higher molecular weight of the secreted form of DGFC5 with CMK treatment compared to the cell lysate form represents the inclusion of the uncleaved peptide (amino acid residues 30–312) plus the additional glycosylation as the protein proceeds through the secretory pathway. This proteolytic processing of the N-terminal domain is thought to be a common feature of DG posttranslational modification for the following reasons: We found a molecular weight shift in α -DG on SDS-PAGE when mouse myoblast C2C12 and rat Schwannoma RT4 cells were cultured in the presence of CMK. This CMK effect was not observed in mouse tissue preparations. The *in vitro* furin treatment of purified α -DG did not show any changes in its molecular weight. Although the physiological role of the N-terminal domain processing is unclear, it provides clear evidence that the N-terminal domain is not directly involved in ligand binding. We did not observe significant differences in ligand binding affinity and maximum binding for DGFC5 after CMK treatment (data not

shown). The N-terminal domain of α -DG has been reported to interact with some extracellular matrix proteins itself (Hall et al., 2003); therefore, the processed N-terminal domain might still be associated in the ECM. Another possibility is that even after processing *in vivo*, the N-terminal domain might still be associated with the core protein since rotary shadowing electron microscopy suggested the dumbbell-like architecture of α -DG consists of two globular structures (Brancaccio et al., 1995, 1997).

Implication of the Laminin Binding Activity of DG in the Pathogenesis of Muscular Dystrophy

Our data demonstrate that the C-terminal half of DG is involved in the assembly of the sarcoglycan-sarcospan complex (Figure 5). Formation of the sarcoglycan complex has been proposed to take place during trafficking from the Golgi to plasma membrane (Noguchi et al., 2000). Our results suggest that full restoration of DGC assembly in MCK-DG null skeletal muscle is not sufficient for the rescue of dystrophic pathology and, rather, the α -DG-laminin linkage is crucial to prevent the dystrophic phenotype (Figures 5 and 6). In addition, the data provide clear *in vivo* evidence that lack of protein recognition by LARGE, and perhaps other glycosyltransferases, through the N-terminal domain of α -DG and the subsequent absence of α -DG-laminin interactions are sufficient to cause muscular degeneration in these disorders. Although mutations of the DG gene have not been reported, it is possible that mutation in the N-terminal domain of α -DG causes abnormal glycosylation of α -DG.

Interestingly, the expression of non-laminin binding DG proteins exacerbated the dystrophic pathology in MCK-DG null mice. MCK-DG null mice have low level expression of endogenous DG due to the re-expression of DG from satellite cells undergoing regeneration. This expression of DG in satellite cells allows efficient regeneration, causing the nonprogressive phenotype of these mice (Cohn et al., 2002). The regenerating capacities of *mdx* (dystrophin null) and sarcoglycan null mice are less than MCK-DG null mice (Cohn et al., 2002). It is possible that these AdV constructs also transduced satellite cells and truncated DG proteins affected regenerative capacity. Overexpression of DG mutants without laminin binding activity may be competing with the residual endogenous DG for intracellular binding partners, which are involved in signal transduction (Henry and Campbell, 1999). This dominant-negative effect in satellite cells may impair the repair mechanism and eventually lead to fibrosis and fat replacement in skeletal muscle.

In the last decade, primary genetic defects in the DGC were identified in several forms of muscular dystrophy. These include dystrophin, α -, β -, γ -, and δ -sarcoglycan, which cause Duchenne/Becker muscular dystrophy (DMD/BMD), limb-girdle muscular dystrophies (LGMD) type 2D, 2E, 2C, and 2F, respectively (for review, see Cohn and Campbell [2000]). However, the exact molecular mechanisms through which these primary defects lead to muscle cell death remain elusive because other DGC components including α -DG are lost en bloc or are at least severely reduced as a result of each primary defect in these disorders (Ervasti et al., 1990; Ohlendieck

et al., 1993; Duclos et al., 1998a, 1998b; Holt et al., 1998; Durbeej et al., 2000; Matsumura et al., 2003). Thus, it is possible that the secondary disruption of the DG-laminin linkage due to the concomitant reduction of α -DG, rather than primary deficiency of dystrophin or sarcoglycans, is causative for the muscle cell degeneration in DMD and LGMD. To facilitate the understanding of the pathogenesis of these muscular dystrophies with secondary DGC disruption, it is of particular importance to generate animal models in which a certain protein component or certain protein linkage alone is selectively disrupted within a normally expressed DGC. From this point of view, our MCK-DG null mice expressing mutant DG proteins provide an example of a unique model in which α -DG-laminin interactions are selectively lost and provide the opportunity to specifically define the critical role of these interactions in the prevention of muscle disease. Taken together, we propose that the disruption of the DG-laminin linkage is a common, central pathway leading to the muscle cell pathogenesis in the muscular dystrophies. Overall, we propose that molecular recognition of DG by LARGE is a key determinant in the biosynthetic pathway to produce a mature and functional DG.

Experimental Procedures

Antibodies

Antibodies to α -DG (IIH6, FPD, and GT20ADG), and β -DG were described previously (Ibraghimov-Beskrovnaia et al., 1992; Michele et al., 2002; Williamson et al., 1997). Antibodies against α -sarcoglycan, δ -sarcoglycan, sarcospan, dystrophin and β -sarcoglycan were described elsewhere (Duclos et al., 1998b). Antibodies against LARGE and α -DG N-terminal fragment (rabbit 73) were raised in rabbits. Synthetic peptides corresponding to the C-terminal sequence of mouse LARGE (C-YGFAALKYLTAENN) and the N-terminal sequence of rabbit DG (HWPSEPSEAVRDWEN) were used as antigens, respectively. Specificity of anti-LARGE antibody was confirmed by Western blotting using TSA201 cell lysates prepared before and after LARGE overexpression. Monoclonal antibody against γ -sarcoglycan, 21B5, was generated in collaboration with Dr. Louise Anderson. Anti-laminin and anti-heparan sulfate proteoglycan (perlecan) antibodies were purchased from Sigma and Chemicon International Inc, respectively. Antibodies against laminin α 2-chain, laminin α 1-chain and α 1 syntrophin were kind gifts from Drs. Peter Yurchenco, Lydia Sorokin and Stanley Froehner, respectively. Peroxidase conjugated secondary antibodies were obtained from Roche. Biotinylated anti-human IgG was from Vector. Cy3 and FITC conjugated secondary antibodies were from Jackson Immuno Research. Peroxidase conjugated avidin was from Pierce.

Expression and Purification of Recombinant α -DG-IgG Fc Fusion Proteins

Construction of α -DG-IgG Fc fusion proteins were described elsewhere (Kunz et al., 2001). Construction of DGFc6 is shown in the Supplemental Data available on the Cell website. TSA201 cells were grown in Dulbecco's modified eagle media (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and penicillin-streptomycin (GIBCO-BRL). Cells were transiently transfected using FuGene 6 (Roche). The conditioned medium and the cells were collected 4 days after transfection unless stated otherwise. For CMK-treatment, after the transfection, cells were cultured in the presence of 20 μ M CMK (ALEXIS) for 2-4 days. The cells were lysed in Buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.6 μ g/ml of pepstatin A, 0.5 μ g/ml of aprotinin, 0.5 μ g/ml of leupeptin, 0.75 mM of benzamide, 0.1 mM of PMSF, 0.4 μ g/ml of calpain inhibitor and 0.4 μ g/ml of calpeptin) for 2 hr at 4°C and the extracts were collected by centrifugation at 20,000 \times g for 10 min. The cell extracts and conditioned medium were incubated with protein A-agarose (Santa Cruz) overnight at 4°C. The beads were washed

extensively with Buffer A and the bound DGFC proteins were eluted by 100 mM glycine-HCl, pH 2.8, or by boiling in LSB.

Deglycosylation Experiments

Thirty μ l of purified DGFC5 preparations were boiled for 5 min in the presence of 0.7% SDS, then Triton X-100 was added to a final concentration of 1% and pH was adjusted to 5.5 by adding 50 mM sodium acetate. The mixture was incubated with 100 mU of *Vibrio cholerae* neuraminidase (Roche) and 2 mU of O-glycosidase (Roche) at 37°C for 16 hr. The pH was neutralized by adding 100 mM sodium phosphate buffer and the mixture was then incubated with 10 mU of N-glycosidase (Glyko) at 37°C for 16 hr. Chemical deglycosylation was performed as previously described (Ervasti and Campbell, 1993).

LARGE Expression

Generation of E1-deficient recombinant adenovirus Ad5 LARGE/eGFP was described elsewhere (Barresi, et al., 2004). TSA201 cell cultures were infected with viral vector for 2 hr with multiplicity of infection (MOI) of 10 in DMEM supplemented with 2% FBS and then the culture medium was changed to 10% FBS-containing DMEM. Cell lysates were prepared after 2 days post-infection as described above. For detection of DGFC-LARGE interaction, LARGE-expressed cell lysates were incubated with DGFC-protein A-beads at 4°C for 3 hr. After extensive washing with buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Triton X-100, bound materials were eluted with LSB.

Adenovirus-Mediated Gene Transfer into ES Cell

Generation of replication-deficient adenoviral (AdV) vectors and α -DG deletion constructs were described elsewhere (Kunz et al., 2001). DG^{+/+} clone (354.C3) and DG^{-/-} clone (354.B11) of ES cells were cultured as previously described (Henry and Campbell, 1998). ES cells were infected by AdV constructs with MOI of 3 in DMEM supplemented with 2% FBS overnight. The next day, the cells were processed for a laminin and perlecan clustering assay as described elsewhere (Henry et al., 2001). For biochemical analysis, the cells were harvested 48 hr after infection and lysed with buffer A. After centrifugation, the supernatants were incubated with Jacalin-agarose (Vector) overnight at 4°C. The beads were washed 3 times with buffer A and bound proteins were eluted by LSB.

Adenovirus-Mediated Gene Transfer into Mouse Skeletal Muscle

One week old MCK-DG null mice and Cre-negative homozygous floxed mice as control animals were injected percutaneously with 3×10^9 particles in 10 μ l of saline solution into the hamstring. As mock injections, only the saline without viruses was injected. Five weeks after injection, the muscles were harvested for histological and immunofluorescence analysis.

Histological Analysis

Hematoxylin-eosin staining was performed as described elsewhere (Cohn et al., 2002). For the calculation of the percentage of myofibers with centrally located nuclei, 700 to 1200 myofibers from 5 different visual fields were observed and centrally nucleated fibers were counted. To assess the variability of myofiber diameter, the shortest diameter was measured in 250 myofibers. The statistical difference was evaluated using t test.

Miscellaneous

Native α -DG was purified from rabbit skeletal muscle as described previously (Rambukkana et al., 1998). Western blotting, immunofluorescence analysis, ligand overlay, and solid-phase binding assays were described previously (Michele et al., 2002).

Acknowledgments

We would like to thank Aaron M. Beedle, Daniel Beltrán-Valero De Bernabé and all members of the Campbell laboratory for the critical reading of the manuscript and fruitful discussion. We thank the following for their contribution to this work; Ronald Cohn for the colony of MCK-DG null mice; Shanna Sawatzki and Melissa Hasse-

brock for expert technical assistance; Charles Lovig and The University of Iowa Hybridoma Facility for preparing monoclonal antibody IIH6; Peter Yurchenco (Robert Johnson Medical School), Lydia Sorokin (University of Erlangen), Stanley Froehner (University of Washington) and Louise Anderson (Novocastra) for providing antibodies; The University of Iowa Gene Transfer Vector Core, supported by NIH P30 DK54759, for adenovirus purification. There are no potential financial conflicts of interest for any of the authors. This work was supported in part by the Muscular Dystrophy Association (K.P.C.) and by US Public Health Service grant AI 45927 (S.K. and M.B.O.). K.P.C. is an investigator of the Howard Hughes Medical Institute.

Received: September 29, 2003

Revised: March 9, 2004

Accepted: May 11, 2004

Published online: June 3, 2004

References

- Barresi, R., Michele, D.E., Kanagawa, M., Harper, H.A., Dovico, S.A., Satz, J.S., Moore, S.A., Zhang, W., Schachter, H., Dumanski, J.P., et al. (2004). LARGE can functionally bypass α -dystroglycan glycosylation defects in distinct congenital muscular dystrophy. *Nat. Med.*
- Bowe, M.A., Deyst, K.A., Leszyk, J.D., and Fallon, J.R. (1994). Identification and purification of an agrin receptor from Torpedo postsynaptic membranes: a heteromeric complex related to the dystroglycans. *Neuron* 12, 1173–1180.
- Brancaccio, A., Schulthess, T., Gesemann, M., and Engel, J. (1995). Electron microscopic evidence for a mucin-like region in chick muscle alpha-dystroglycan. *FEBS Lett.* 368, 139–142.
- Brancaccio, A., Schulthess, T., Gesemann, M., and Engel, J. (1997). The N-terminal region of α -dystroglycan is an autonomous globular domain. *Eur. J. Biochem.* 246, 166–172.
- Burton, E.A., and Davies, K.E. (2002). Muscular dystrophy—reason for optimism? *Cell* 108, 5–8.
- Chiba, A., Matsumura, K., Yamada, H., Inazu, T., Shimizu, T., Kusunoki, S., Kanazawa, I., Kobata, A., and Endo, T. (1997). Structures of sialylated O-linked oligosaccharides of bovine peripheral nerve α -dystroglycan. *J. Biol. Chem.* 272, 2156–2162.
- Cohn, R.D., and Campbell, K.P. (2000). Molecular basis of muscular dystrophies. *Muscle Nerve* 23, 1456–1471.
- Cohn, R.D., Henry, M.D., Barresi, R., Saito, F., Moore, S.A., Flanagan, J.D., Skwarchuk, M.W., Robbins, M.E., Williamson, R., and Campbell, K.P. (2002). Disruption of *Dag1* in differentiated skeletal muscle reveals a role for dystroglycan in muscle regeneration. *Cell* 110, 639–648.
- Colognato, H., Winkelmann, D.A., and Yurchenco, P.D. (1999). Laminin polymerization induces a receptor-cytoskeleton network. *J. Cell Biol.* 145, 619–631.
- Duclos, F., Broux, O., Bourg, N., Straub, V., Feldman, G.L., Sunada, Y., Lim, L.E., Piccolo, F., Cutshall, S., Gary, F., et al. (1998a). Beta-sarcoglycan: genomic analysis and identification of a novel missense mutation in the LGMD2E Amish isolate. *Neuromuscul. Disord.* 8, 30–38.
- Duclos, F., Straub, V., Moore, S.A., Venzke, D.P., Hrstka, R.F., Crosbie, R.H., Durbeej, M., Lebakken, C.S., Ettinger, A.J., van der Meulen, J., et al. (1998b). Progressive muscular dystrophy in alpha-sarcoglycan-deficient mice. *J. Cell Biol.* 142, 1461–1471.
- Durbeej, M., Cohn, R.D., Hrstka, R.F., Moore, S.A., Allamand, V., Davidson, B.L., Williamson, R.A., and Campbell, K.P. (2000). Disruption of the beta-sarcoglycan gene reveals pathogenetic complexity of limb-girdle muscular dystrophy type 2E. *Mol. Cell* 5, 141–151.
- Durbeej, M., Henry, M.D., Ferletta, M., Campbell, K.P., and Ekblom, P. (1998). Distribution of dystroglycan in normal adult mouse tissues. *J. Histochem. Cytochem.* 46, 449–457.
- Ervasti, J.M., Ohlendieck, K., Kahl, S.D., Gaver, M.G., and Campbell, K.P. (1990). Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. *Nature* 345, 315–319.
- Ervasti, J.M., and Campbell, K.P. (1991). Membrane organization of the dystrophin-glycoprotein complex. *Cell* 66, 1121–1131.

- Ervasti, J.M., and Campbell, K.P. (1993). A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. *J. Cell Biol.* **122**, 809–823.
- Esapa, C.T., Benson, M.A., Schroder, J.E., Martin-Rendon, E., Brockington, M., Brown, S.C., Muntoni, F., Kroger, S., and Blake, D.J. (2003). Functional requirements for fukutin-related protein in the Golgi apparatus. *Hum. Mol. Genet.* **11**, 3319–3331.
- Grewal, P.K., Holzfeind, P.J., Bittner, R.E., and Hewitt, J.E. (2001). Mutant glycosyltransferase and altered glycosylation of alpha-dystroglycan in the myodystrophy mouse. *Nat. Genet.* **28**, 151–154.
- Hall, H., Bozic, D., Michel, K., and Hubbell, J.A. (2003). N-terminal alpha-dystroglycan binds to different extracellular matrix molecules expressed in regenerating peripheral nerves in a protein-mediated manner and promotes neurite extension of PC12 cells. *Mol. Cell. Neurosci.* **24**, 1062–1073.
- Hayashi, Y.K., Ogawa, M., Tagawa, K., Noguchi, S., Ishihara, T., Nonaka, I., and Arahata, K. (2001). Selective deficiency of alpha-dystroglycan in Fukuyama-type congenital muscular dystrophy. *Neurology* **57**, 115–121.
- Henry, M.D., and Campbell, K.P. (1998). A role for dystroglycan in basement membrane assembly. *Cell* **95**, 859–870.
- Henry, M.D., and Campbell, K.P. (1999). Dystroglycan inside and out. *Curr. Opin. Cell Biol.* **11**, 602–607.
- Henry, M.D., Satz, J.S., Brakebusch, C., Costell, M., Gustafsson, E., Fassler, R., and Campbell, K.P. (2001). Distinct roles for dystroglycan, beta1 integrin and perlecan in cell surface laminin organization. *J. Cell Sci.* **114**, 1137–1144.
- Hohenester, E., Tisi, D., Talts, J.F., and Timpl, R. (1999). The crystal structure of a laminin G-like module reveals the molecular basis of alpha-dystroglycan binding to laminins, perlecan, and agrin. *Mol. Cell* **4**, 783–792.
- Holt, K.H., Lim, L.E., Straub, V., Venzke, D.P., Duclos, F., Anderson, R.D., Davidson, B.L., and Campbell, K.P. (1998). Functional rescue of the sarcoglycan complex in the BIO 14.6 hamster using delta-sarcoglycan gene transfer. *Mol. Cell* **1**, 841–848.
- Ibraghimov-Beskrovnaya, O., Ervasti, J.M., Leveille, C.J., Slaughter, C.A., Sernett, S.W., and Campbell, K.P. (1992). Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* **355**, 696–702.
- Kano, H., Kobayashi, K., Herrmann, R., Tachikawa, M., Manya, H., Nishino, I., Nonaka, I., Straub, V., Talim, B., Voit, T., et al. (2002). Deficiency of alpha-dystroglycan in muscle-eye-brain disease. *Biochem. Biophys. Res. Commun.* **291**, 1283–1286.
- Kunz, S., Sevilla, N., Campbell, K.P., and Oldstone, M.B.A. (2001). Molecular analysis of the interaction of LCMV with its cellular receptor α -dystroglycan. *J. Cell Biol.* **155**, 301–310.
- Longman, C., Brockington, M., Torelli, S., Jimenez-Mallebrera, C., Kennedy, C., Khalil, N., Feng, L., Saran, R.K., Voit, T., Merlini, L., et al. (2003). Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of alpha-dystroglycan. *Hum. Mol. Genet.* **12**, 2853–2861.
- Manya, H., Chiba, A., Yoshida, A., Wang, X., Chiba, Y., Jigami, Y., Margolis, R.U., and Endo, T. (2004). Demonstration of mammalian protein O-mannosyltransferase activity: coexpression of POMT1 and POMT2 required for enzymatic activity. *Proc. Natl. Acad. Sci. USA* **101**, 500–505.
- Matsumura, K., Arai, K., Zhong, D., Saito, F., Fukuta-Ohi, H., Mae-kawa, R., Yamada, H., and Shimizu, T. (2003). Disruption of dystroglycan axis by beta-dystroglycan processing in cardiomyopathic hamster muscle. *Neuromuscul. Disord.* **13**, 796–803.
- Michele, D.E., Barresi, R., Kanagawa, M., Saito, F., Cohn, R.D., Satz, J.S., Dollar, J., Nishino, I., Kelley, R.I., Somer, H., et al. (2002). Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. *Nature* **418**, 417–422.
- Michele, D.E., and Campbell, K.P. (2003). Dystrophin-glycoprotein complex: post-translational processing and dystroglycan function. *J. Biol. Chem.* **278**, 15457–15460.
- Moore, S.A., Saito, F., Chen, J., Michele, D.E., Henry, M.D., Messing, A., Cohn, R.D., Ross-Barta, S.E., Westra, S., Williamson, R.A., et al. (2002). Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy. *Nature* **418**, 422–425.
- Muntoni, F., Brockington, M., Torelli, S., and Brown, S.C. (2004). Defective glycosylation in congenital muscular dystrophies. *Curr. Opin. Neurol.* **17**, 205–209.
- Noguchi, S., Wakabayashi, E., Imamura, M., Yoshida, M., and Ozawa, E. (2000). Formation of sarcoglycan complex with differentiation in cultured myocytes. *Eur. J. Biochem.* **267**, 640–648.
- Ohlendieck, K., Matsumura, K., Ionasescu, V.V., Towbin, J.A., Bosch, E.P., Weinstein, S.L., Sernett, S.W., and Campbell, K.P. (1993). Duchenne muscular dystrophy: deficiency of dystrophin-associated proteins in the sarcolemma. *Neurology* **43**, 795–800.
- Peng, H.B., Ali, A.A., Daggett, D.F., Rauvala, H., Hassell, J.R., and Smalheiser, N.R. (1998). The relationship between perlecan and dystroglycan and its implication in the formation of the neuromuscular junction. *Cell Adhes. Commun.* **5**, 475–489.
- Peyrard, M., Seroussi, E., Sandberg-Nordqvist, A.C., Xie, Y.G., Han, F.Y., Fransson, I., Collins, J., Dunham, I., Kost-Alimova, M., Imreh, S., and Dumanski, J.P. (1999). The human LARGE gene from 22q12.3-q13.1 is a new, distinct member of the glycosyltransferase gene family. *Proc. Natl. Acad. Sci. USA* **96**, 598–603.
- Rambukkana, A., Yamada, H., Zanazzi, G., Mathus, T., Salzer, J.L., Yurchenco, P.D., Campbell, K.P., and Fischetti, V.A. (1998). Role of alpha-dystroglycan as a Schwann cell receptor for *Mycobacterium leprae*. *Science* **282**, 2076–2079.
- Saito, F., Moore, S.A., Barresi, R., Henry, M.D., Messing, A., Ross-Barta, S.E., Cohn, R.D., Williamson, R.A., Sluka, K.A., Sherman, D.L., et al. (2003). Unique role of dystroglycan in peripheral nerve myelination, nodal structure, and sodium channel stabilization. *Neuron* **38**, 747–758.
- Sugita, S., Saito, F., Tang, J., Satz, J., Campbell, K.P., and Südhof, T.C. (2001). A stoichiometric complex of neuroligins and dystroglycan in brain. *J. Cell Biol.* **154**, 435–445.
- Sunada, Y., Bernier, S.M., Kozak, C.A., Yamada, Y., and Campbell, K.P. (1994). Deficiency of merosin in dystrophic dy mice and genetic linkage of laminin M chain gene to dy locus. *J. Biol. Chem.* **269**, 13729–13732.
- Tomé, F.M.S. (1999). The Peter Emil Becker award lecture 1998. The sage of congenital muscular dystrophy. *Neuropediatrics* **30**, 55–65.
- Williamson, R.A., Henry, M.D., Daniels, K.J., Hrstka, R.F., Lee, J.C., Sunada, Y., Ibraghimov-Beskrovnaya, O., and Campbell, K.P. (1997). Dystroglycan is essential for early embryonic development: disruption of Reichert's membrane in Dag1-null mice. *Hum. Mol. Genet.* **6**, 831–841.
- Yamada, H., Chiba, A., Endo, T., Kobata, A., Anderson, L.V.B., Hori, H., Fukuta-Ohi, H., Kanazawa, I., Campbell, K.P., Shimizu, T., and Matsumura, K. (1996). Characterization of dystroglycan-laminin interaction in peripheral nerve. *J. Neurochem.* **66**, 1518–1524.

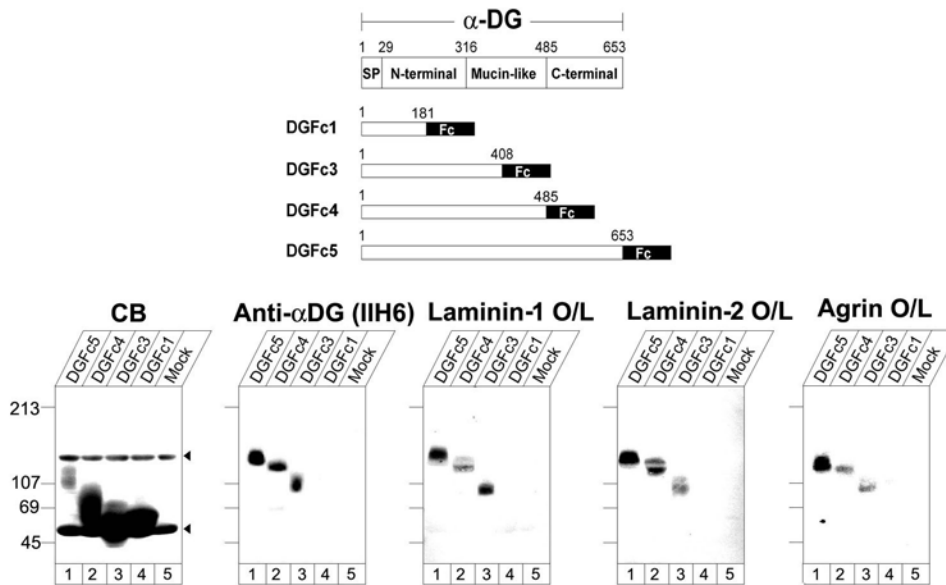
Note Added in Proof

The data referred to throughout as “Barresi et al., 2004 (in press)” are now published online: Barresi, R., Michele, D.E., Kanagawa, M., Harper, H.A., Dovico, S.A., Satz, J.S., Moore, S.A., Zhang, W., Schachter, H., Dumanski, J.P., et al. (2004). LARGE can functionally bypass α -dystroglycan glycosylation defects in distinct congenital muscular dystrophies. *Nat. Med.*, in press. Published online June 6, 2004. 10.1038/nm1059.

Supplemental Experimental Procedures

Construction of DGFc6

The DGFc6 construct was engineered as follows: by using the DGFc5 construct (DGFc5pcDNA3) as a template, the following primers were used to generate PCR fragments of α -DG (T7, 5'-GTAATACGACTCACTATAGGG-3'; DGSSR, 5'-TTCTCGAGTTTCGCGACTGAGCCACGCCACACAGA-3'; Dgmucin, 5'-AAGGCGCCACCCACACCTGTAC-3'; DGXhol, 5'-CACCTCGAGTCCGAGTGGT-3'). A T7-DGSSR PCR fragment (digested with KpnI and NruI, encoding the signal sequence of DG) and a Dgmucin-DGXhol PCR fragment (digested with SfoI and XhoI, encoding the mucin-like domain and the first half of C-terminal domains) were ligated along the XhoI fragment from DGFc5pcDNA3 (encoding the second half of C-terminal domain and the Fc portion).



Supplemental Figure S1. Binding of Extracellular Matrix Ligands to the N-Terminal Half of DGFc Proteins

Schematic representation of DGFc deletion mutant proteins is shown. CB-stained gel of expressed purified proteins is also shown. Western blot and ligand overlay assays were performed on DGFc5, DGFc4, DGFc3, and DGFc1 prepared from conditioned media. IIH6, laminin-1, laminin-2, and agrin bind only to the higher molecular weight purified protein product of DGFc5, DGFc4, and DGFc3, each of which contain the first half of mucin-like domain, but do not bind to DGFc1, which lacks the mucin-like domain. Arrowheads indicate contaminating immunoglobulin light chain, heavy chain, and heavy chain dimers in protein preparations. Abbreviations: O/L, overlay.

