Ⅲ.　研究成果の刊行に関する一覧表

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Ⅳ.　研究成果の刊行物・別刷

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POMT1 mutation results in defective glycosylation and loss of laminin-binding activity in α-DG

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Abstract—Walker–Warburg syndrome (WWS) is a congenital muscular dystrophy associated with neuronal migration disorder and structural eye abnormalities. The mutations in the *O*-mannosyltransferase 1 gene (*POMT1*) were identified recently in 20% of patients with WWS. The authors report on a patient with WWS and a novel *POMT1* mutation. Their patient expressed α-dystroglycan (α-DG) core protein, but fully glycosylated α-DG antibody epitopes were absent, associated with the loss of laminin-binding activity.

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Walker–Warburg syndrome (WWS; MIM 236670), Fukuyama-type congenital muscular dystrophy (FCMD; MIM 253800), and muscle-eye-brain disease (MEB; MIM 253280) are closely related congenital muscular dystrophies (CMDs) with cobblestone lissencephaly and eye abnormalities. Although they are known to be caused by the mutations of different genes encoding putative glycosyltransferases,[1] it now is clear that the mutations of each gene produce overlapping clinical phenotypes.[2,3] In addition, they share a similar pattern of selective loss of α-dystroglycan (α-DG) on immunohistochemical study.[1] A recent study showed hypoglycosylation of α-DG and loss of binding activity of α-DG to laminin, neurexin, and agrin in FCMD, MEB, and the mutant myodystrophy (Large^{myd}) mouse, suggesting a defect in the same post-translational modification pathway of glycosylation in α-DG.[4]

Mutations in the *O*-mannosyltransferase 1 gene (*POMT1*) were implicated recently in 20% of patients with WWS.[5] The laminin-binding site in α-DG is thought to reside in *O*-mannosyl-linked carbohydrate side chains, which may require POMT1 for synthesis.[6]

We report our experience with a Japanese boy with WWS and a novel *POMT1* mutation, who showed reduced glycosylation and loss of laminin-binding activity of α-DG in skeletal muscle.

Additional material related to this article can be found on the Neurology Web site. Go to www.neurology.org and scroll down the Table of Contents for the March 23 issue to find the title link for this article.

Methods. *Patient.* The patient was a Japanese boy aged 3.5 years from apparently nonconsanguineous parents. No other family member was affected. Prenatal ultrasonography showed that the patient had a meningoencephalocele. He was born at gestational week 38 by Cesarean section with a body weight of 2,042 g. He was floppy with an enlarged head. He underwent surgery to remove a meningoencephalocele, and a ventriculoperitoneal shunt was added 21 days after birth. Mild microphthalmia and corneal clouding also were observed. Serum creatine kinase levels were markedly elevated to 600 to 31,000 IU/L (upper normal limit, 70 IU/L). He exhibited markedly delayed milestones. He could not control his head, roll over, or sit. He showed lack of facial expression with an inability to smile and never developed the ability to speak. Brain MRI revealed agyric frontal and temporo-occipital lobes mixed with pachygyric parietal cortex. Hypoplasia of brain stem and cerebellum also was observed (figure 1). EEG showed multifocal spikes, and the muscle biopsy showed marked increase in fatty tissue with evidence of necrosis and regeneration. The mutational analysis for fukutin and protein *O*-mannose β-1,2-N-acetylglucosaminyl-transferase gene (*POMGnT1*) did not show any abnormalities.

Immunohistochemistry and immunoblotting studies. The following antibodies were used: monoclonal anti-α-DG (VIA4-1, Upstate Biotechnology, Lake Placid, NY), polyclonal goat anti-α-DG (GT20ADG),[4] monoclonal anti-β-DG (43DAG1/8D5, Novocastra Laboratories, Newcastle upon Tyne, UK), monoclonal anti-laminin-α2 chain (5H2, Chemicon, Temecula, CA), monoclonal anti-dystrophin C-terminal (Dy8/6C5, Novocastra Laboratories), and monoclonal antisarcoglycan antibodies (Novocastra Laboratories). The detailed techniques of the immunohistochemistry, immunoblotting, and laminin overlay assays have been described previously.[4,7]

Mutation analysis. Genomic DNA was extracted from frozen muscle tissue using standard method with informed consent. Primer

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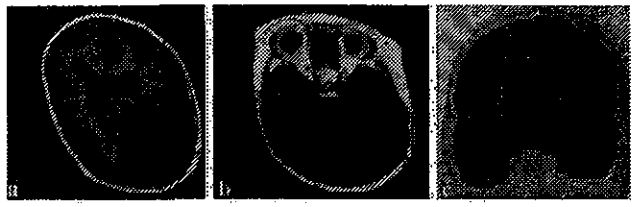

Figure 1. Brain MRI of patient at age 3 years shows agyric frontal and temporo-occipital lobes mixed with pachygyric parietal cortex, hypoplasia of brain stem and cerebellum, and defect of septum pellucidum. The periventricular white matter change (A and B, TR540/TE15; C, TR5400/TE90) also is seen.

pairs were designed to amplify all coding exons and flanking intronic sequences of *POMT1*. The amplified products were sequenced using an ABI PRISM 3100 (Applied Biosystems, Foster City, CA). For the detection and screening of L421del (1260 to 1262 delCCT) in exon 13 of *POMT1*, primers F-CAGTAGCAGCAACTCATGGG, R-ACGGTTGTGGCTGCTATAGC, and restriction enzyme *AvaI* were used. One hundred healthy Japanese individuals served as control subjects.

Results. *Immunohistochemical and immunoblotting analyses.* The immunohistochemical analysis revealed an almost complete loss of immunoreactivity with VIA4-1 anti-α-DG antibody in the patient, whereas anti-α-DG core protein GT20ADG showed membrane staining in each muscle fiber (figure 2). Immunoreaction against the laminin-α2 chain was reduced slightly, but β-DG (see figure 2), dystrophin, and sarcoglycans (not shown) were well preserved.

Immunoblotting analysis using GT20ADG showed a band with a reduced molecular mass, whereas VIA4-1 showed no detectable band for α-DG in the patient (figure 3, A and B). The molecular weight shift observed in our patient (>60 kDa) was almost identical to those reported in FCMD and MEB.[4] On laminin overlay assay, the patient's muscle showed an almost complete loss of laminin-binding activity of α-DG (figure 3C).

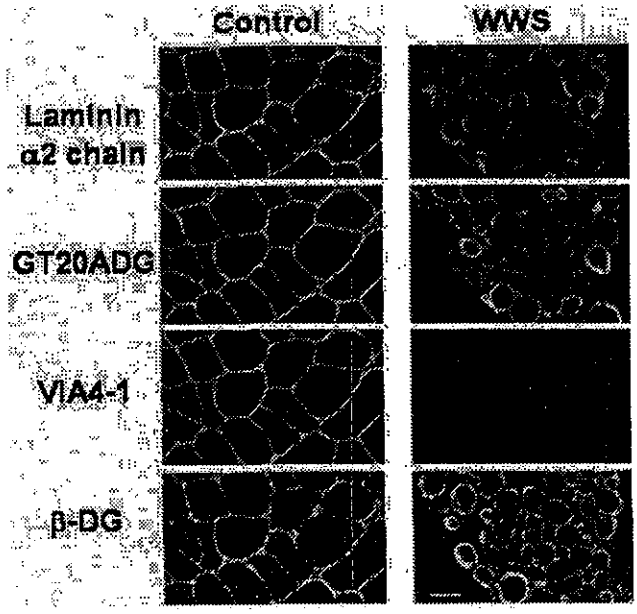

Figure 2. In the patient (with Walker–Warburg syndrome [WWS]), a complete loss of immunoreactivity is observed with the monoclonal antibody VIA4-1 against α-dystroglycan (α-DG), whereas it appears normal around muscle fibers when the polyclonal antibody GT20ADG against α-DG was used. β-DG is well preserved, but the laminin-α2 chain shows mild reduction; bar = 20 μm.

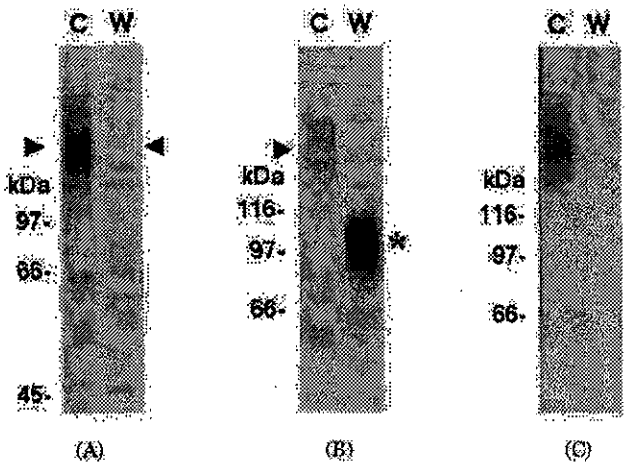

Figure 3. (A) The immunoblotting study using the antibody VIA4-1 showed a broad band around 156 kDa (arrowheads) in control skeletal muscle (C) that is undetectable in the patient (W). (B) The immunoblotting study using the antibody GT20ADG showed a band with a reduced molecular mass (≅90 kDa, asterisk) in the patient, whereas the normal band of α-dystroglycan (α-DG) at 156 kDa was detected in the control. (C) The laminin overlay assay showed loss of band in the patient, suggesting there is an almost complete loss of laminin-binding activity in α-DG from the patient's muscle. M = molecular mass.

Mutation analysis. We found a homozygous deletion of three base pairs (1260 to 1262 delCCT) in *POMT1*, which is expected to delete single amino acid leucine at position 421 (see figure E-1A on the *Neurology* Web site). No identical mutation was present in 100 normal Japanese control subjects (see figure E-1B on the *Neurology* Web site). The amino acid sequence alignment showed that the deleted amino acid leucine and surrounding primary sequence are highly conserved among different species (see figure E-1C on the *Neurology* Web site).

Discussion. In this study, we identified a deletion of the single amino acid leucine at position 421 of *POMT1* from the patient's DNA. This is considered to be a causative mutation for several reasons. First, the same change was not found among 100 Japanese control subjects. Next, the deleted amino acid leucine is located within a highly conserved region of the gene and is conserved among different species. A previously reported V428D mutation also is only seven amino acids downstream to ours.[5] These findings suggest that this conserved region plays an important role in the proper function of the protein.

Our patient showed exceptionally long survival for WWS because most patients with WWS die during infancy and rarely survive beyond age 3

years. Because complete agyria is common in patients with WWS, the pattern of the cortical dysplasia in our patient—agyria mixed with parietal pachygyria in MRI—could be considered milder than typical WWS. Thus, our patient showed intermediate phenotype between WWS and MEB in terms of clinical severity and MRI finding. However, the diagnosis of WWS seems more accurate than MEB or FCMD in our patient because he had a meningoencephalocele, which is almost exclusively seen in WWS.[8] There are some recent reports documenting the remarkable clinical variability originating from the mutation of the same genes causing CMDs, and thus, it is possible for *POMT1* mutations to produce a more benign WWS phenotype like that seen in our patient.[2,3,9]

Although the immunoreactivity against the antibody VIA4-1 was lost completely in our patient, the reaction against the antibody GT20ADG was well preserved. Because the antibody GT20ADG recognizes the core protein of α-DG, our results indicate that α-DG localizes at the surface membrane of skeletal muscle but that the epitope for VIA4-1 antibody was specifically disrupted or masked.[4] Because the antibody VIA4-1 is thought to recognize, at least in part, the carbohydrate epitope of α-DG, the glycosylation status of α-DG is likely to be altered in our patient.[4] The results of immunoblotting and laminin overlay assays further support this speculation. The α-DG from normal skeletal muscle is a heavily glycosylated protein with a molecular weight of 156 kDa. Thus, the reduction of molecular weight, seen only by GT20ADG, is likely to be related to the loss of glycoconjugates from α-DG. Accordingly, the loss of laminin-binding activity shown in the laminin overlay assay most likely is caused by the loss of glycoconjugate, which is thought to be a laminin-binding ligand of α-DG.[6] A brain-selective deletion of dystroglycan in mice was shown recently to cause CMD-like brain malformations and defective laminin binding, giving strong evidence that abnormalities of dystroglycan underlie the neuronal migration disorder seen in this group of disorders.[10] Because similar pattern of glycosylation-deficient disruption of dystroglycan function has been observed in FCMD, MEB, and Large^{myd} mice,[4] it is likely that WWS shares a similar pathomechanism with them. In addition, the complete loss of the laminin-binding activity of α-DG in our patient with WWS is almost identical to that observed in FCMD.[4]

Our study proves that WWS caused by the mutation of *POMT1* coexists with other types of CMDs in the Japanese population. We also demonstrated that WWS is a member of the group of CMDs associated with defective glycosylation of α-DG that results in the loss of function of α-DG as a matrix receptor.

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A new diagnostic test for VLCAD deficiency using immunohistochemistry

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Abstract—*Background:* Muscle pathology is often unhelpful in elucidating the specific underlying abnormality in patients with metabolic myopathy with rhabdomyolysis, including very-long chain acyl-CoA dehydrogenase (VLCAD) deficiency. Biochemical analyses require large amounts of biopsy samples for each enzyme assay. *Objective*: To develop a more efficient diagnostic method for VLCAD deficiency. *Methods*: The authors performed immunohistochemical analysis using an antibody to VLCAD on muscles from 344 patients (226 men and 118 women) without a specific diagnosis who had at least one of the following symptoms: myoglobinuria, high CK level, muscle pain, muscle stiffness, sudden infant death syndrome, and Reye-like syndrome. *Results*: Immunoreactivity to VLCAD was absent or markedly reduced in 13 patients. Biochemical analyses confirmed that all these patients had low enzymatic activity and reduced amount of protein. They all had the myopathic phenotype. The authors identified homozygous or compound heterozygous mutations in all of them. All recombinant proteins had reduced enzymatic activity except for 128G>A (G43D) and 796C>G (P266A) mutants, indicating that they are neutral polymorphisms. *Conclusions*: The new screening method for the detection of VLCAD deficiency using an immunohistochemical technique identified 13 new Japanese patients with VLCAD deficiency.

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Muscle biopsies are often performed to look for the underlying metabolic abnormalities in patients with rhabdomyolysis. However, pathologic findings are frequently nonspecific and extensive biochemical analyses are necessary to make a specific diagnosis of metabolic myopathy including glycogenosis, β-oxidation enzyme defects, and carnitine palmityl transferase (CPT) deficiency. Because large amounts of muscle tissue are required for each enzyme assay, biochemical screening is sometimes incomplete.

Mitochondrial fatty acid oxidation is carried out by the integrated action of the carnitine-dependent transport of long-chain fatty acids across the mitochondrial membrane and the intramitochondrial β-oxidation cycle. The first step of this cycle is catalyzed by four fatty acyl-CoA dehydrogenases, which have different substrate chain-length specificities. Short-chain, medium-chain, and long-chain acyl-CoA dehydrogenases are matrix proteins with specificity for C_4-C_6, C_4-C_{12}, and C_{10}-C_{18} fatty acyl-CoAs, while very-long chain acyl-CoA dehydrogenase (VLCAD) is associated with the mitochondrial inner membrane and is specific for C_{14}-C_{24} fatty acyl-CoAs.[1,2] In 1999, 54 cases of genetically confirmed VLCAD deficiency were reviewed and 19 cases have been reported thereafter in the English language literature (table 1).[3]

VLCAD deficiency is clinically classified into three forms: severe form, or severe childhood form, with early onset, high mortality, and a high incidence of cardiomyopathy; an intermediate form, or milder childhood form, with later onset, usually with hypoketotic hypoglycemia as the main presenting feature in which cardiac involvement is rare and outcome is more favorable; and a myopathic form, or adult form, with isolated skeletal muscle involvement, rhabdomyolysis, and myoglobinuria, usually triggered by exercise or fasting.[3] Their muscle biopsies usually show nonspecific changes.

We therefore thought that there may be patients with VLCAD deficiency among our cases with such symptoms whose muscle biopsies were not diagnostic. To identify patients with VLCAD deficiency and also to develop an efficient screening method, we

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Table 1 Clinical classification and number of genetically confirmed patients

Form	Onset	Main manifestation	Number (%) of genetically confirmed patients	
			Previous reports	This report
Severe	Neonatal to early childhood	Cardiomyopathy	34 (47)	0
Intermediate	Childhood	Hypoketotic hypoglycemia	24 (33)	0
Myopathic	Adolescence to adulthood	Rhabdomyolysis	15 (20)	13
Total			73	13

performed immunohistochemistry for VLCAD on sections of skeletal muscle from such patients.

Methods. *Patients.* As candidates, we selected muscle biopsies from 344 patients (226 men and 118 women) with one or more of the following: myoglobinuria, high CK level, muscle pain, muscle stiffness, sudden infant death syndrome (SIDS), and Reye-like syndrome which showed no diagnostic change after microscopic examinations with a full battery of histochemical and immunohistochemical stains against a variety of sarcolemmal proteins, including dystrophin, sarcoglycans, dystroglycans, merosin, and collagen VI. Biopsy specimens were frozen in liquid nitrogen-cooled isopentane. We performed indirect immunohistochemical staining with the rabbit polyclonal antibody to VLCAD on 7 μm cryosections of the skeletal muscle.[4] All sections were stained using an automated immunohistochemical stainer (Nex, Bentana Medical Systems, Tucson, AZ). As disease control, we used muscle from a patient with genetically confirmed VLCAD deficiency reported previously.[5]

Enzyme assay. Acyl-CoA dehydrogenase activities toward octanoyl–CoA (C8-DH) and palmitoyl-CoA (C16-DH), as substrates, were measured according to the ferricenium method described by Lehman et al.[6]

Immunoblot analysis. Thirty micrograms of protein from muscle extracts was applied to a 10% SDS-PAGE gel, and Western blotting was performed on a nitrocellulose sheet using an anti-human VLCAD antibody. Protein was detected with anti-rabbit antibody HRP-linked IgG (Cell Signaling, Beverly, MA) and ECL Western blotting detection reagents (Amersham, Buckinghamshire, UK). Anti-human trifunctional protein (TP) antibody served as positive controls, and we compared the amount of VLCAD protein with TP-α.[7]

Mutation analysis. Human VLCAD gene is located on the short arm of chromosome 17, spanning a 5.3 kb region, and contains 20 exons.[8,9] To identify mutations, we sequenced the VLCAD gene in DNA from patients with VLCAD deficiency identified in this study. Genomic DNA was extracted from the muscle biopsies by a standard method.[10] We designed intron-based oligonucleotide primers and amplified two overlapping fragments which cover the entire VLCAD gene region. PCR conditions were as follows: the first fragment, denaturation at 94 °C for 1 minute; annealing at 63 °C for 2 minutes; and extension at 72 °C for 2 minutes, and 30 cycles of PCR, and the other, denaturation at 94 °C for 1 minute; annealing at 67 °C for 2 minutes; and extension at 72 °C for 2 minutes, and 30 cycles of PCR. LA Taq polymerase (Takara, Tokyo, Japan) was used throughout our study. We directly sequenced the amplified fragments using 13 primers on an automated DNA sequencer (Applied Biosystems 3100, Foster, CA) by the Big Dye cycle sequencing kits (Applied Biosystems) by following the manufacturer's protocol. Nucleotide and amino acid numbering were carried out according to the VLCAD cDNA sequence deposited in GenBank.

Enzymatic assay of recombinant proteins. To determine which missense mutations are actually pathogenic in patients with more than two identified "mutations," we measured the enzyme activity of recombinant proteins with those mutations. We extracted total muscle RNA from patients who had three or four mutations using ISOGEN (Nippon Gene, Tokyo, Japan), synthesized first-strand cDNA by Super Script III First-Strand Synthesis System (Invitrogen, Carlsbad, CA), and PCR amplified the VLCAD cDNA using exon-based oligonucleotide primers. PCR products were then cloned into the pGEM-T easy vector (Promega, Madison, WI).

Seven mutant VLCAD cDNAs, 128G>A, 790A>G, 1748C>T, 796C>G, 1153C>T, 128G>A + 790A>G, and 796C>G + 1153C>T, as well as the wild type of the VLCAD cDNA were prepared by in vitro mutagenesis, using QuickChange Site-Directed mutagenesis kits (Stratagene, La Jolla, CA). These cDNAs were inserted separately into a pCAGGS expression vector. We used the LipofectAMINE PLUS Reagent according to the protocol. Briefly, 3.5 μg of the expression vectors were transfected into 5×10^5 COS 7 transformed fibroblasts. One point five micrograms of pCAGGS expression vector for human cytosolic thiolase cDNA was cotransfected to monitor transfection efficiency. The cells were harvested after 48 hours incubation at 37 °C, and the enzyme activities of C16-dehydrogenase and acetoacetyl-CoA thiolase were assayed.

Results. *Immunochemical analysis.* Immunoreactivity was markedly reduced in five patients and absent in nine patients (see supplementary figure E-1 at www.neurology.org, table 2), including the patient previously reported.

Enzyme assay. The C16-DH activity in all 13 patients identified by immunohistochemistry as well as the disease control was reduced compared to normal controls, whereas the C8-DH activity was all within the normal range (see table 2), confirming that all 13 patients had VLCAD deficiency. There was no correlation between the residual activity of the long chain acyl-CoA esters and the immunostaining pattern.

Immunoblot analysis. All 13 patients had decreased amounts of VLCAD as compared with TP-α (see table 2). There was no significant correlation between the amount of VLCAD protein and the immunostaining pattern.

Mutation analysis. Sequence analysis of the entire VLCAD gene revealed homozygous or compound heterozygous mutations in all 13 patients (see table 2). Among them, 997insG, 419G>A (G140E), 1800A>C (K600N), 796C>G (P266A), 1748C>T (S583L), 1153C>T (R385W), 1801delA, and 642–643delCT have not been previously reported. These mutations were absent in 100 genomic DNA samples from control Japanese individuals, suggesting that these mutations were pathogenic. An unexpected finding was the presence of the 128G>A (G43D) in addition to other homozygous or compound heterozygous mutations in six patients, raising the possibility of a polymorphism. Among them, Patient 10 had four missense mutations and Patients 2, 11, 16, and 17 had three mutations. By cloning the PCR products, we confirmed that 128G>A and 790A>G were present in the same allele, as were 128G>A and 1801delA. We also confirmed that 796C>G and 1153C>T were in the same allele.

Enzymatic assay of recombinant proteins. Each transfection gave similar levels of acetoacetyl-CoA thiolase ac-

Table 2 Enzyme activities, protein amount, and identified mutations

Patient	Immunohistochemistry	Enzyme activities C16/C8	Relative protein amount VLCAD/TP-α	Nucleotide change	Location (exon)	Amino acid change	Zygosity
1	A	0.89	2.00	128G>A*	2	G43D	Hetero
				790A>G	9	K264E	Hetero
				1349G>A	14	R450H	Hetero
2	A	0.68	0.91	419G>A	6	G140E	Hetero
				1800A>C	19	K600N	Hetero
3	A	0.77	1.24	128G>A*	2	G43D	Homo
				790A>G	9	K264E	Homo
4	R	0.80	2.55	128G>A*	2	G43D	Hetero
				790A>G	9	K264E	Hetero
				1246G>A	12	A416T	Hetero
5	A	0.69	1.30	1144A>C	11	K382Q	Homo
6	A	0.67	1.47	128G>A*	2	G43D	Hetero
				790A>G	9	K264E	Hetero
				1748C>T	18	S583L	Hetero
7	A	0.48	0.75	644–647 del GTCT	8	Frame shift	Hetero
				1144A>C	11	K382Q	Hetero
8	R	0.46	0.83	1144A>C	11	K382Q	Homo
9	R	0.67	1.65	128G>A*	2	G43D	Hetero
				1801 del A	19	Frame shift	Hetero
				1246G>A	12	A416T	Hetero
10	R	0.69	1.70	128G>A*	2	G43D	Hetero
				790A>G	9	K264E	Hetero
				796C>G*	9	P266A	Hetero
				1153C>T	11	R385W	Hetero
11	A	0.43	2.40	642–64 del CT	8	Frame shift	Hetero
				1349G>A	14	R450H	Hetero
12	A	0.79	1.10	1349G>A	14	R450H	Homo
13	R	0.72	3.05	1246G>A	12	A416T	Hetero
				997 ins G	10	Frame shift	Hetero
DC†	A	0.61	2.24	1246G>A	12	A416T	Hetero
				1349G>A	14	R450H	Hetero
NC		2.23 ± 0.17 (n = 8)	5.05 ± 1.56 (n = 8)				

* Neutral polymorphism based on enzymatic assay of recombinant proteins.
† Previously reported case.[17]

A = completely absent; R = reduced; DC = disease control; NC = normal control.

tivity, thereby confirming the transfection efficiency to be similar among each transfection (data not shown). All recombinant proteins showed reduced enzymatic activity except for 128G>A (G43D) and 796C>G (P266A) mutants (see supplementary figure E-2 at www.neurology.org), clearly demonstrating that they are neutral polymorphisms. The recombinant proteins with two mutations, 128G>A (G43D) and 790A>G (K264E), and 796C>G (P266A) and 1153C>T (R385W), also demonstrated similar reduction in enzymatic activity as in 790A>G and 1153C>T single mutants, confirming that 128G>A and 796C>G have no effect on enzymatic activity.

Clinicopathologic features. The clinical features of the 13 patients with VLCAD deficiency identified in this study and the one used as a disease control are summarized in table 3. There were six men and eight women. Ages at onset varied from 3 to 29 years. The mean age at biopsy was 23 years with a range of 8 to 40 years. The main clinical features at presentation were muscle pain, muscle stiffness, muscle weakness, and general fatigue, usually with triggers such as exercise, infection, and cold temperature, but sometimes without any trigger. Although one patient had no trigger, all patients had had more than one episode. Ten patients had myoglobinuria, and two of them

Table 3 Clinical features

Patient	Sex	Age at onset, y	Age at biopsy, y	Trigger	Main symptoms	Myoglobin uria	CK level	Forearm exercise test	Hemodialysis	Family history
1	M	Adolescence	32	E	P	+	50,000	No lactate increase	+	–
2	M	Adolescence	34	E	P, G	+	Unknown	Not done	+	–
3	M	Adolescence	33	E, I	P	+	10,000	Not done	–	–
4	M	Adolescence	27	E, C	P, S	+	44,000	Normal	–	–
5	M	16	16	E	P	+	100,000	Not done	–	+
6	M	16	34	E	S	+	77,000	No lactate increase	–	+
7	F	8	20	F	P, W	+	10,000	No lactate increase	–	–
8	F	3	22	E	P, S	+	130,000	No lactate increase	–	–
9	F	10	19	E	P, G	+	26,000	Normal	–	–
10	F	14	17	None	P, W	+	25,000	Not done	–	+
11	F	15	16	E	P	–	19,000	No lactate increase	–	–
12	F	29	40	E	P, G	–	90,000	Normal	–	–
13	F	13	14	E, I, C	P, W	–	44,000	Not done	–	–
DC	F	6	8	E, F	P, G	–	10,000	Not done	–	–

E = exercise; P = muscle pain; G = general fatigue; I = infection; C = cold temperature; S = muscle stiffness; W = muscle weakness; F = fatigue; DC = disease control.

needed hemodialysis for acute renal failure. None had cardiomyopathy or hypoglycemia. All 14 patients had the myopathic form with normal development and normal intelligence.

Laboratory tests showed markedly elevated serum CK levels from 10,000 to 130,000 IU/L (normal range: <220) during the attacks. However, CK levels normalized within 2 weeks. Blood glucose, ammonium, and lactate levels were within the normal range. Forearm exercise test was performed in eight patients, and five patients showed no elevation in the lactate level.

Three patients had a positive family history. Patient 5 had a younger brother with similar muscle pain. The older sister of Patient 6 had died of heart failure at the age of 23 years. Patient 11 had an older sister with repeated episodes of rhabdomyolysis.

In most patients muscle biopsy showed mild fiber size variation, especially of type1 fibers (see supplementary table E-1 at www.neurology.org). In more than 50% of the patients there were type 2C fibers. A few necrotic and regenerating fibers were found in only three patients.

Discussion. We identified 13 new patients with VLCAD deficiency by immunohistochemistry. All 13 patients had various homozygous or compound heterozygous mutations, including 8 novel mutations. Among them, 128G>A (G43D) and 796C>G (P266A) were neutral polymorphisms, as evidenced by the preserved enzymatic activities in recombinant proteins. Point mutations were frequently found in exon 9 (790A>G, 21.4%), exon 11 (1144A>C, 17.9%), and exon 14 (1349G>A 17.9%). Because five of six patients with 128G>A had 790A>G and since they existed in the same allele, 128G>A should be considered to be a polymorphism tightly linked to 790A>G, suggesting the presence of a common founder effect.

Previous reports indicated that severe and intermediate forms are frequent while the myopathic form is rare.[3] To our knowledge, 73 patients with genetically confirmed VLCAD deficiency have been reported in the English literature, with severe, intermediate, and myopathic forms comprising 47%, 33%, and 20% (see table 1).[3] Interestingly, all 13 patients as well as the disease control who was identified before this study had the myopathic form, suggesting that the myopathic form may be more prevalent in Japan. Alternatively, a substantial number of patients with the myopathic form may have been unrecognized.

Five patients had no lactate increase on ischemic forearm exercise test even though β-oxidation disorders theoretically should have a normal response. This was most likely due to improper test procedure.

The older sister of Patient 6 had died of heart failure at age 23. Although there is a report of a 5-year-old boy with VLCAD deficiency with acute cardiomyopathy,[11] she may not have had VLCAD deficiency because cardiomyopathy in adults with VLCAD deficiency is very rare.

Immunoblot analysis showed that the VLCAD protein was reduced, but not absent in any patient. In the severe phenotype, no VLCAD protein or only a trace amount, if any, is present, indicating the inverse correlation between the residual amount of the VLCAD protein and clinical severity.[12,13] Our patients may have sufficient residual VLCAD activity to maintain metabolic status when receiving adequate nourishment without any stress. Most likely during exercise, infection, fasting, or exposure to cold, the residual VLCAD activity is insufficient to sustain the demands of muscle fatty acid oxidation, causing rhabdomyolysis.

Immunostaining pattern did not show clear corre-

lation either with the residual protein amount on immunoblot or to residual enzymatic activity. This may be attributed to the non-quantitative nature of indirect immunohistochemical methods. Although we did not find any equivocal case, the sensitivity and specificity of our method should be investigated further.

Biochemical examination, organic acid analysis in urine, and acyl-carnitine analysis in dried blood spots or serum using tandem mass spectrometry are useful for initial screening.[14] VLCAD deficiency can be diagnosed by enzyme activity in fibroblasts, lymphocytes, or muscle.[15] Muscle biopsy is not necessary for the diagnosis when VLCAD deficiency is specifically suspected. However, since symptoms of VLCAD deficiency resemble those in other metabolic myopathies including glycogenosis and CPT deficiency and because muscle biopsy is a safe procedure, albeit more invasive than blood sampling, muscle biopsies are frequently performed in such patients. Therefore, our immunohistochemical method is useful to make a specific diagnosis of VLCAD deficiency. In addition, our method requires only a single section of frozen muscle in contrast to biochemical enzymatic assays. According to previous reports, muscle biopsy sometimes shows lipid storage.[12,16] However, our patients did not show any specific changes on muscle pathology, but they were not completely normal. This may be because all our patients had the myopathic form and, therefore, milder symptoms. Absence of specific histopathologic findings emphasizes the importance of our immunohistochemical method in identifying the possibly unrecognized patients with VLCAD deficiency.

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ARTICLES

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LARGE can functionally bypass α-dystroglycan glycosylation defects in distinct congenital muscular dystrophies

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Several congenital muscular dystrophies caused by defects in known or putative glycosyltransferases are commonly associated with hypoglycosylation of α-dystroglycan (α-DG) and a marked reduction of its receptor function. We have investigated changes in the processing and function of α-DG resulting from genetic manipulation of LARGE, the putative glycosyltransferase mutated both in Large^{myd} mice and in humans with congenital muscular dystrophy 1D (MDC1D). Here we show that overexpression of LARGE ameliorates the dystrophic phenotype of Large^{myd} mice and induces the synthesis of glycan-enriched α-DG with high affinity for extracellular ligands. Notably, LARGE circumvents the α-DG glycosylation defect in cells from individuals with genetically distinct types of congenital muscular dystrophy. Gene transfer of *LARGE* into the cells of individuals with congenital muscular dystrophies restores α-DG receptor function, whereby glycan-enriched α-DG coordinates the organization of laminin on the cell surface. Our findings indicate that modulation of LARGE expression or activity is a viable therapeutic strategy for glycosyltransferase-deficient congenital muscular dystrophies.

Fukuyama congenital muscular dystrophy (FCMD), muscle-eye-brain disease (MEB), and Walker-Warburg syndrome (WWS) are congenital muscular dystrophies with similar associated defects in brain development[1–3]. The genes involved in these diseases encode putative or known glycosyltransferases: mutations in *FCMD* (encoding fukutin) are responsible for FCMD, mutations in *POMGNT1* for MEB, and mutations in *POMT1* for a percentage of WWS[2,4,5]. WWS can also be caused by severe mutations in *FCMD*, in *POMGNT1* or in *FKRP*, the gene encoding fukutin-related protein, which is another putative glycosyltransferase responsible for MDC1C and limb-girdle muscular dystrophy 2I (LGMD2I)[6]. The common biochemical feature in these disorders is the abnormal glycosylation of α-DG, a ubiquitous external membrane protein, which suggests that α-DG may be a potential target of these enzymes[7–9].

Dystroglycan, which is present in skeletal muscle as part of the dystrophin-glycoprotein complex (DGC)[10], comprises two subunits, the extracellular α-DG and the transmembrane β-DG[11,12], derived from post-translational cleavage of a precursor polypeptide encoded by the *DAG1* gene. Both subunits undergo glycosylation, but whereas β-DG is consistently detected with a molecular mass (M_r) of 43 kDa, the mass of α-DG varies from 120 kDa to 200 kDa, owing to developmental and tissue-specific glycosylation of a 74-kDa core polypeptide[12]. We have previously shown that the inability of aberrantly glycosylated α-DG to bind extracellular matrix ligands such as laminin, agrin and neurexin causes muscle degeneration and abnormal neuronal migration in individuals with MEB and FCMD[13].

Animal models of these diseases are not available or are embryonically lethal[14], although the spontaneous mouse model Large^{myd} closely resembles the human diseases[13,15]. The convergence of clinical and biochemical phenotypes of persons with congenital muscular dystrophy and Large^{myd} mice suggests that the same glycosylation pathway of α-DG may be affected[13]. *LARGE*, the gene mutated in Large^{myd} mice and in persons with MDC1D[16,17], encodes a putative glycosyltransferase with two structurally distinct domains that are homologous to bacterial α-glycosyltransferase and mammalian β-1,3-*N*-acetylglucosaminyltransferase[16,18]. However, because the activity of glycosyltransferases of this class has not been shown to modify α-DG, it is possible that LARGE affects the glycosylation pathway of α-DG by modulating the activity of other enzymes.

Here we have evaluated the effect of LARGE on glycosylation of α-DG by genetic manipulation of *LARGE in vivo* and *in vitro*. In particular, we have investigated whether glycosylation in cells from individuals with congenital muscular dystrophy can be modulated or enhanced by expression of LARGE. Unexpectedly, we show that expression of

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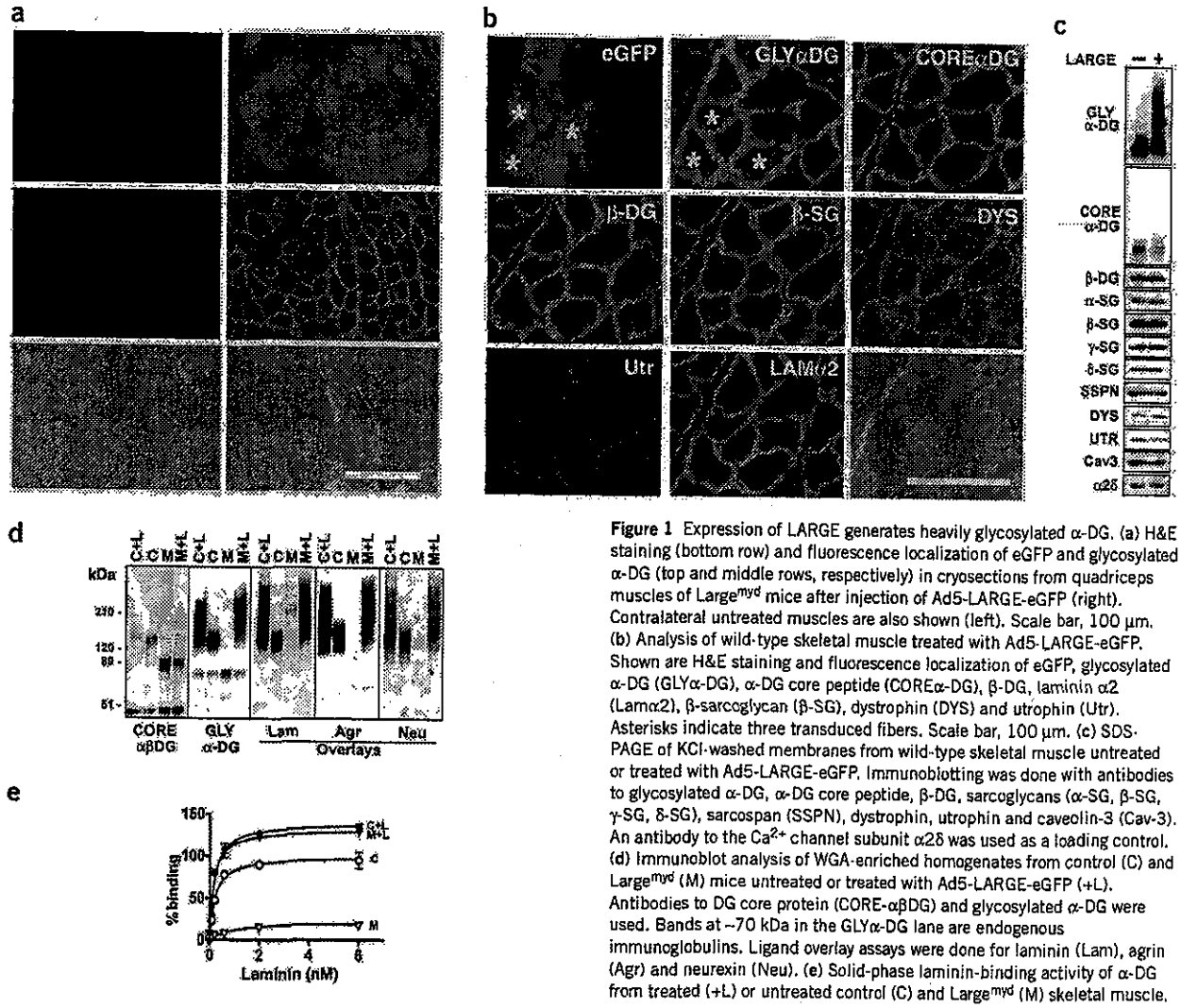

Figure 1 Expression of LARGE generates heavily glycosylated α-DG. (a) H&E staining (bottom row) and fluorescence localization of eGFP and glycosylated α-DG (top and middle rows, respectively) in cryosections from quadriceps muscles of Large^{myd} mice after injection of Ad5-LARGE-eGFP (right). Contralateral untreated muscles are also shown (left). Scale bar, 100 μm. (b) Analysis of wild-type skeletal muscle treated with Ad5-LARGE-eGFP. Shown are H&E staining and fluorescence localization of eGFP, glycosylated α-DG (GLYα-DG), α-DG core peptide (COREα-DG), β-DG, laminin α2 (Lamα2), β-sarcoglycan (β-SG), dystrophin (DYS) and utrophin (Utr). Asterisks indicate three transduced fibers. Scale bar, 100 μm. (c) SDS-PAGE of KCl-washed membranes from wild-type skeletal muscle untreated or treated with Ad5-LARGE-eGFP. Immunoblotting was done with antibodies to glycosylated α-DG, α-DG core peptide, β-DG, sarcoglycans (α-SG, β-SG, γ-SG, δ-SG), sarcospan (SSPN), dystrophin, utrophin and caveolin-3 (Cav-3). An antibody to the Ca^{2+} channel subunit α2δ was used as a loading control. (d) Immunoblot analysis of WGA-enriched homogenates from control (C) and Large^{myd} (M) mice untreated or treated with Ad5-LARGE-eGFP (+L). Antibodies to DG core protein (CORE-αβDG) and glycosylated α-DG were used. Bands at ~70 kDa in the GLYα-DG lane are endogenous immunoglobulins. Ligand overlay assays were done for laminin (Lam), agrin (Agr) and neurexin (Neu). (e) Solid-phase laminin-binding activity of α-DG from treated (+L) or untreated control (C) and Large^{myd} (M) skeletal muscle.

LARGE leads to a recovery of α-DG function as a receptor not only in Large^{myd} mouse muscle but also in cell cultures derived from individuals with FCMD, MEB and WWS.

RESULTS

LARGE prevents muscular dystrophy in Large^{myd} mice

To investigate the effect of LARGE on α-DG glycosylation, we generated an adenovirus expressing LARGE and enhanced green fluorescent protein (Ad5-LARGE-eGFP). Gene transfer was done in Large^{myd} and control littermate pups (2–4 days of age). Adenovirus-injected and non-injected contralateral leg muscles were examined for expression of DG by using antibodies to core and glycosylated α-DG. The transduction efficiency estimated by eGFP expression was 40–80% (Fig. 1a). All transduced muscle fibers reacted with antibodies to glycosylated α-DG. The expression of other DGC components did not change (Fig. 1b, Supplementary Fig. 1 online and data not shown). Dystrophic histological features, such as internally placed nuclei, were reduced by more than 70% in the muscles of transduced Large^{myd} mice, and a significant reduction in fiber size variation was observed after treatment ($P = 0.007$; Supplementary Fig. 2 online). Analysis of Large^{myd} mice treated with Ad5-LARGE-eGFP at older ages (12 d to 5 weeks) did not produce conclusive results, owing to an inflammatory response to the virus and rapid loss of expression of eGFP and glycosylated α-DG.

Notably, *LARGE* gene transfer in healthy muscle did not cause histological abnormalities (Fig. 1b and Supplementary Fig. 2 online). Unexpectedly, we detected stronger expression of α-DG glycoepitopes at the sarcolemma of transduced control fibers, suggesting that *LARGE* gene transfer leads to the synthesis of α-DG species that are more enriched in glycans (Fig. 1b). Indeed, immunoblots of KCl-washed membranes from control skeletal muscle showed that after treatment with Ad5-LARGE-eGFP there were no changes in the DGC, aside from more heavily glycosylated α-DG (Fig. 1c).

Immunoblot analysis of wheat germ agglutinin (WGA)-enriched fractions from treated control and Large^{myd} muscles detected glycosylated α-DG as a broad band with an M_r of 150–300 kDa (Fig. 1d). Core peptide antibodies recognized α-DG with a high M_r only poorly, probably because of epitope masking by the additional carbohydrate moieties. Residual hypoglycosylated α-DG (90 kDa) from nontransduced fibers was also detected in treated Large^{myd} muscle[13]. Ligand overlay assays showed the rescue of α-DG receptor function in treated

Large^{myd} muscle and the persistent ligand-binding activity of heavily glycosylated α-DG (Fig. 1d).

Quantitative solid-phase laminin-binding assays of WGA-enriched fractions showed enhanced activity of nondenatured α-DG in Large^{myd} and control muscles after viral transduction (Fig. 1e). We obtained similar results in analogous experiments done on primary myoblast cultures from control and Large^{myd} mice. Notably, forced expression of LARGE induced synthesis of glycosylated α-DG in all transduced cell types: myotubes, myoblasts and fibroblasts (data not shown).

To determine whether functional benefit is conferred by *LARGE* gene transfer, we exercised Large^{myd} mice and control littermates that had been injected with Ad5-LARGE-eGFP in their hamstring muscles, where the transduction efficacy was higher. Uptake of Evans blue dye (EBD) showed that skeletal muscles of Large^{myd} mice were susceptible to exercise-induced sarcolemmal injury. Quantitative image analysis indicated that uptake of EBD in Large^{myd} muscles decreased, on average, from 11.3% to 1.4% after *LARGE* gene transfer. We did not observe EBD uptake in transduced areas of Large^{myd} muscle or control skeletal muscle (**Supplementary Fig. 2** online and not shown), indicating improved functional features in treated muscles and an absence of pathological effects from the overexpression of LARGE.

LARGE generates functional α-DG in FCMD myoblasts

Our findings in control and Large^{myd} muscle suggested that LARGE is an essential component of the glycosylation machinery of α-DG. Genetic and biochemical analysis of individuals with dystroglycanopathy indicates that residual glycosylation activity is present[13,19]. We therefore investigated whether the residual activity could be modulated or enhanced by expressing LARGE in cells from individuals with congenital muscular dystrophy. We first examined the expression and biochemical characteristics of α-DG in myoblasts obtained from three individuals with FCMD and one control subject.

The expression of functionally glycosylated α-DG increased throughout the differentiation of control myoblasts into myotubes; by contrast, highly reduced and patchy expression of glycosylated α-DG was observed in FCMD cultures (Fig. 2a), indicating a considerable reduction in fukutin activity in FCMD cells. Immunoblot analysis of WGA-enriched lysates of control myoblasts using antibodies to DG core peptide showed a smaller developmental α-DG isoform of 110–125 kDa that showed binding activity for laminin, neurexin and agrin (Fig. 2b). In FCMD myoblasts, α-DG was identified with antibodies to core DG as a broad band of 90–125 kDa, indicating the expression of variably glycosylated α-DG species (Fig. 2b).

The presence of residual glycosylated α-DG in FCMD myoblasts as judged by immunohistochemistry contrasts with what has been reported in FCMD muscle biopsies[13] and is similar to a phenomenon of transient re-expression of functional α-DG observed in individuals with a mild form of LGMD2I, suggesting that fukutin may be expressed differentially at earlier stages of muscle development. However, antibodies to glycoepitopes of α-DG did not detect the protein on immunoblots. Consistently, no binding of laminin to α-DG was observed, and ligand-binding activity for neurexin and agrin was considerably diminished and only present in a α-DG fraction with an M_r similar to that seen in control cultures (Fig. 2b).

After treatment with Ad5-LARGE-eGFP, all transduced cells in control and FCMD cultures expressed glycosylated α-DG (Fig. 2c). Cell viability, proliferation rate, and the time and degree of fusion did not vary after transduction with Ad5-LARGE-eGFP. Biochemical changes in α-DG glycosylation were similar to those observed *in vivo*: core-DG antibodies weakly detected α-DG with a higher M_r, and did not recognize heavily glycosylated forms (≥200 kDa; Fig. 2d). Notably, overlay assays showed that *LARGE*-transfected FCMD myoblasts gained laminin, agrin and neurexin binding to a highly glycosylated α-DG with an M_r similar to that seen in control cultures

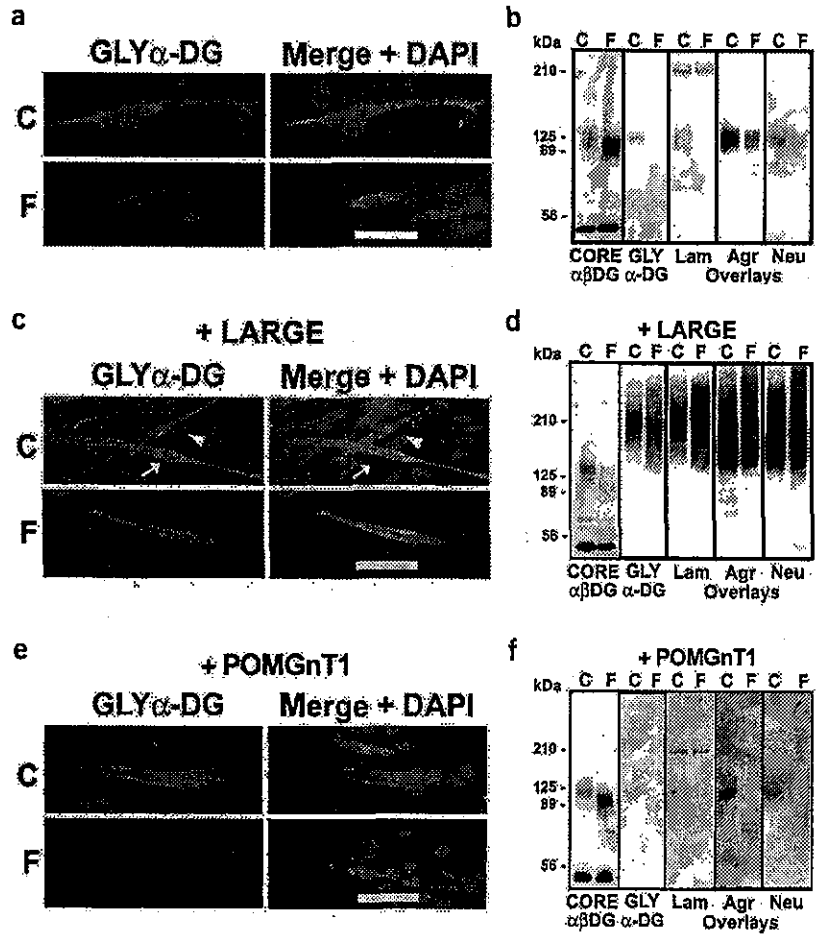

Figure 2 LARGE generates functional α-DG in FCMD myoblasts. (a–f) Analysis of untreated (a,b), or infected with Ad5-LARGE-eGFP (c,d) or Ad5-POMGnT1-eGFP (e,f) control (C) and FCMD (F) cultures. Immunofluorescence shows localization of desmin (a) or eGFP (c,e). Arrows indicate myotubes and arrowheads indicate myoblasts. Scale bars, 10 μm. WGA-enriched lysates of treated cultures were also analyzed by SDS-PAGE followed by immunoblot analysis with antibodies to DG core protein (CORE-αβDG) and glycosylated α-DG (b,d,f). Laminin (Lam), neurexin (Neu) and agrin (Agr) were used for ligand overlay assays. Bands at ~210 kDa in b and f are endogenous laminin. Data are representative of three individuals with FCMD.

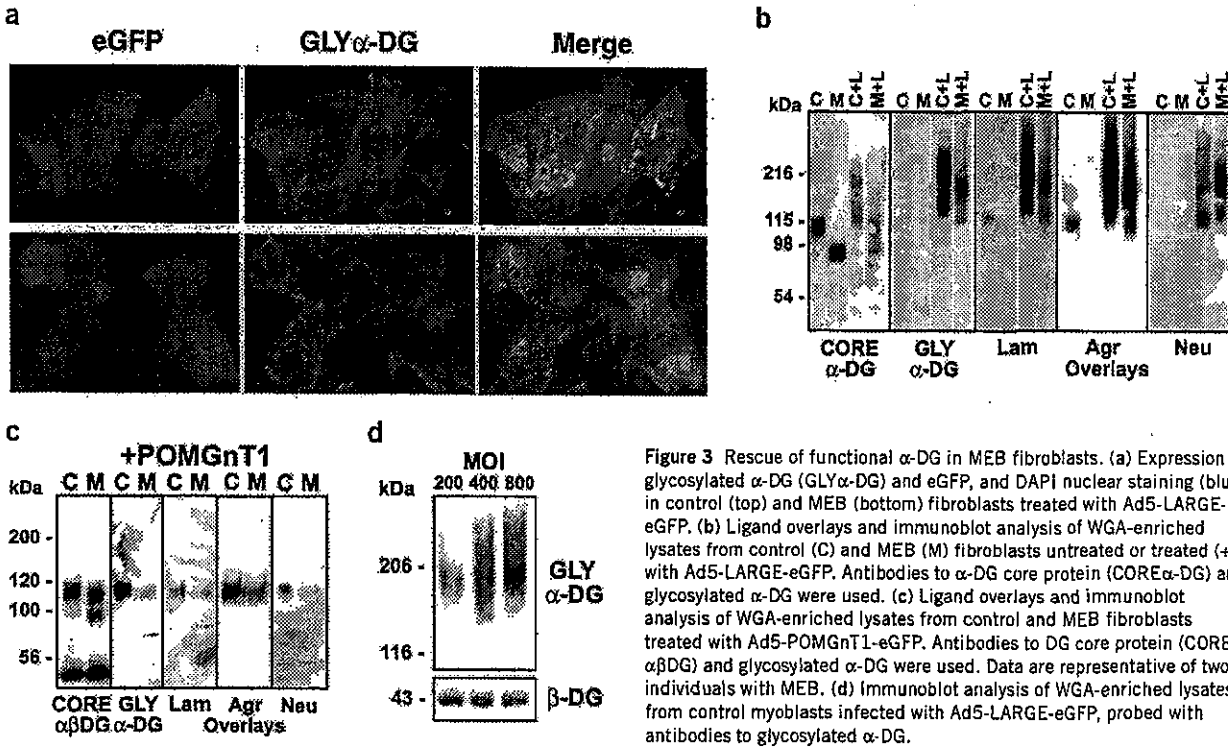

Figure 3 Rescue of functional α-DG in MEB fibroblasts. (a) Expression of glycosylated α-DG (GLYα-DG) and eGFP, and DAPI nuclear staining (blue) in control (top) and MEB (bottom) fibroblasts treated with Ad5-LARGE-eGFP. (b) Ligand overlays and immunoblot analysis of WGA-enriched lysates from control (C) and MEB (M) fibroblasts untreated or treated (+L) with Ad5-LARGE-eGFP. Antibodies to α-DG core protein (COREα-DG) and glycosylated α-DG were used. (c) Ligand overlays and immunoblot analysis of WGA-enriched lysates from control and MEB fibroblasts treated with Ad5-POMGnT1-eGFP. Antibodies to DG core protein (COREαβDG) and glycosylated α-DG were used. Data are representative of two individuals with MEB. (d) Immunoblot analysis of WGA-enriched lysates from control myoblasts infected with Ad5-LARGE-eGFP, probed with antibodies to glycosylated α-DG.

(Fig. 2d). Analysis at different stages of myoblast differentiation did not detect substantial differences (data not shown).

Given that the retrotransposon insertions in the *FCMD* gene in individuals with FCMD lead to a marked reduction in, but not absence of, fukutin[21], we reasoned that the observed synthesis of functionally glycosylated α-DG species in FCMD myoblasts following overexpression of LARGE might be due to either enhancement of the residual activity of fukutin or activation of compensatory glycosylation pathways. To test whether the observed changes were a specific consequence of LARGE overexpression, we treated our cultures with an analogous adenoviral vector expressing eGFP and full-length human protein *O*-mannosyl β-1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1). Although infection resulted in more than a 34-fold increase in POMGnT1 enzyme activity, as assessed with the substrate Man(α1-)*O*-benzyl[22], we did not observe notable variations in α-DG (Fig. 2e,f).

LARGE functionally rescues α-DG in MEB fibroblasts

The rare oligosaccharide NeuAcα2,3 Galβ1,4 GlcNAcβ1,2 Manα–O-Ser/Thr is important in the interaction of α-DG with its ligands[23,24]. POMGnT1 has been shown to be active in this pathway, catalyzing the second step of the synthesis of the tetrasaccharide[4]. To investigate the mechanism of LARGE-dependent glycosylation, we transferred *LARGE* into fibroblasts from two siblings affected with MEB. Expression of glycosylated α-DG was detected in control and MEB fibroblasts by immunofluorescence analysis only after treatment with Ad5-LARGE-eGFP (Fig. 3a). Immunoblotting with antibodies to DG core peptide detected α-DG species of 115–125 kDa in control and 90 kDa in MEB fibroblasts, whereas glycosylated α-DG with laminin- and agrin-binding activity was detected only in control cells (Fig. 3b). Treatment with Ad5-LARGE-eGFP generated glycosylated α-DG expression in every transduced control and MEB cell, and produced highly glycosylated, functional α-DG (Fig. 3b).

The low but significant levels of POMGnT1 detected in individuals with MEB are consistent with a proportion of proper glycosylation occurring in the presence of mutated POMGnT1 (refs 13,25). To assess whether LARGE modulates the partially functional enzyme, we evaluated POMGnT1 activity in control and MEB fibroblasts infected with Ad5-LARGE-eGFP or Ad5-eGFP. The 5.9% residual activity of POMGnT1 found in MEB fibroblasts did not vary after *LARGE* gene transfer (data not shown). In MEB fibroblasts, *POMGNT1* gene transfer resulted in expression of α-DG with a M_r comparable to that seen in control cells and restored ligand-binding activity (Fig. 3c). However, overexpression of POMGnT1 did not cause changes as marked as those caused by LARGE overexpression.

The relative abundance of *LARGE* transcripts in tissues where α-DG is more heavily glycosylated[18], coupled with the finding that high concentrations of LARGE generate heavily glycosylated α-DG species, suggest that levels of LARGE may be a limiting factor in α-DG glycosylation. Indeed, treating human control cells with Ad5-LARGE-eGFP at increasing multiplicity of infection (MOI) resulted in the synthesis of α-DG forms with an increasing M_r (Fig. 3d). These results show that induction of LARGE can activate compensatory mechanisms for the glycosylation of α-DG in a dose-dependent fashion.

LARGE generates functional α-DG in WWS cells

To further investigate whether the extensive glycosylation of α-DG after *LARGE* gene transfer is due to the synthesis of novel glycan chains that are normally absent or less represented in skeletal muscle, we transferred *LARGE* into myoblast and fibroblast cells from four individuals with WWS. Myoblasts from one of the individuals were mutated in the gene encoding protein *O*-mannosyltransferase 1 (POMT1), which catalyzes the first step of the *O*-mannose linked glycan motif of α-DG[26].

As also described for WWS skeletal muscle[9], glycosylated α-DG was not seen at any stage in WWS cell cultures, and immunoblot analysis

detected hypoglycosylated α-DG (Fig. 4a and data not shown). After treatment with Ad5-LARGE-eGFP, but not Ad5-POMGnT1-eGFP, staining for glycosylated α-DG was observed in transduced cells (Fig. 4a and Supplementary Fig. 3 online). In all of the WWS cell lines examined, treatment with Ad5-LARGE-eGFP led to the synthesis of α-DG species with a higher range of M_r values than were seen in control cultures, although residual hypoglycosylated α-DG was still found (Fig. 4b). Notably, hyperglycosylation correlated with functional rescue of α-DG as a receptor for its extracellular ligands (Fig. 4b). These results indicate that induction of LARGE leads to functional glycosylation of α-DG on alternative *O*-linked residues.

Because the sialyl *N*-acetyllactosamine moiety involved in the interaction of α-DG with its ligands is a common constituent of many *N*-glycans, we subjected WGA-enriched fractions from treated control and WWS cells to enzymatic deglycosylation by *N*-glycanase. Enzyme activity was verified by complete *N*-deglycosylation of β-DG. The M_r of α-DG did not change after enzymatic treatment, confirming the predominance of *O*-linked oligosaccharides (Fig. 4c). Reactivity for glycosylated α-DG also disappeared after alkaline *O*-deglycosylation (data not shown). Our attempts at enzymatic deglycosylation with exoglycosidases for sugars that are putatively added by LARGE did not release any sugars. Collectively, these data suggest either that LARGE modulates the activities of other as yet undetermined enzymes, thereby affecting an alternative glycosylation pathway of α-DG, or that LARGE activity may differ from that predicted by homology of its catalytic domains.

α-DG-laminin affinity facilitates laminin clustering

The perturbation of the basement membrane described in FCMD and WWS muscle is an important pathogenetic event common to many congenital muscular dystrophies[27,28]. We examined the ability of glycan-enriched α-DG to coordinate the assembly of extracellular matrix proteins by analyzing the formation of exogenous laminin-1 clusters on differentiating myoblasts. DG-mediated laminin clustering is a dynamic process in which small dot-shaped clusters merge into complex structures[29,30].

Very little endogenous laminin-1 was on the surface of control cells before treatment (data not shown). Within 2 h of incubation with laminin-1, only a few cells showed dot-like clusters. By 16 h, more cells were found to be positive for laminin clusters and a few myoblasts bearing linear and small plaque-like clusters were observed (Fig. 5a,b). In FCMD and WWS cultures, a small amount of exogenous laminin bound to the cell surface but did not organize into complex structures (Fig. 5a).

A marked change in clustering was observed after *LARGE* viral treatment: complex plaques formed within 2 h in all cultures. In control and FCMD cultures, plaques were the predominant type of cluster and the distribution of cells with dot, linear and plaque-like clusters remained stable over 16 h. In WWS myoblasts, the punctate pattern of laminin progressed more slowly into organized arrays (Fig. 5b). Notably, laminin always colocalized with glycosylated α-DG. Similar results were obtained in Large^{myd} myoblasts after *LARGE* gene transfer (data not shown). The laminin binding activity in control myoblast cultures was very low and we were unable to measure substantial differences among control, FCMD and WWS samples by quantitative solid-phase assays. Forced expression of LARGE markedly increased maximum laminin binding and affinity in control and FCMD cells, and to a lesser degree in WWS cells (Fig. 5c).

We conclude that very low binding activity is adequate for α-DG to act as a receptor for extracellular matrix proteins; however, the efficiency of assembling and remodeling the extracellular matrix increases with a higher affinity of α-DG for its ligands. As the receptor activity of α-DG is restored by inducing LARGE expression, it seems likely that the mechanical properties of diseased muscle fibers will be improved by this induction.

DISCUSSION

The pathogenetic mechanisms underlying glycosyltransferase-deficient muscular dystrophies are becoming increasingly clear. Our study provides evidence that LARGE has a regulatory role in α-DG

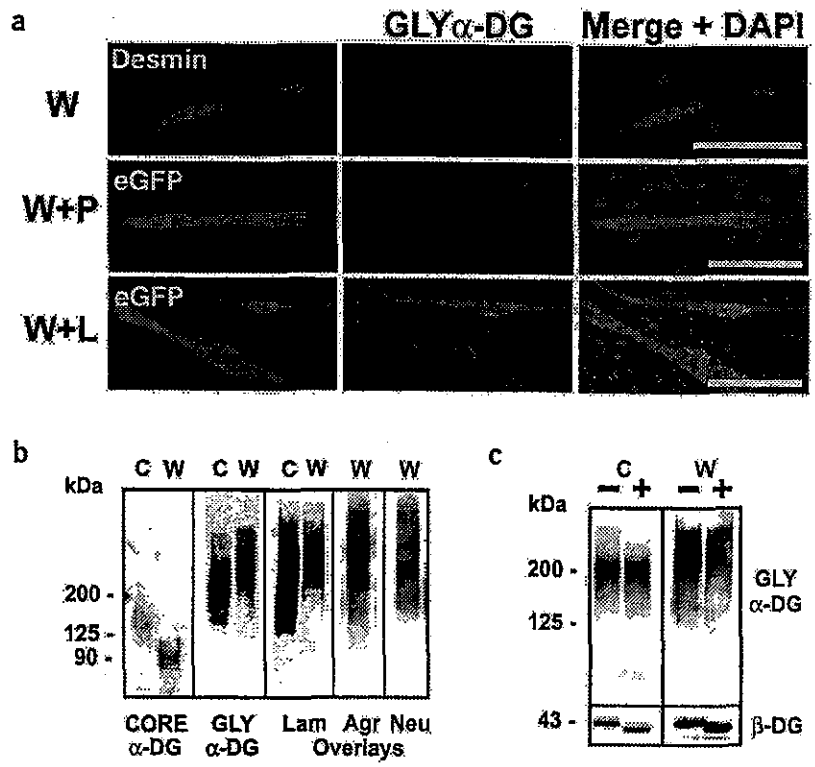

Figure 4 LARGE induces the synthesis of functional α-DG in WWS myoblasts and myotubes. (a) Expression of eGFP, desmin (green, upper row) and glycosylated α-DG (GLYα-DG), and nuclear labeling (DAPI, blue) in WWS myoblasts either untreated (W) or treated with Ad5-LARGE-eGFP (W+L) or Ad5-POMGnT1-eGFP (W+P). Scale bar, 20 μm. (b) Biochemical analysis of WGA-enriched fractions from control (C) and WWS myoblasts (W) treated with Ad5-LARGE-eGFP. Immunoblotting was done with antibodies to glycosylated α-DG and α-DG core protein (COREα-DG). Ligand overlay assays were done for laminin (Lam), agrin (Agr) and neurexin (Neu). (c) *N*-glycanase deglycosylation of WGA-enriched fractions from control (C) and WWS myoblasts (W) treated with Ad5-LARGE-eGFP. Immunoblotting was done with antibodies to β-DG and glycosylated α-DG. Untreated samples (–) are shown. Data are representative of four individuals with WWS.

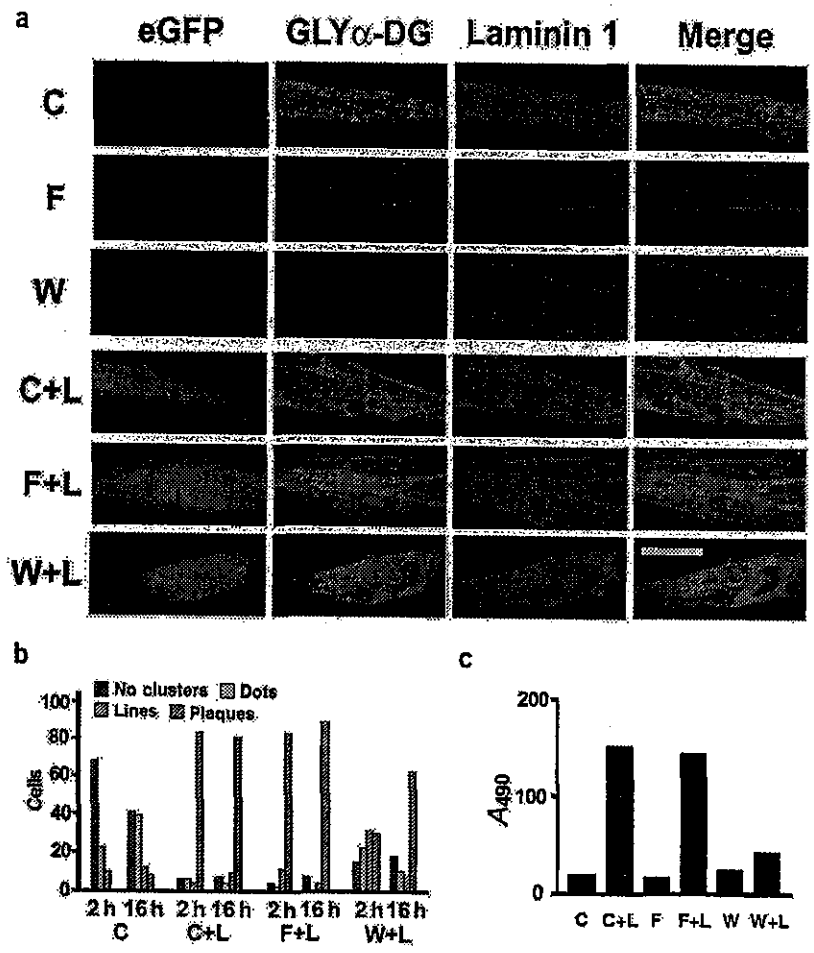

Figure 5 Laminin clustering correlates with high α-DG–laminin affinity. (a) Immunofluorescence localization of glycosylated α-DG (GLYα-DG), laminin-1 and eGFP after 16 h of incubation with laminin-1. Scale bar, 20 μm. Shown are untreated and Ad5-LARGE-eGFP–treated (+L) control (C), FCMD (F) and WWS (W) myoblasts. (b) Kinetic analysis of laminin-1 cluster formation after 2 and 16 h of incubation with laminin-1. About 100 cells were evaluated for each group. (c) Solid-phase assay of maximum binding between α-DG and laminin at 0.6 nM laminin-1 in control (C), FCMD (F) and WWS (W) WGA-enriched glycoprotein fractions from untreated and Ad5-LARGE-eGFP–treated (+L) myoblasts.

glycosylation. We have shown that *LARGE* gene transfer restores α-DG function in Large^{myd} skeletal muscle and ameliorates muscular dystrophy, indicating that adjusting the glycosylation status of α-DG can improve the muscle phenotype.

Another mechanism for preventing muscular dystrophy, involving upregulation of utrophin through the transgenic expression of an endogenous glycosyltransferase, has been explored in the *mdx* mouse model of Duchenne muscular dystrophy. Ectopic expression of the cytotoxic T cell (CT) GalNAc transferase, which is normally present at the neuromuscular junction, leads to extrasynaptic localization of the utrophin-glycoprotein complex and consequently improves the dystrophic phenotype in the transgenic *mdx*-CT mouse[31,32]. α-DG was glycosylated with the CT carbohydrate in *mdx*-CT muscle. CT GalNAc transferase has not, however, been shown to modify the ligand-binding activity of α-DG. In addition, overexpression of this enzyme in transgenic mice causes a marked reduction in the diameter of myofibers and alterations in the neuromuscular junction architecture[31].

By contrast, forced expression of LARGE in wild-type muscle does not result in morphological or pathological changes. In addition, Large^{myd} mice and individuals with dystroglycanopathy have normal expression of the DGC at the sarcolemma, showing that correct localization of the DGC is necessary but not sufficient for the protection and stability of the myofibers. *LARGE* gene transfer did not change the expression of any of the DGC components and ameliorated the pathological phenotype by restoring the function of the DGC via glycosylation of α-DG.

Unexpectedly, we found that the effect of LARGE was similar in all of the cell types and tissues that we analyzed, suggesting that induction of LARGE may be effective in the treatment not only of MDC1D but also of FCMD, MEB, WWS, LGMD2I (R.B. and K.P.C., unpublished data) and other glycosyltransferase-deficient muscular dystrophies. Because the rescue effect of LARGE is similar in cells from individuals with distinct diseases, it seems unlikely that LARGE is directly or indirectly activating the mutant enzyme in each disorder. In addition, there is evidence that a direct interaction between the amino-terminal domain of α-DG and LARGE is essential for the functional post-translational modification of α-DG (M.K. and P.K.C., unpublished data).

Our findings point toward the existence of dual, concentration-dependent functions of LARGE. At physiological concentration, LARGE may regulate the *O*-mannosylation pathway of α-DG, whereas forced expression of LARGE may activate alternative pathways for the *O*-glycosylation of α-DG that possibly generate a type of repeating polymer of variable lengths, such as glycosaminoglycan-like or core 1 or core 2 structures. This alternative glycan mimics the *O*-mannose glycan in its ability to bind α-DG ligands and can compensate for the defective tetrasaccharide (Fig. 6).

The finding that an endogenous protein can modulate the glycosylation of α-DG in genetically distinct diseases without undesirable effects arising from its upregulation makes LARGE an attractive target for the design of therapies intended to manipulate α-DG glycosylation. Several reported muscle pathologies associated with defective glycosylation of α-DG and unlinked to known genes[33] might particularly benefit from such therapeutic approaches. Glycotherapies and treatments aimed at modulating the expression or the activity of LARGE may be the basis of an adequate therapeutic option for the whole group of glycosyltransferase-deficient muscular dystrophies.

METHODS

Antibodies. VIA4-1 and IIH6 are monoclonal antibodies to fully glycosylated species of α-DG[10]. We used the antibodies interchangeably and refer to them as GLYα-DG in the figure legends; however, most of the data shown represent staining obtained with IIH6. GT20ADG (COREα-DG) is from goat

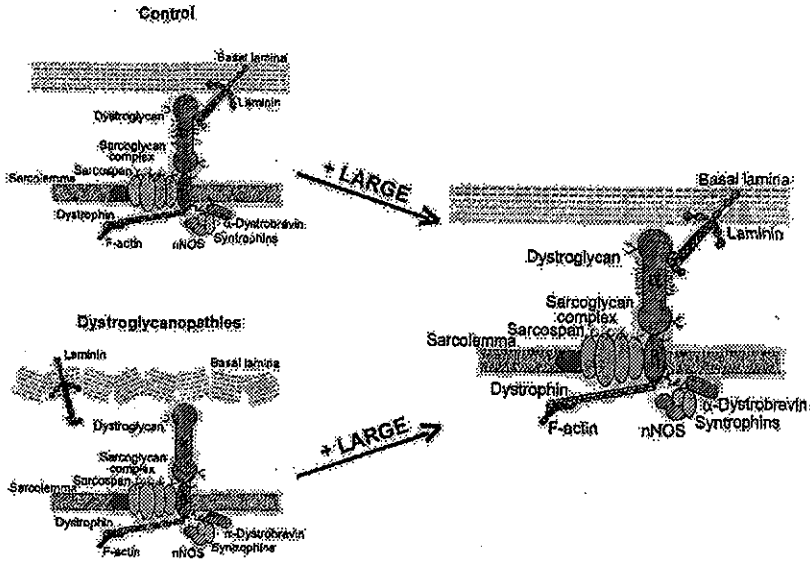

Figure 6 Effect of LARGE on α-DG glycosylation. Representation of the effect of overexpressing LARGE in skeletal muscle from control and affected individuals. See text for details.

antiserum raised against the whole DGC and purified against a hypoglycosylated full-length α-DG–human IgGFc fusion protein[13,34]. CORE-αβDG is purified from sheep polyclonal antiserum raised against the whole DGC and recognizes both α- and β-DG[12]. Polyclonal antibodies to DGC components were used as described[35]. Rabbit polyclonal antibody to laminin-1 and mouse monoclonal antibody to desmin were from Sigma, and mouse monoclonal antibodies to agrin and caveolin-3 were from Chemicon and Transduction Laboratories, respectively.

Mice. We obtained myodystrophy mice (Large^{myd}) and control littermate mice (Large$^{+/+}$ or Large$^{myd/+}$) by mating heterozygous pairs provided by Jackson Laboratories. All mice were maintained at the University of Iowa Animal Care Unit in accordance with animal usage guidelines.

Adenovirus generation and infection. E1-deficient recombinant adenoviruses, Ad5-LARGE-eGFP and Ad5-POMGnT1-eGFP, which contain eGFP plus the complete open reading frame of human *LARGE* or human *POMGNT1* in separate expression cassettes, respectively, were generated by the University of Iowa Gene Transfer Vector Core. Transcription of *LARGE* or *POMGNT1* was initiated by the cytomegalovirus promoter (CMV), and transcription of eGFP was initiated by the Rous sarcoma virus promoter (RSV). Translation of the transcripts yielded LARGE or POMGnT1 and eGFP as separate proteins. *In vivo* gene transfer was done on Large^{myd} and control littermate pups, aged 2-4 d, as described[36]. Injected and non-injected contralateral leg muscles were examined after 4 weeks. Cell cultures were infected with viral vector for 12 h at an MOI of 200–800. At this concentration, cell cytotoxicity was <15%. We examined cultures 3–10 d after treatment.

Immunohistochemical analysis. Cryosections (7 μm) were processed for immunofluorescence as described[35]. Cultured cells were fixed in 4% paraformaldehyde before being incubated with primary antibodies. Slides were observed with an MRC-600 laser scanning confocal microscope (Bio-Rad). Digitized images were captured under identical conditions.

Glycoprotein enrichment and biochemical analysis. Frozen samples and cultured cells were processed as described[13]. Immunoblots and ligand overlay assays were done on polyvinylidene difluoride membranes as described[13]. Blots were developed by horseradish peroxidase (HRP) enhanced chemiluminescence (Pierce). *N*-deglycosylation was done by using *N*-glycanase (ProZyme) according to the manufacturer's instructions. We did the solid-phase binding assay as described[13].

Treadmill exercise. Mice were exercised by an Omnipacer Treadmill (Model LC4/M-MGA/AT; Accuscan Instruments) at a 15° downward angle with increasing speed up to 19 m/min for 30 min. Large^{myd} and littermate mice, aged 5–7 weeks, injected with *LARGE* adenovirus in the right hamstrings were tested ($n = 4$). All mice were injected with EBD intraperitoneally 5 h before exercise. Mice were killed 24 h after exercise and sections of right and left hamstrings were compared for EBD uptake and expression of glycosylated α-DG. Quantification of EBD-positive areas in sections of skeletal muscle was done by using ImageJ software (National Institutes of Health; NIH). The percentage of positively stained areas was calculated by dividing the area stained by the total area of the analyzed skeletal muscle section.

Human cells. The MEB fibroblasts were from two compound heterozygote siblings with a G1908→A transversion in exon 21 (Arg605His) in one allele and a single-base-pair insertion in exon 11 in the other allele (1106insT, causing a frameshift and premature termination at codon 338) of the *POMGNT1* gene[25]. The FCMD myoblasts were from three Japanese individuals: one was homozygous for the retrotransposon insertion in *FCMD*[37]; the other two were heterozygous for the insertion and either Cys250Gly or Leu353Stop (plus polymorphism Arg203Glu). The WWS myoblasts were from a Japanese boy homozygous for Leu421del in *POMT1* and from a Hispanic male aged 6 months[38]. The WWS fibroblasts were from a 3-year-old female and a 4-year-old male with clinical diagnosis of WWS. Analysis of the full-length *POMT1* and *POMGNT1* complementary DNA in these individuals did not identify mutations. The regions of DG cDNA corresponding to the laminin-binding domains and glycosylated domains were normal. We obtained and tested all tissues in agreement with the Human Subjects Institutional Review Board of the University of Iowa; informed consent was obtained from all subjects.

Cell cultures. Cells were maintained at 37 °C and 5% CO_2 in DMEM medium plus 20% fetal bovine serum, 0.18 μg/ml of insulin and 0.5% penicillin-streptomycin (Gibco). The myoblast/fibroblast ratio estimated by desmin staining was 1/7 in control and FCMD cultures, and 1/5 in WWS cultures. Myoblast fusion was done by decreasing the serum concentration to 2% (differentiation medium). For the laminin clustering assay, myoblasts were switched to fresh differentiation medium containing 7.5 nM mouse EHS laminin-1 and incubated for 2, 5 or 16 h. Confocal immunofluorescence images were compiled from a 5-μm *z*-series extending from the dorsal surface of the cells in 0.5-μm steps. Morphological evaluation of clusters was done as described[39].

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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