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

Y. Ohashi, MD; Y. Hasegawa, MD; K. Murayama, BS; M. Ogawa, MS; T. Hasegawa, MD, PhD; M. Kawai, MD; N. Sakata, MD; K. Yoshida, MD, PhD; H. Yarita, MD, PhD; K. Imai, MD, PhD; I. Kumagai, MD, PhD; K. Murakami, MD, PhD; H. Hasegawa, MD; S. Noguchi, PhD; I. Nonaka, MD, PhD; S. Yamaguchi, MD, PhD; and I. Nishino, MD, PhD

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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From the Department of Neuromuscular Research, National Institute of Neuroscience (Drs. Ohashi, Noguchi, and Nishino, and K. Murayama and M. Ogawa) and the National Hospital of Mental, Nervous and Muscular Disorders (Dr. Nonaka), National Center of Neurology and Psychiatry (NCNP), Kodaira, Tokyo; the Department of Pediatrics, Keio University (Dr. T. Hasegawa), Shinjuku, Tokyo; the Department of Neurology and Clinical Neuroscience, Yamaguchi University (Drs. Kawai and Sakata), Ube, Yamaguchi; Shinshu University Hospital (Dr. Yoshida), Matsumoto, Nagano; Gunma Central Hospital (Dr. Imai), Maebashi Gunma; Teraoka Memorial Hospital (Dr. Kumagai), Fukuyama, Hiroshima; Tamashima Central Hospital (Dr. Murakami), Kurashiki, Okayama; the Department of Pediatrics, Shimane University (Drs. Y. Hasegawa and Yamaguchi), Izumo, Shimane; the Department of Pediatrics, Kyorin University (Dr. Ohashi), Mitaka, Tokyo; the Division of Clinical and Molecular Genetics, Tatebayashi Kosei Hospital (Dr. Yarita), Tatebayasi, Gunma; and Kitasato Institute Medical Center Hospital (Dr. H. Hasegawa), Kitamoto, Saitama, Japan.

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Address correspondence and reprint requests to Dr. Ichizo Nishino, Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), 4-1-1 Ogawahigashi-cho, Kodaira, Tokyo 187–8502, Japan; e-mail: nishino@ncnp.go.jp

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

Rita Barresi[1], Daniel E Michele[1], Motoi Kanagawa[1], Hollie A Harper[1], Sherri A Dovico[1], Jakob S Satz[1], Steven A Moore[2], Wenli Zhang[3], Harry Schachter[3], Jan P Dumanski[4], Ronald D Cohn[1], Ichizo Nishino[5] & Kevin P Campbell[1]

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

[1]Howard Hughes Medical Institute, Department of Physiology and Biophysics, Department of Neurology, [2]Department of Pathology, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242, USA. [3]Hospital for Sick Children, Department of Biochemistry, University of Toronto, Ontario M5G 1X8, Canada. [4]Department of Genetics and Pathology, Uppsala University, 75185 Uppsala, Sweden. [5]Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan. Correspondence should be addressed to K.P.C. (kevin-campbell@uiowa.edu).

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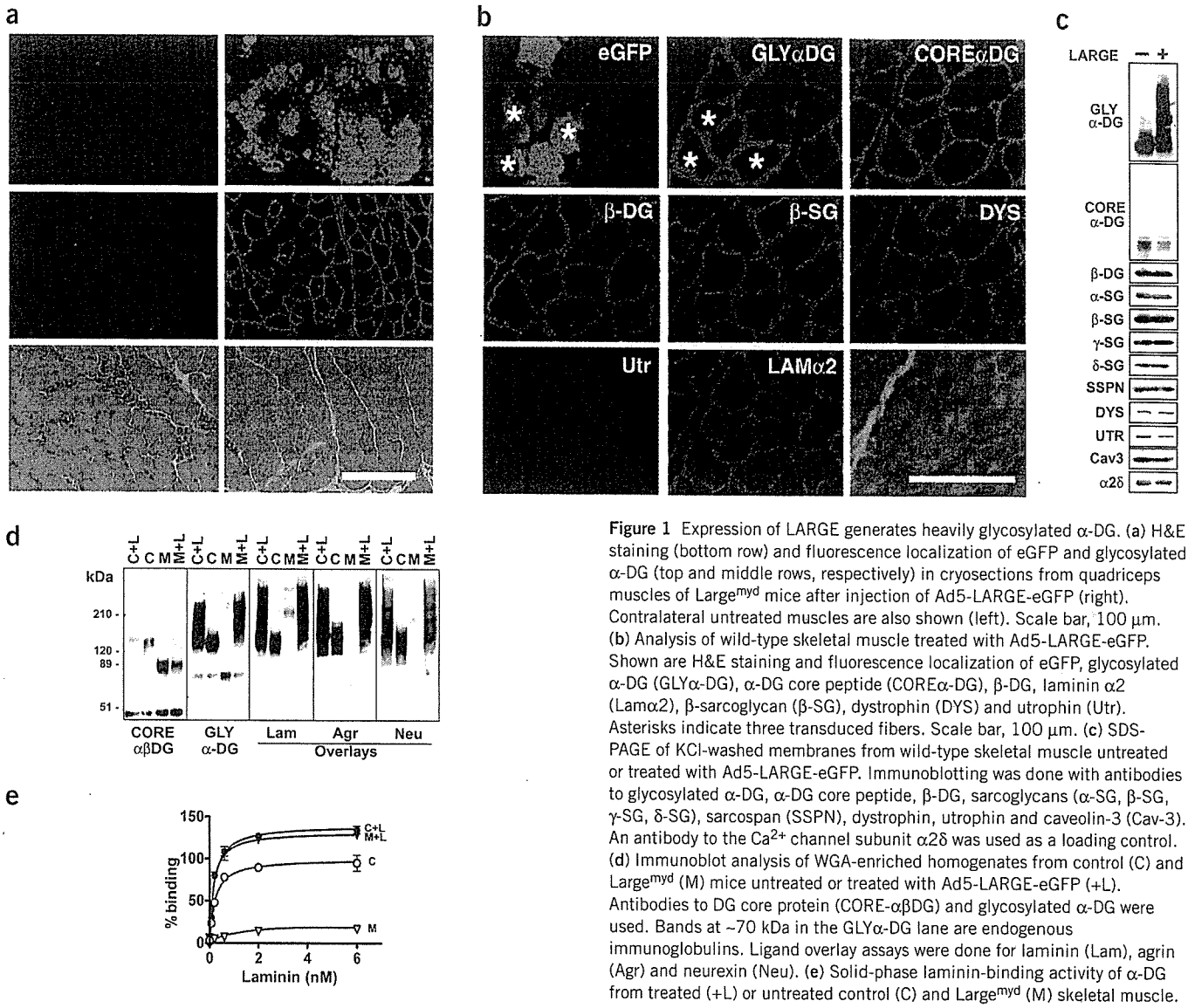

Figure 1 Expression of LARGE generates heavily glycosylated α-DG. (**a**) H&E staining (bottom row) and fluorescence localization of eGFP and glycosylated α-DG (top and middle rows, respectively) in cryosections from quadriceps muscles of Large^{myd} mice after injection of Ad5-LARGE-eGFP (right). Contralateral 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 LGMD[20], 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

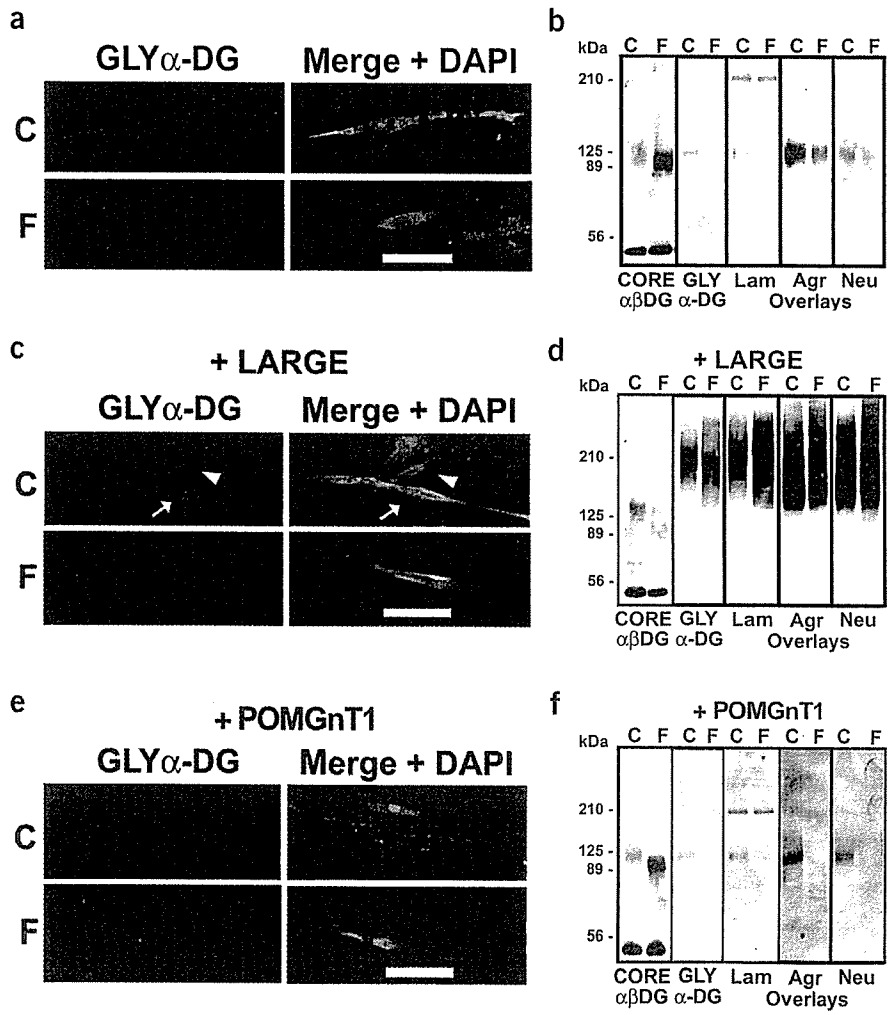

Figure 2 LARGE generates functional α-DG in FCMD myoblasts. (**a–f**) Analysis of untreated (**a,b**), or infected with Ad5-LARGE-eGFP (**c,d**) or Ad5-POMGnT1-eGFP (**e,f**) control (C) and FCMD (F) cultures. Immunofluorescence shows localization of desmin (**a**) or eGFP (**c,e**). Arrows indicate 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.

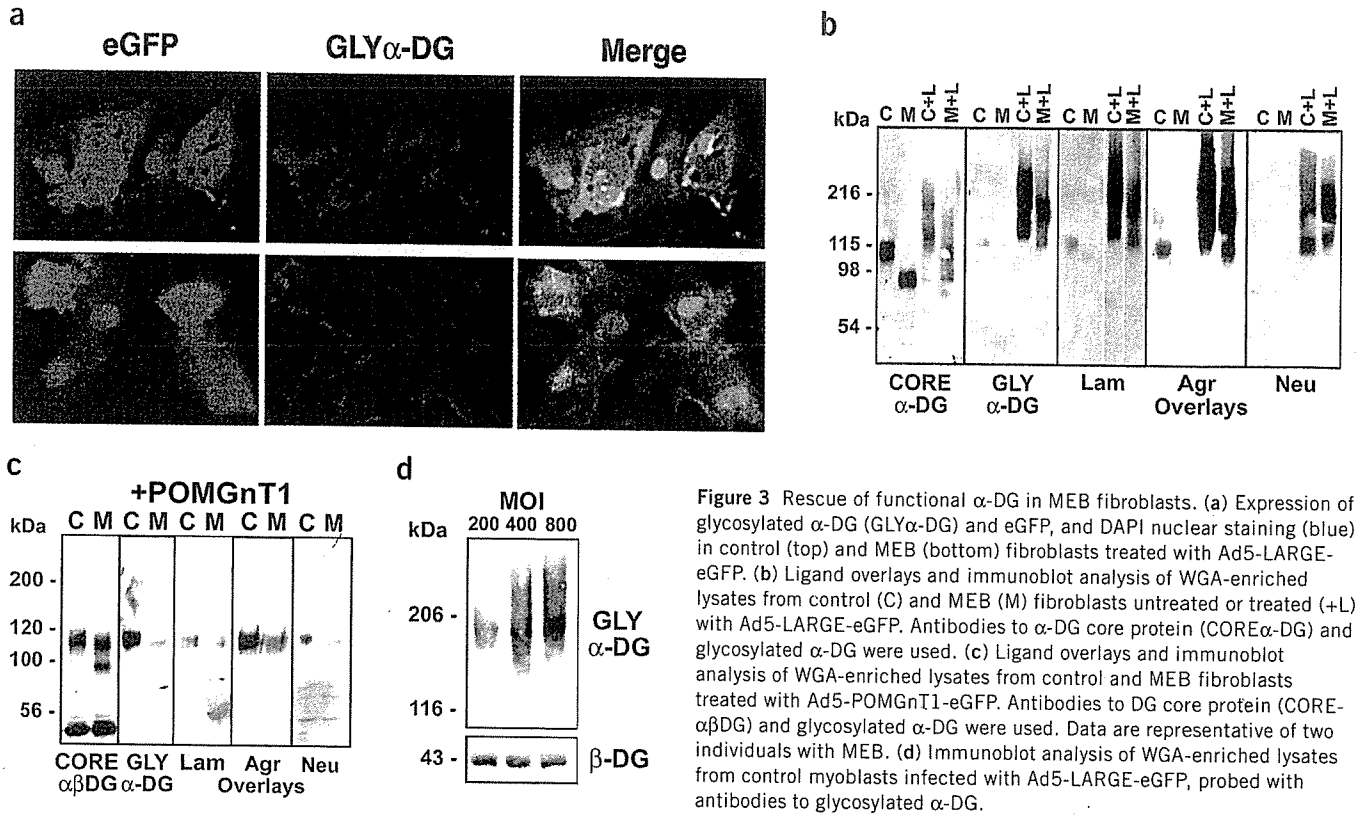

Figure 3 Rescue of functional α-DG in MEB fibroblasts. (**a**) Expression of glycosylated α-DG (GLYα-DG) and eGFP, and DAPI nuclear staining (blue) in control (top) and MEB (bottom) fibroblasts treated with Ad5-LARGE-eGFP. (**b**) Ligand overlays and immunoblot analysis of WGA-enriched lysates from control (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 (CORE-αβDG) 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

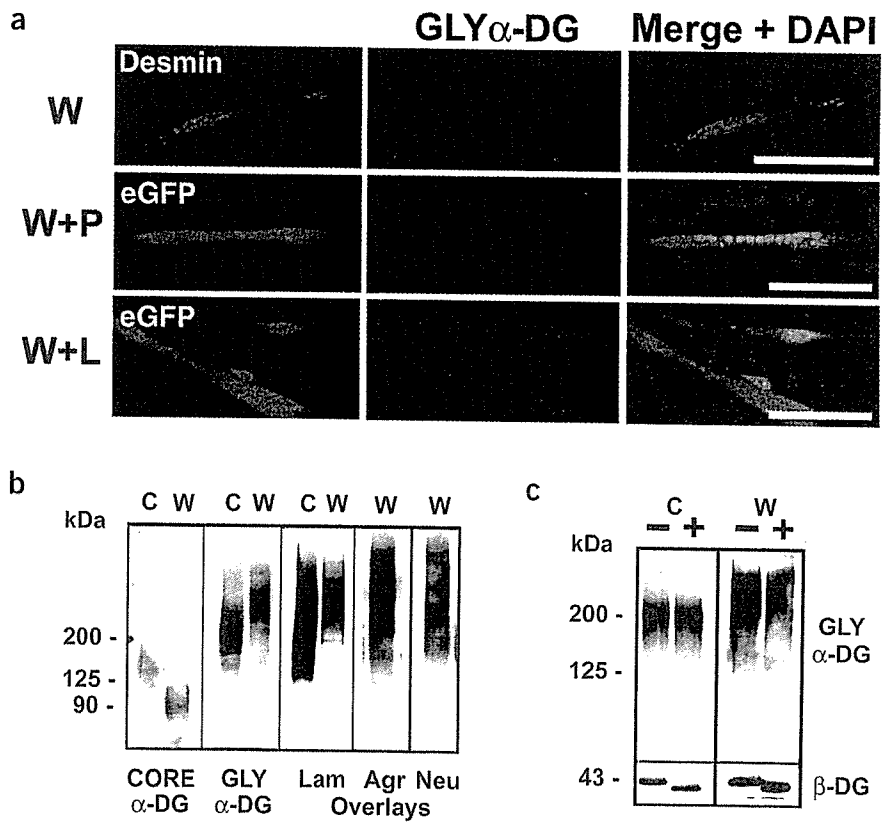

Figure 4 LARGE induces the synthesis of functional α-DG in WWS myoblasts and myotubes. (a) Expression of eGFP, desmin (green, upper row) and glycosylated α-DG (GLYα-DG), and nuclear labeling (DAPI, blue) in WWS myoblasts either untreated (W) or treated with Ad5-LARGE-eGFP (W+L) or Ad5-POMGnT1-eGFP (W+P). 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.

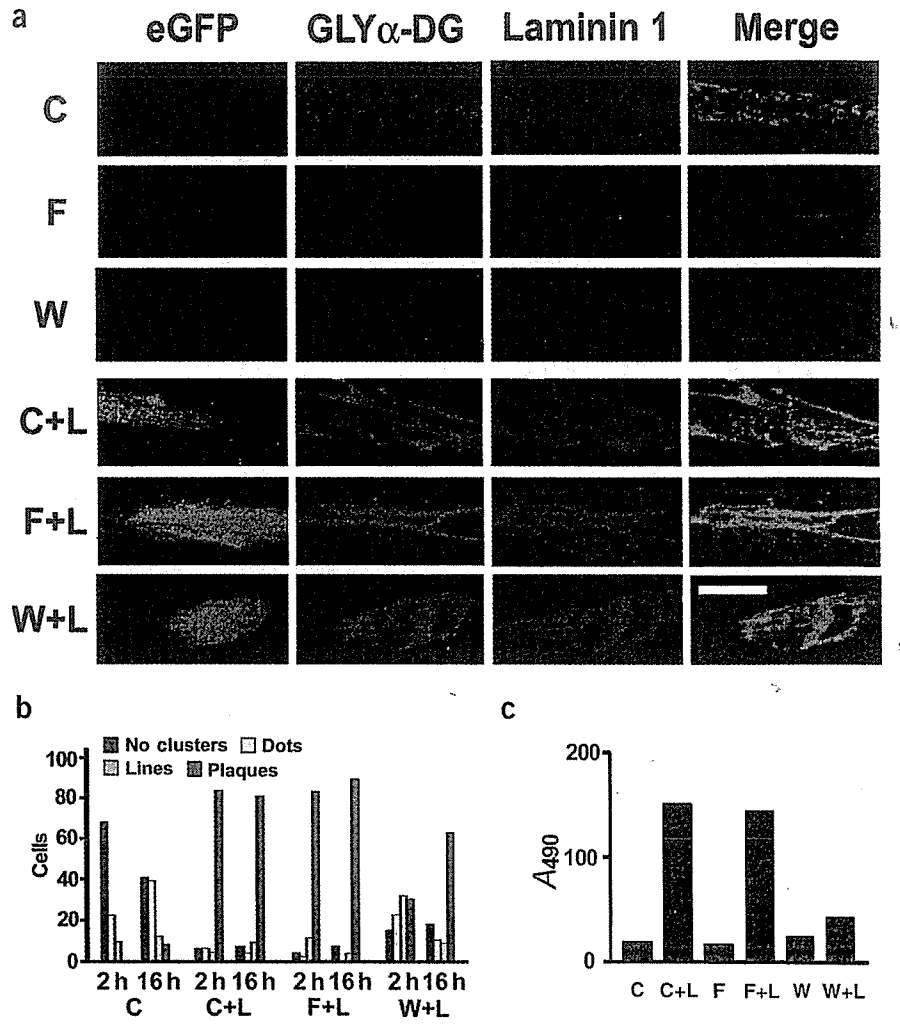

Figure 5 Laminin clustering correlates with high α-DG–laminin affinity. (a) Immunofluorescence localization of glycosylated α-DG (GLYα-DG), laminin-1 and eGFP after 16 h of incubation with laminin-1. Scale bar, 20 μm. Shown are untreated and Ad5-LARGE-eGFP–treated (+L) control (C), FCMD (F) and WWS (W) 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

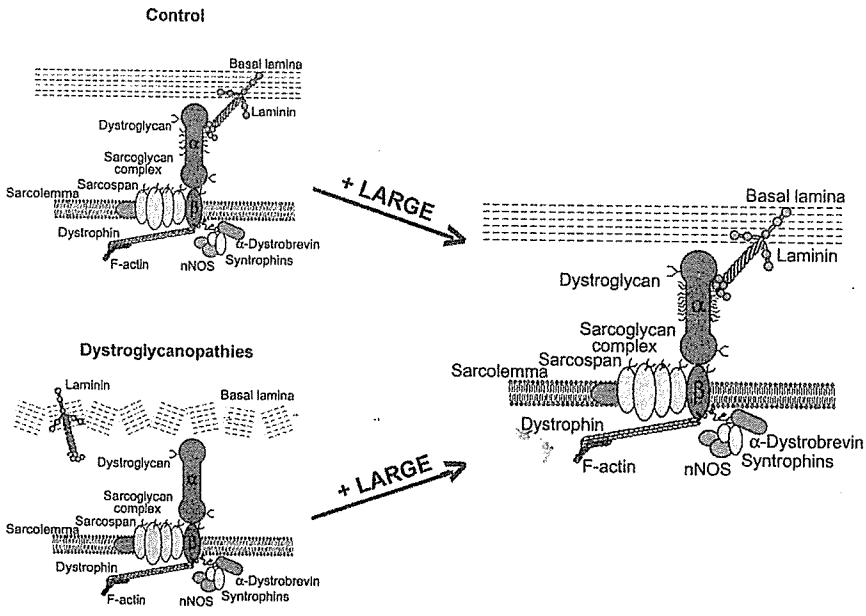

Figure 6 Effect of LARGE on α-DG glycosylation. Representation of the effect of overexpressing LARGE in skeletal muscle from control and affected individuals. See text for details.

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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Subcellular Localization of Fukutin and Fukutin-Related Protein in Muscle Cells

Hiroshi Matsumoto[1,2], Satoru Noguchi[1,3,*], Kazuma Sugie[1], Megumu Ogawa[1,3], Kumiko Murayama[1], Yukiko K. Hayashi[1] and Ichizo Nishino[1,3]

[1]Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Tokyo 187-8502; [2]Department of Pediatrics, National Defense Medical College, Saitama, 359-8513; [3]Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Saitama 332-0012.

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Fukuyama-type congenital muscular dystrophy and congenital muscular dystrophy 1C are congenital muscular dystrophies that commonly display reduced levels of glycosylation of α-dystroglycan in skeletal muscle. The genes responsible for these disorders are *fukutin* and *fukutin-related protein* (*FKRP*), respectively. Both gene products are thought to be glycosyltransferases, but their functions have not been established. In this study, we determined their subcellular localizations in cultured skeletal myocytes. FKRP localizes in rough endoplasmic reticulum, while fukutin localizes in the *cis*-Golgi compartment. FKRP was also localized in rough endoplasmic reticulum in skeletal muscle biopsy sample. Our data suggest that fukutin and FKRP may be involved at different steps in *O*-mannosylglycan synthesis of α-dystroglycan, and FKRP is most likely involved in the initial step in this synthesis.

Key words: *cis*-Golgi, fukutin, fukutin-related protein, rough, endoplasmic reticulum, skeletal muscle.

Abbreviations: DG, dystroglycan; ER, endoplasmic reticulum; FCMD, Fukuyama-type congenital muscular dystrophy; FKRP, fukutin-related protein; MDC1C, congenital muscular dystrophy 1C; POMT1, protein *O*-mannosyltransferase 1.

Congenital muscular dystrophy comprises a genetically heterogeneous group of disorders. Fukuyama-type congenital muscular dystrophy (FCMD), the most common congenital muscular dystrophy in Japan, is characterized by muscle weakness and hypotonia from early infancy, and is associated with mental retardation and a brain anomaly called type II lissencephaly. The causative gene was cloned in 1998 and named *fukutin*, and a 3-kb retrotransposal insertion in the 3′-untranslated region of *fukutin* is found in most patients with FCMD (*1*). Fukutin-related protein (FKRP) was characterized as a homolog of fukutin in 2001 (*2*), and mutations in *FKRP* were also shown to cause muscular dystrophies (congenital muscular dystrophy 1C (MDC1C) and limb-girdle muscular dystrophy 2I) (*2*, *3*).

Skeletal muscle biopsy samples from FCMD and MDC1C patients show a marked reduction in α-dystroglycan (α-DG) by immunostaining with an antibody against the glycosylated epitope (*2*, *5*), whereas α-DG could still be visualized even in FCMD skeletal muscle with an antibody that recognizes the peptide epitope (*6*). The reduction in α-DG glycosylation in skeletal muscle leads to a loss in its ability to bind to extracellular ligands such as laminin, agrin and neurexin (*6*). Thus, the abnormality in posttranslational glycosylation of α-DG plays a crucial role in the pathogenesis of FCMD and MDC1C. The functions of fukutin and FKRP have not been clarified. However, they are supposed to act as glycosyltransferases, because they both are type-II transmembranous proteins and possess a DXD motif in the C-terminal side that is often found in glycosyltransferases (*4*). These facts strongly indicate that these two proteins may participate in the glycosylation of α-DG.

Protein glycosylation is a highly organized orderly process. Newly synthesized naked proteins are sequentially modified *en route* by glycosyltransferases during transport from the rough endoplasmic reticulum (ER) to the *trans*-Golgi network (*7*). Therefore, the expression and localization of a glycosyltransferase must be precisely regulated to synthesize the specific glycostructure in each tissue and cell. Determining the localization of putative glycosyltransferases will help to predict their functions. In this study, we determined the precise localization of fukutin and FKRP in muscle cells.

MATERIALS AND METHODS

Antibodies—Anti-FKRP and anti-fukutin antibodies were raised in rabbits against the recombinant fragments comprising the carboxyl-terminal 112 and 121 amino acids, respectively. Expression vectors for the FKRP and fukutin fragments with a glutathione-*S*-transferase tag at the N-terminus were constructed by inserting the cDNA fragments into pGEX-4T (Amarsham). Both antibodies were affinity-purified on antigen-immobilized activated thiol-Sepharose 4B (Amersham Pharmacia Biotec). Other antibodies used in this study are anti-emerin (Novocastra Laboratories), anti-BiP, anti-calnexin, anti-

*To whom correspondence should be addressed. Tel: +81-423412712; Fax: +81-423461742 Email: noguchi@ncnp.go.jp

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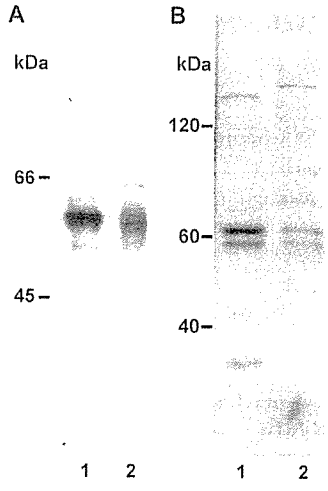

Fig. 1. **Characterization of newly raised polyclonal antibodies by Western blotting.** A: The anti-FKRP antibody detected a band at approximately 60 kDa in COS cell lysates with transiently expressed myc-FKRP (lane 1) and in human skeletal muscle cells (lane 2). B: Western blotting of a C2C12 cell lysate transfected with myc-fukutin. Both anti-fukutin (lane 1) and anti-myc (lane 2) antibodies detected doublet bands at approximately 60 kDa.

GM130 (BD Biosciences Pharmingen), and anti-myc 9E10 (Oncogene Science). Primary antibodies were used at the following dilutions: anti-FKRP (1:200), anti-fukutin (1:100), anti-myc (1:100), anti-BiP (1:100), anti-calnexin (1:100), anti-GM130 (1:500) and anti-emerin (1:500). Alexa 488-labeled anti-rabbit IgG and Alexa 568-labeled anti-mouse IgG (Molecular probes) were used as secondary antibodies.

cDNA Transfection and Immunocytochemistry—The open reading frames of FKRP and fukutin were amplified by PCR and subcloned into the expression vector CMV-myc (BD Clontech). These expression vectors encoding myc-epitope tagged FKRP and fukutin were transfected into the C2C12 cell line using Lipofectamine as described in the manufacturer's instructions (Invitrogen Carlsbad). The C2C12 cells and human skeletal muscle cells were induced to differentiate as described in a previous report (*8*). The immunostaining of myocytes was performed as described earlier (*9*). Laser confocal fluorescence images were obtained using an Olympus FLUOVIEW confocal microscope (Olympus).

Western Blotting and Immunohistostaining of Human Skeletal Muscles—Western blotting and immunohistostaining of human skeletal muscle were performed as described previously (*5*, *9*). The immunoreactive bands on Western blot were visualized using an ECL kit (Amersham Pharmacia Biotec). The immunostained sections were observed under a Zeiss Axiophot2 microscope (Carl Zeiss)

Muscle Biopsy Sample—Control muscle was obtained from surgical discards from a 24-yr-old male patient. Informed consent was obtained using the form approved by the Ethical Review Board at NCNP.

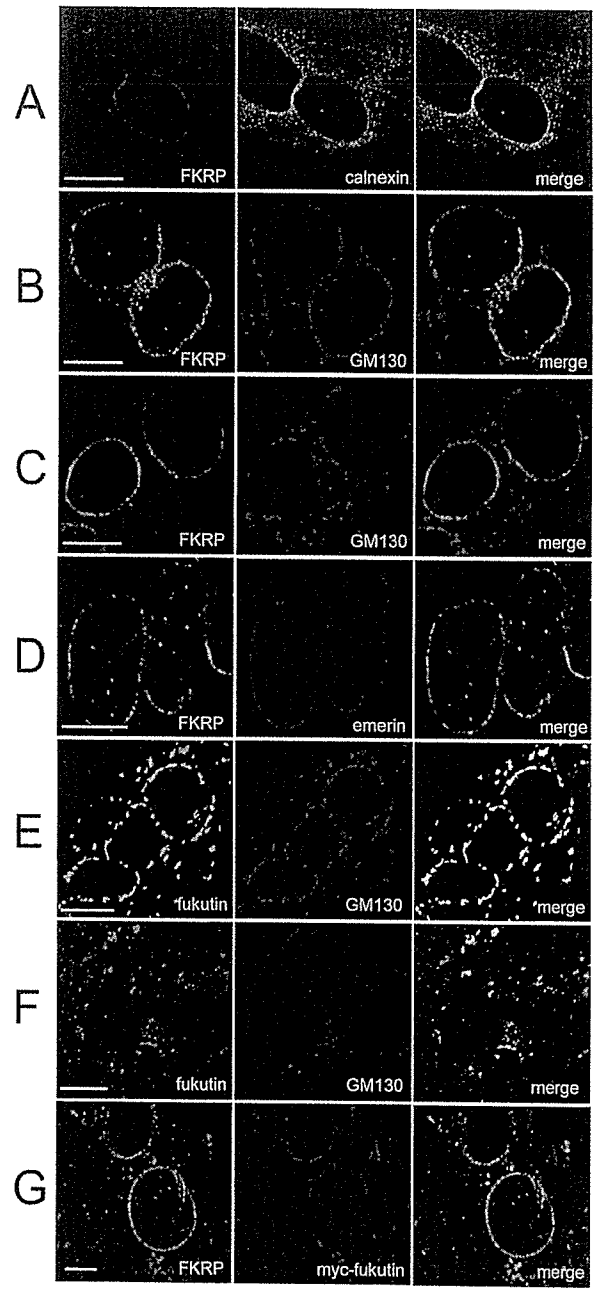

Fig. 2. **Subcellular localization of FKRP and fukutin.** A: FKRP and calnexin staining of human skeletal myotubes. FKRP (red) was continuously stained around nuclei with the innermost part of calnexin staining (green). B, C: FKRP and GM130 staining in mouse C2C12 myotubes. FKRP (green) was stained in the inner aspect of GM130 localization (red). Treatment with brefeldin A induced the dissociation of the Golgi apparatus, but FKRP remained localized around nuclei (C). D: FKRP and emerin staining in human skeletal myotubes. FKRP (green) was stained on the outer aspect of emerin staining (red). E, F: Immunostaining of transfected fukutin and GM130 in C2C12 myotubes. The merged image shows the co-localization of transfected fukutin and GM130. Treatment with brefeldin A dispersed both stainings into the cytosol (F). G: Spatial relationship between FKRP and fukutin. Endogenous FKRP (green) and transfected fukutin (red) in C2C12 myotubes showed different localizations. Bars denote 10 μm.

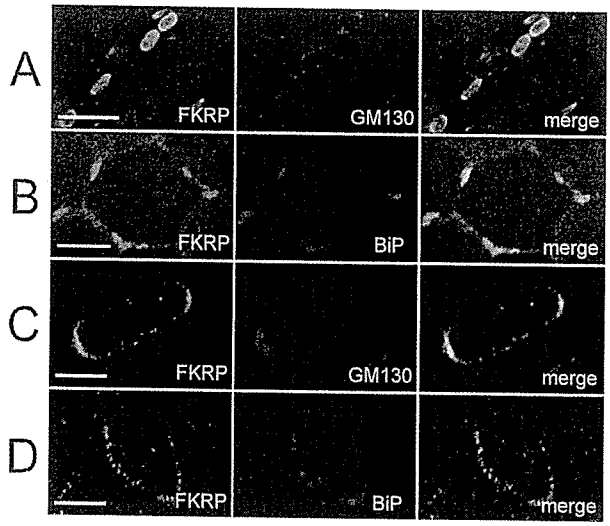

Fig. 3. **Immunohistochemical staining of FKRP in skeletal muscle.** The anti-FKRP antibody stained continuously around nuclei (A–D, green), while the anti-GM130 antibody stained nuclear poles and granular concentrates in cytosol (A, C, red). On higher magnifications, FKRP and GM130 are seen to overlap partially at both nuclear poles (C). BiP staining merges with FKRP in muscles (D). Bars denote 50 μm (A, B) and 5 μm (C, D).

RESULTS

Characterization of Newly Raised Antibodies against FKRP and Fukutin—Polyclonal antibodies against FKRP and fukutin were raised using recombinant fragments comprising the carboxyl-terminal 112 and 121 amino acids, respectively; the fragments share no homology to each other. Western blot analysis of a homogenate from cultured human skeletal muscle cells using the anti-FKRP antibody detected a band migrating at approximately 60 kDa (lane 2 in Fig. 1A), which matches the size of transiently expressed myc-FKRP in COS cells (lane 1).

The anti-fukutin antibody was not adequately sensitive to detect endogenous fukutin in skeletal muscle or in cultured muscle cell homogenates. In C2C12 muscle cells, in which myc-fukutin was transiently expressed, the anti-fukutin antibody detected two adjacent bands migrating at 60 kDa (lane 1 in Fig. 1B). The anti-myc antibody also detected two same sized bands (lane 2), suggesting that some posttranslational modification, such as glycosylation, generates the two bands. The specificity of each antibody was also examined by cross Western blot experiments in which the anti-FKRP antibody was allowed to react with the recombinant fukutin protein expressed in C2C12 cells, and the anti-fukutin antibody to react with the recombinant FKRP expressed in COS cells. No band was detected in either blotting, demonstrating the specificity of each antibody.

Subcellular Localization of FKRP and Fukutin in Differentiated Myocytes—To determine the subcellular localization of FKRP and fukutin in differentiated myocytes, human myocytes or C2C12 cells were immunostained with the two antibodies (Fig. 2). FKRP staining was detected surrounding the nuclei only in myotubes, but not in myoblasts. We compared this localization with those of organelle marker proteins against calnexin, a chaperone protein in rough ER (*10*). Calnexin localizes around the nuclei and merges with FKRP staining (Fig. 2A), although calnexin is also diffusely distributed in the cytosol. Double staining with antibodies for GM130, a matrix protein in the *cis*-Golgi compartment (*11*) or emerin, an inner nuclear membranous protein, showed that FKRP co-localizes with neither of them, the inner aspect of GM130 and outer aspect of emerin (Fig. 2B, D). After treating of the cells with brefeldin A, which induces the dissociation of the Golgi apparatus (*12*), GM130 staining was dispersed in the cytoplasm while FKRP staining was unaffected (Fig. 2C). These results suggest that FKRP is not localized in the Golgi or inner nuclear membrane but in the rough ER.

The staining of transfected C2C12 cells with antibodies against fukutin and its introduced tag showed the same distribution in the perinuclear area in a discontinuous dot-like pattern under confocal microscopy (data not shown). When transfected C2C12 cells were stained for fukutin and GM130, the two stains precisely overlapped (Fig. 2E), and treatment with brefeldin A dispersed both proteins (Fig. 2F). These phenomena indicate that fukutin is localized in the *cis*-Golgi in differentiated myocytes.

We compared the localization of FKRP and fukutin in C2C12 cells in which myc-fukutin was transiently expressed. FKRP and myc-fukutin displayed different localizations in myocytes (Fig. 2G).

Localization of FKRP in human skeletal muscle—In skeletal muscle biopsy specimens, FKRP was detected continuously around the nuclei of skeletal muscle fibers (Fig. 3A–D, green). After absorption of the FKRP antibody with recombinant FKRP protein, no staining was observed (data not shown). We compared this localization with those of organelle marker proteins against BiP, a chaperone protein in rough ER (*10*), and GM130. BiP was localized around the nuclei and merged with FKRP staining (Fig. 3B, D). GM130 was present at the poles of the nuclei and, in addition, a few concentrated granules were present within the cytosol and did not overlap with FKRP (Fig. 3A, C). These findings suggest that FKRP is localized in rough ER *in vivo*. Interestingly, in some nuclear poles, GM130 and FKRP staining was found to be partly merged (Fig. 3C). It is important to note that ER and the Golgi apparatus form an essentially continuous structure in the intracellular transport system, and that this is a possible explanation for the partial co-localization of FKRP and GM130.

DISCUSSION

The localization of FKRP is distinctly different from that of fukutin. Recently, Esapa et al. reported the subcellular localization of FKRP and fukutin in the medial-Golgi apparatus using rat kidney fibroblasts (*13*). Our data do not support the localization of FKRP in the Golgi apparatus, although immunohistochemical staining of skeletal muscle biopsy sample showed the partial incorporation of FKRP into other Golgi resident proteins. We assume that this difference is a result of the different cell types used in these two studies because FKRP colocalized with fukutin in CHO cells in which FKRP and myc-fukutin were transiently expressed (data not shown). In our experiments using C2C12 cells, endogenous FKRP was seen

only in differentiated myotubes, and the localization was different from those of GM130 and fukutin. Thus, we believe during muscle cell development, FKRP is expressed and functions in the rough ER after myotube formation. In addition, preliminary observations by immunoelectron microscopy demonstrated FKRP localization on the outer nuclear membrane or in transporting vesicles between the nuclear membrane and the Golgi apparatus, close to the distribution of ribosomes (unpublished data). It is unlikely that the localization of extrinsic fukutin is an artefact due to overexpression of this protein, because the extrinsic fukutin localizes only in the *cis*-Golgi apparatus in all transfected cells despite of its variable expression level.

Newly synthesized α-DG in the rough ER is thought to be modified by a series of glycosyltransferases, finally forming unique sugar chains consisting of *O*-mannosyl tetrasaccharide (Siaα2–3Galβ1–4GlcNAcβ1–2Man-*O*-protein) (*14*, *15*). The first *O*-mannosylation step is catalyzed by protein *O*-mannosyltransferase 1 (POMT1) (*16*), and this step may occur in rough ER. In fact, in yeast, several O-mannosyltransferases and their donor substrate, dolichyl-*P*-mannose, are indeed localized in rough ER (*17*). A mutation in *POMT1* causes Walker-Warburg syndrome, the most severe form of congenital muscular dystrophy with brain involvement, and it is also known that skeletal muscle biopsy samples from patients with Walker-Warburg syndrome show markedly reduced levels of α-DG (*16*, *18*). Our observations predict that FKRP localizes in rough ER, suggesting that FKRP may play a role in the first *O*-mannosylation step of α-DG with POMT1. Further analyses of the relationship between POMT1 and FKRP may clarify the role of FKRP in the *O*-mannosylation of α-DG.

In this study, we have clarified the precise subcellular localization of fukutin and FKRP in myocytes. The localization of both proteins suggests that they are involved in the glycosylation of α-DG, but in different manners. The biological characterization of fukutin and FKRP as glycosyltransferases and structural analyses of the α-DG sugar chains in patients are necessary to gain a complete understanding of the glycosylation process of α-DG, and, hence, the possible elucidation of the pathomechanism of FCMD and MDC1C.

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Proteolysis of β-dystroglycan in muscular diseases

Kiichiro Matsumura[a,*], Di Zhong[a], Fumiaki Saito[a], Ken Arai[a], Katsuhito Adachi[b], Hisaomi Kawai[c], Itsuro Higuchi[d], Ichizo Nishino[e], Teruo Shimizu[a]

[a]*Department of Neurology and Neuroscience, Teikyo University School of Medicine,2-11-1 Kaga Itabashi-ku, Tokyo 173-8605, Japan*
[b]*Department of Internal Medicine, Tokushima National Hospital, 1354 Shikiji, Kamojima-cho, Oe-gun, Tokushima 776-8585, Japan*
[c]*Takamatsu Municipal Hospital,2-36-1 Miyawaki-cho, Takamatsu, Kagawa 760-8538, Japan*
[d]*Third Department of Internal, Medicine, Kagoshima University of Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima, Kagoshima 890-8544, Japan*
[e]*Department of Neuromuscular, Research, National Institute of Neuroscience, 4-1-1 Ogawahigashi-cho, Kodaira, Tokyo187-8502, Japan*

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Abstract

α-Dystroglycan is a cell surface peripheral membrane protein which binds to the extracellular matrix (ECM), while β-dystroglycan is a type I integral membrane protein which anchors α-dystroglycan to the cell membrane via the N-terminal extracellular domain. The complex composed of α-and β-dystroglycan is called the dystroglycan complex. We reported previously a matrix metalloproteinase (MMP) activity that disrupts the dystroglycan complex by cleaving the extracellular domain of β-dystroglycan. This MMP creates a characteristic 30 kDa fragment of β-dystroglycan that is detected by the monoclonal antibody 43DAG/8D5 directed against the C-terminus of β-dystroglycan. We also reported that the 30 kDa fragment of β-dystroglycan was increased in the skeletal and cardiac muscles of cardiomyopathic hamsters, the model animals of sarcoglycanopathy, and that this resulted in the disruption of the link between the ECM and cell membrane via the dystroglycan complex. In this study, we investigated the proteolysis of β-dystroglycan in the biopsied skeletal muscles of various human muscular diseases, including sarcoglycanopathy, Duchenne muscular dystrophy (DMD), Becker muscular dystrophy, Fukuyama congenital muscular dystrophy, Miyoshi myopathy, LGMD2A, facioscapulohumeral muscular dystrophy, myotonic dystrophy and dermatomyositis/polymyositis. We show that the 30 kDa fragment of β-dystroglycan is increased significantly in sarcoglycanopathy and DMD, but not in the other diseases. We propose that the proteolysis of β-dystroglycan may contribute to skeletal muscle degeneration by disrupting the link between the ECM and cell membrane in sarcoglycanopathy and DMD.
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Keywords: Dystroglycan; Sarcoglyan; Dystrophin; Laminin; Extracellular matrix; Matrix metalloproteinase; Sarcoglycanopathy; Duchenne muscular dystrophy

1. Introduction

The dystroglycan complex is a cell membrane-spanning complex composed of α-and β-dystroglycan, which are encoded by a single gene *Dag1* and cleaved into two proteins by posttranslational processing [1]. α-Dystroglycan is a cell surface peripheral membrane protein which binds to laminin in the basement membrane, while β-dystroglycan is a type I integral membrane protein which anchors α-dystroglycan to the cell membrane via the N-terminus of the extracellular domain and binds to the cytoskeletal protein dystrophin via the C-terminal cytoplasmic domain [1–5]. Thus, the dystroglycan complex provides a tight link between the extracellular matrix (ECM) and intracellular cytoskeleton. Recently, we reported a matrix metalloproteinase (MMP) activity that disrupts the dystroglycan complex by cleaving the extracellular domain of β-dystroglycan specifically [6]. This MMP creates a characteristic 30 kDa fragment of β-dystroglycan (β-DG_{30}) that is detected by the monoclonal antibody 43DAG/8D5 directed against the C-terminus of β-dystroglycan [6].

In the previous study, we showed that β-DG_{30} was increased in the skeletal and cardiac muscles of cardiomyopathic hamsters [7], the model animals of sarcoglycanopathy (SGCP) [8,9], and that this resulted in the disruption of the link

* Corresponding author. Tel.: +81 3 3964 1211x1915; fax: +81 3 3964 6394.
E-mail address: k-matsu@med.teikyo-u.ac.jp (K. Matsumura).

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between the ECM and cell membrane via the dystroglycan complex in these tissues [7]. In the present study, we investigated the proteolysis of β-dystroglycan in the biopsied skeletal muscles of various human muscular diseases. We show that β-DG_{30} is increased significantly in SGCP and Duchenne muscular dystrophy (DMD), but not in the other diseases.

2. Materials and methods

2.1. Patients

Tables 1 and 2 summarize the patients investigated in this study. The skeletal muscle specimens were obtained by diagnostic biopsy. The diseases include SGCP, DMD, Becker muscular dystrophy (BMD), Fukuyama congenital muscular dystrophy (FCMD), Miyoshi myopathy (MM), LGMD2A, facioscapulohumeral muscular dystrophy (FSHD), myotonic dystrophy (DM) and dermatomyositis/

Table 1
Summary of the patients and results of immunoblot analysis of β-dystroglycan in the skeletal muscle biopsy specimens

Diagnosis	No.	Age	Sex	β-DG_{30}/ β-DG_{full}	Average ± SE
Normal control	1	13	M	0.0358	0.0538 ± 0.0165
	2	14	M	0.0496	
	3	15	M	0.0411	
	4	15	M	0.0127	
	5	16	M	0.0823	
	6	40	M	0.0228	
	7	41	M	0.0253	
	8	43	F	0.0125	
	9	59	M	0.0687	
	10	62	M	0.1869	
SGCP	1	10M	F	2.1467	0.6801 ± 0.2299
	2	7	F	0.3227	
	3	8	M	0.2978	
	4	13	F	0.6819	
	5	15	F	1.0325	
	6	17	F	0.2721	
	7	18	F	0.2500	
	8	31	F	0.4374	
DMD	1	4M	M	0.3729	0.4540 ± 0.0944
	2	1	M	0.4553	
	3	1	M	0.4868	
	4	3	M	0.6436	
	5	3	M	0.3541	
	6	4	M	0.4800	
	7	4Y10M	M	0.3303	
	8	5	M	0.3762	
	9	5	M	0.5457	
	10	6	M	0.5272	
	11	7Y1M	M	0.5011	
	12	8Y9M	M	0.3745	
BMD	1	3	M	0.0309	0.1030 ± 0.0253
	2	3Y10M	M	0.1340	
	3	4Y7M	M	0.0585	
	4	5	M	0.1236	
	5	13	M	0.1681	
FCMD	1	7M	F	0.0218	0.0336 ± 0.0069
	2	8M	F	0.0213	
	3	9M	M	0.0599	
	4	9M	M	0.0421	
	5	9M	M	0.0204	
	6	1Y	F	0.0166	
	7	1Y5M	F	0.0658	
	8	3Y	F	0.0209	
MM	1	25	F	0.0991	0.0779 ± 0.0164
	2	27	M	0.0652	
	3	30	M	0.1313	
	4	38	M	0.0504	
	5	39	M	0.0436	
LGMD2A	1	7Y3M	F	0.0752	0.0646 ± 0.0148
	2	11	F	0.0794	
	3	20	M	0.0206	
	4	26	F	0.0832	
FSHD	1	8	M	0.1307	0.1048 ± 0.0234
	2	19	F	0.0966	
	3	25	M	0.1363	
	4	41	M	0.0170	
	5	48	M	0.1437	
DM	1	14	M	0.0652	0.0722 ± 0.0226
	2	28	M	0.0701	
	3	37	F	0.1541	
	4	50	F	0.0557	
	5	60	M	0.0161	
DM/PM	1	2Y5m	M	0.0762	0.0589 ± 0.0135
	2	3	F	0.0951	
	3	4	F	0.0602	
	4	4	M	0.0000	
	5	10	F	0.1340	
	6	23	M	0.0168	
	7	30	F	0.0114	
	8	33	F	0.0477	
	9	46	M	0.0512	
	10	51	F	0.0957	

The skeletal muscle biopsy specimens were analyzed by immunoblotting using the monoclonal antibody 43DAG/8D5 and the β-DG_{30}/β-DG_{full} ratio was obtained for each patient as described in Materials and Methods. SE, standard error.

Table 2
Genetic analysis of SGCP patients

Patient no	Genetic analysis
1	β-SG, 325 C to T (R109X), homozygous
2	β-SG, 325 C to T (R109X), homozygous
3	α-SG, 229 C to T (R77C), homozygous
4	γ-SG, 630-702 base deletion, homozygous
5	Not done
6	α-SG, 229 C to T (R77C), homozygous
7	α-SG, 220 C to T (R74W), homozygous
8	α-SG, 410 A to G (E137G)/409-423 bases insertion

Patient 5 was diagnosed as SGCP, based on the clinical profile and the specific deficiency of the components of the sarcoglycan complex in the biopsied skeletal muscle as revealed by immunohistochemical analysis (not shown).