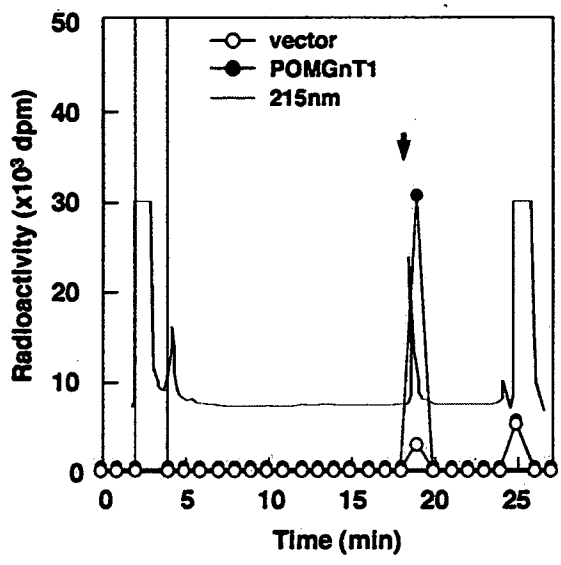

Fig. 4. POMGnT activity of human POMGnT1. UDP-[^{3}H]GlcNAc and mannosylpeptide were reacted with membrane fraction in POMGnT1 reaction buffer and then subjected to reversed-phase high-performance liquid chromatography. The mobile phase consists of (1) 100% A for 5 min, (2) a linear gradient to 75% A, 25% B for 20 min, (3) a linear gradient to 100% B for 1 min, and (4) 100% B for 5 min. The peptide separation was monitored at 214 nm and the radioactivity of each 1-mL fraction was measured by liquid scintillation counting. Arrow indicates the elution position of the mannosylpeptide. Vector (○), cells transfected with vector alone; POMGnT1 (●), cells transfected with human *POMGnT1*.

2. The POMGnT reaction buffer is added to the microsomal membrane fraction at a protein concentration of 2 mg/mL. The fraction is suspended with a bath-type sonicator on ice and solubilized by moderate pipetting until transparent. After centrifugation at 10,000*g* for 10 min, 20 μL of the supernatant is added to the dried substrate (prepared in **step 1**), vortexed gently, and incubated at 37°C for 2 h. The reaction is stopped by boiling at 100°C for 3 min. 180 μL of water is added to the reaction mixture and filtered with a centrifugal filter device.
3. The filtrate is analyzed by reversed-phase HPLC on the following condition: the gradient solvents are aqueous 0.1% TFA (solvent A) and acetonitrile containing 0.1% TFA (solvent B). The mobile phase consists of (1) 100% A for 5 min, (2) a linear gradient to 75% A, 25% B for 20 min, (3) a linear gradient to 100% B for 1 min, and (4) 100% B for 5 min. The peptide separation is monitored by measuring the absorbance at 214 nm, and the radioactivity of each 1-mL fraction is measured by liquid scintillation counting (*see* **Fig. 4**).
4. The reaction product is characterized by two different methods: (1) The product is dried up by an evaporator and then incubated with 50 mU of streptococcal

β-*N*-acetylhexosaminidase at 37°C for 48 h. After incubation, the enzyme is inactivated by boiling at 100°C for 3 min. The enzyme-digested sample is rechromatographed as described in **step 3**. (2) β-Elimination is performed as follows: the product is dissolved in 500 µL of 0.05 *N* NaOH and 1 *M* of $NaBH_4$, and incubated for 18 h at 45°C. After adjusting the pH to 5.0 by adding 4 *N* of acetic acid, the solution is applied to a column containing 1 mL of AG-50W-X8 (H^+ form) and the column is then washed with 10 mL of water. The effluent and the washing are combined and evaporated. After the remaining borate is removed by repeated evaporation with methanol, the residue is analyzed by high-pH anion-exchange chromatography with pulsed amperometric detection (*see* **ref. *14)***.

4. Notes

1. Do not dry completely; approx 10 µL of solvent should remain.
2. Synthesis of mannosylpeptide substrate: Mannosylpeptide (Ac-Ala-Ala-Pro-Thr(Man)-Pro-Val-Ala-Ala-Pro-NH_2) is synthesized in a solid-phase manner using 9-fluorenyloxymethylcarbonyl (Fmoc) chemistry. Fmoc-Thr(Man)-OH is synthesized as follows: the reaction of phenyl 2,3,4,6-tetra-*O*-benzyl-1-thio-D-mannopyranoside and *N*-benzyloxycarbonyl-L-threonine benzyl ester (Z-Thr-OBzl) in the presence of *N*-iodosuccinimide and trifluoromethanesulfonic acid give the desired protected mannosyl threonine derivative (Z-Thr(Man(OBzl)$_4$)-OBzl) with a 77% yield. After deprotection of all benzyl groups and the Z group by catalytic hydrogenation, Fmoc-OSu is reacted with the residue to give the desired Fmoc-Thr(Man)-OH with a 75% yield. The product is easily purified by solid-phase extraction using a polymeric adsorbent, such as Dianion HP-20 (Nippon Rensui Co., Tokyo, Japan) or Amberlite XAD-2 (Organo, Tokyo, Japan).

   After the final deprotection from the glycopeptide resin, the crude mannosyl peptide is purified on a C18-preparative reversed-phase column (Inertsil ODS-3, 20X 250 mm, GL Sciences Inc., Tokyo, Japan) eluted by mixing solvent A (0.1% TFA in water) with solvent B (0.1% TFA in acetonitrile) at 45°C at a flow rate of 10 mL/min as follows: 25 min at 5% solvent B, linear gradient to 10 min at 35% solvent B. The glycopeptide separation is monitored continuously by measuring the absorbance at 214 nm. The structure of the product is identified by ^{1}H-NMR, amino acid analysis (6 *M* HCl, 110°C, 24 h), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Distilled water is referred to as water in this text.
3. Cell pellets can be stored at –80°C after removal of PBS.
4. Typical sonication conditions to reach semitranslucent cell suspensions are: 10 cycles of 0.6-s pulse with 0.4-s intervals, and these procedures are repeated again.
5. The precipitate can be stored at –80°C after removal of supernatant.
6. Semitranslucent cell suspensions are obtained by 3-s sonication with 3-s intervals for 5–10 min.
7. POMT activity is inactivated in the presence of Triton X-100.

8. When using a screw-cap tube, a packing seal is required to prevent the leakage of radioactivity.

Acknowledgments

This study was supported by a Research Grant for Nervous and Mental Disorders (17A-10) from the Ministry of Health, Labour and Welfare of Japan, and a Grant-in-Aid for Scientific Research on Priority Area (14082209) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Chiba, A., Matsumura, K., Yamada, H., et al. (1997) Structures of sialylated *O*-linked oligosaccharides of bovine peripheral nerve α-dystroglycan. The role of a novel *O*-mannosyl-type oligosaccharide in the binding of α-dystroglycan with laminin. *J. Biol. Chem.* **272,** 2156–2162.
2. Yuen, C. T., Chai, W., Loveless, R. W., Lawson, A. M., Margolis, R. U., and Feizi, T. (1997) Brain contains HNK-1 immunoreactive *O*-glycans of the sulfoglucuronyl lactosamine series that terminate in 2-linked or 2,6-linked hexose (mannose). *J. Biol. Chem.* **272,** 8924–8931.
3. Sasaki, T., Yamada, H., Matsumura, K., Shimizu, T., Kobata, A., and Endo, T. (1998) Detection of *O*-mannosyl glycans in rabbit skeletal muscle α-dystroglycan. *Biochim. Biophys. Acta* **1425,** 599–606.
4. Smalheiser, N. R., Haslam, S. M., Sutton-Smith, M., Morris, H. R., and Dell, A. (1998) Structural analysis of sequences *O*-linked to mannose reveals a novel Lewis *X* structure in cranin (dystroglycan) purified from sheep brain. *J. Biol. Chem.* **273,** 23,698–23,703.
5. Chai, W., Yuen, C. T., Kogelberg, H., et al. (1999) High prevalence of 2-mono- and 2,6-di-substituted manol-terminating sequences among O-glycans released from brain glycopeptides by reductive alkaline hydrolysis. *Eur. J. Biochem.* **263,** 879–888.
6. Endo, T. (1999) *O*-mannosyl glycans in mammals. *Biochim. Biophys. Acta* **1473,** 237–246.
7. Michele, D. E., Barresi, R., Kanagawa, M., et al. (2002) Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. *Nature* **418,** 417–422.
8. Michele, D. E. and Campbell, K. P. (2003) Dystrophin-glycoprotein complex: post-translational processing and dystroglycan function. *J. Biol. Chem.* **278,** 15,457–15,460.
9. Montanaro, F. and Carbonetto, S. (2003) Targeting dystroglycan in the brain. *Neuron* **37,** 193–196.
10. Endo, T. (2004) Structure, function and pathology of *O*-mannosyl glycans. *Glycoconj. J.* **21,** 3–7.
11. Yoshida, A., Kobayashi, K., Manya, H., et al. (2001) Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. *Dev. Cell* **1,** 717–724.

12. Manya, H., Chiba, A., Yoshida, A., et al. (2004) Demonstration of mammalian protein *O*-mannosyltransferase activity: coexpression of POMT1 and POMT2 required for enzymatic activity. *Proc. Natl. Acad. Sci. USA* **101,** 500–505.
13. Beltran-Valero De Bernabe, D., Currier, S., Steinbrecher, A., et al. (2002) Mutations in the *O*-mannosyltransferase gene *POMT1* give rise to the severe neuronal migration disorder Walker-Warburg syndrome. *Am. J. Hum. Genet.* **71,** 1033–1043.
14. Takahashi, S., Sasaki, T., Manya, H., et al. (2001) A new β-1,2-*N*-acetylglucosaminyltransferase that may play a role in the biosynthesis of mammalian *O*-mannosyl glycans. *Glycobiology* **11,** 37–45.
15. Strahl-Bolsinger, S., Gentzsch, M., and Tanner, W. (1999) Protein *O*-mannosylation. *Biochim. Biophys. Acta* **1426,** 297–307.
16. Jurado, L. A., Coloma, A., and Cruces, J. (1999) Identification of a human homolog of the *Drosophila* rotated abdomen gene *(POMT1)* encoding a putative protein *O*-mannosyl-transferase, and assignment to human chromosome 9q34.1. *Genomics* **58,** 171–180.
17. Willer, T., Amselgruber, W., Deutzmann, R., and Strahl, S. (2002) Characterization of POMT2, a novel member of the *PMT* protein *O*-mannosyltransferase family specifically localized to the acrosome of mammalian spermatids. *Glycobiology* **12,** 771–783.
18. Ibraghimov-Beskrovnaya, O., Ervasti, J. M., Leveille, C. J., Slaughter, C. A., Sernett, S. W., and Campbell, K. P. (1992) Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* **355,** 696–702.
19. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
20. Zhang, W., Vajsar, J., Cao, P., et al. (2003) Enzymatic diagnostic test for Muscle-Eye-Brain type congenital muscular dystrophy using commercially available reagents. *Clin. Biochem.* **36,** 339–344.

Aberrant glycosylation of α-dystroglycan causes defective binding of laminin in the muscle of chicken muscular dystrophy

Fumiaki Saito[a,*], Martina Blank[b], Jörn Schröder[b], Hiroshi Manya[c], Teruo Shimizu[a], Kevin P. Campbell[d], Tamao Endo[c], Makoto Mizutani[e], Stephan Kröger[b], Kiichiro Matsumura[a]

[a] *Department of Neurology and Neuroscience, Teikyo University, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan*
[b] *Institute for Physiological Chemistry, University of Mainz, Duesbergweg 6, D-55099 Mainz, Germany*
[c] *Glycobiology Research Group, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-chyo, Itabashi-ku, Tokyo 173-0015, Japan*
[d] *Howard Hughes Medical Institute, Department of Physiology and Biophysics and Department of Neurology, The University of Iowa, Roy J. and Lucille A. Carver College of Medicine, 400 Eckstein medical research building, Iowa City, IA 52242-1101, USA*
[e] *Nippon Institute for Biological Science, 3331-114 kamisasao, kobuchisawa-chyo, Yamanashi prefecture 408-0041, Japan*

Received 17 February 2005; revised 9 March 2005; accepted 9 March 2005

Available online 25 March 2005

Edited by Sandro Sonnino

Abstract **Dystroglycan is a central component of dystrophin–glycoprotein complex that links extracellular matrix and cytoskeleton in skeletal muscle. Although dystrophic chicken is well established as an animal model of human muscular dystrophy, the pathomechanism leading to muscular degeneration remains unknown. We show here that glycosylation and laminin-binding activity of α-dystroglycan (α-DG) are defective in dystrophic chicken. Extensive glycan structural analysis reveals that Galβ1-3GalNAc and GalNAc residues are increased while Siaα2-3Gal structure is reduced in α-DG of dystrophic chicken. These results implicate aberrant glycosylation of α-DG in the pathogenesis of muscular degeneration in this model animal of muscular dystrophy.**

Keywords: Dystroglycan; Laminin; Muscular dystrophy; Glycosylation; Dystrophic chicken

1. Introduction

The dystroglycan complex is composed of two proteins, α- and β-dystroglycan (α- and β-DG), which are encoded by a single gene and cleaved by posttranslational processing [1]. α-DG is a highly glycosylated extracellular peripheral membrane protein and binds to several extracellular matrix (ECM) proteins including laminin, agrin, and perlecan [2–4]. In turn, the transmembrane protein β-DG anchors α-DG at the extracellular surface of the plasma membrane, while its cytoplasmic domain interacts with dystrophin, a large cytoplasmic protein that binds to F-actin [5]. Thus, the DG complex plays a crucial role to stabilize the plasma membrane by acting as an axis through which the ECM is tightly linked to the cytoskeleton.

Recently, primary mutations in the genes encoding putative glycosyltransferases have been identified in several types of congenital muscular dystrophies including Fukuyama-type congenital muscular dystrophy, muscle–eye–brain disease, Walker–Warburg syndrome, congenital muscular dystrophy 1C (MDC1C) and 1D (MDC1D) [6–10]. Because glycosylation and laminin-binding activity of α-DG are defective in these diseases [11], they are collectively called α-dystroglycanopathy [12]. However, the precise oligosaccharide structures defective in α-dystroglycanopathy have not been elucidated.

Muscular dystrophy in chicken was first described in 1956 [13]. Although dystrophic chicken has been established as an animal model of muscular dystrophy, the primary mutation has not yet been identified [14] and the pathomechanism leading to muscle cell degeneration remains unknown. We demonstrate here that glycosylation and laminin-binding activity of α-DG are defective in the skeletal muscle of dystrophic chicken. Extensive glycan structural analysis reveals that, compared to control chicken, the amount of Galβ1-3GalNAc and GalNAc residues are increased, whereas Siaα2-3Gal structure is reduced in α-DG of dystrophic chicken.

2. Materials and methods

2.1. Antibodies

Mouse monoclonal antibody against sugar chain moiety of α-DG (IIH6) and sheep polyclonal antibody against core protein of α-DG (sheep anti-α-DG) were described previously [2,15]. Mouse monoclonal antibody against sugar chain moiety of α-DG (IVA4-1) was obtained from Upstate Biotechnology. Mouse monoclonal antibody against β-DG (8D5), β-sarcoglycan (5B1) and γ-sarcoglycan (21B5) were kind gifts from Dr. L.V.B. Anderson (Newcastle General Hospital). Mouse monoclonal anti-dystrophin (MANDRA 1) and affinity isolated rabbit anti-laminin were obtained from Sigma. Mouse monoclonal anti-dystrobrevin was purchased from BD Biosciences.

2.2. Lectin chromatography

Dystrophic chicken used in this study is New Hampshire, line 413, the colony of which is maintained homozygously. Line GSN/1, was used as a control. Pectoralis muscle of dystrophic and control chicken of 3 months of age were used. Skeletal muscle was disrupted with a polytron followed by Daunce homogenization and incubation in 50 mM Tris–HCl, pH 7.4, 500 mM NaCl, 1% Triton X-100, 0.6 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, 0.75 mM benzamidine, and 0.1 mM PMSF. The extract was incubated with lectin

*Corresponding author. Fax: +813 3964 6394.
E-mail address: f-saito@med.teikyo-u.ac.jp (F. Saito).

Abbreviations: DG, dystroglycan; DGC, dystrophin–glycoprotein complex

0014-5793/$30.00
doi:10.1016/j.febslet.2005.03.033

agarose, including wheat germ agglutinin (WGA), concanavalin A (Con A), peanut agglutinin (PNA), *Vicia villosa* agglutinin isolectin B_4 (VVA-B_4), *Maackia amurensis* lectin (MAM) and lentil lectin (LCA). Bound proteins were eluted by boiling the beads in sample buffer (65 mM Tris–HCl, pH 6.9, 3% SDS, 1% β-mercaptoethanol, 115 mM sucrose, and 0.0004% bromophenol blue) and the eluates were analyzed by Western blotting using sheep anti-α-DG.

2.3. Miscellaneous

Chemical deglycosylation was described previously [2]. Sialidase digestion was performed using sialidase from *Clostridium perfringens* (Roche) according to the procedure described elsewhere [16]. Immunofluorescent microscopic analysis, Western blotting and blot overlay assay were performed as described elsewhere [11]. The amount of glycosidically bound sialic acid was compared by periodate–resorcinol method [17] and statistical significance was evaluated by *t* test. Solid-phase assay was performed as previously mentioned [11] except that WGA eluates were coated on 96 wells EIA/RIA plates (Coaster) after measuring the band intensity of α-DG on Western blots so that each well contained the same amount of α-DG.

3. Results

3.1. Decreased immunoreactivity of α-DG in the skeletal muscle of dystrophic chicken

We first performed immunofluorescent microscopic analysis. The immunoreactivity of α-DG revealed by antibody against sugar chain moiety of α-DG was significantly decreased in dystrophic chicken, whereas the immunoreactivity of α-DG was indistinguishable between control and dystrophic chicken when detected by antibody against core protein of α-DG. The other components of dystrophin–glycoprotein complex (DGC) were normally expressed in dystrophic chicken (Fig. 1). Consistent with the immunofluorescent analysis, Western blotting with antibody against sugar chain moiety of α-DG demonstrated reduced immunoreactivity of α-DG in dystrophic chicken (Fig. 2). In addition, α-DG of dystrophic chicken migrated at 160 kD, faster than that of control which migrated at 200 kD (Fig. 2). The expression and molecular mass of the other components of the DGC were not altered (Fig. 2).

3.2. Altered glycosylation of α-DG in the skeletal muscle of dystrophic chicken

The results described above raise the possibility that the glycosylation, rather than expression, of α-DG in dystrophic chicken may be altered. In order to test this possibility, α-DG was enriched by WGA chromatography and chemically deglycosylated with trifluoromethanesulfonic acid. Similar to the antibody against sugar chain moiety of α-DG, antibody against core protein of α-DG recognized α-DG species migrating around 200 and 160 kD in control and dystrophic chicken, respectively (Fig. 3, deglycosylation −). In addition, however, the anti-core protein antibody also detected α-DG species with a lower molecular mass of 110 kD in control and 70–120 kD in dystrophic chicken (Fig. 3, deglycosylation −). In this report, we tentatively call the larger and smaller α-DG species as L-α-dystroglycan (L-α-DG) and S-α-dystroglycan (S-α-DG), respectively. Upon chemical deglycosylation, the molecular mass of α-DG was reduced to 55 kD both in control and dystrophic chicken equally, eliminating the difference in molecular mass (Fig. 3, deglycosylation +). These data indicate that α-DG is aberrantly glycosylated in the skeletal muscle of dystrophic chicken. We also examined various tissues of dystrophic chicken to see if defective glycosylation of α-DG was present. Western blot analysis using antibody against core protein of α-DG demonstrated a

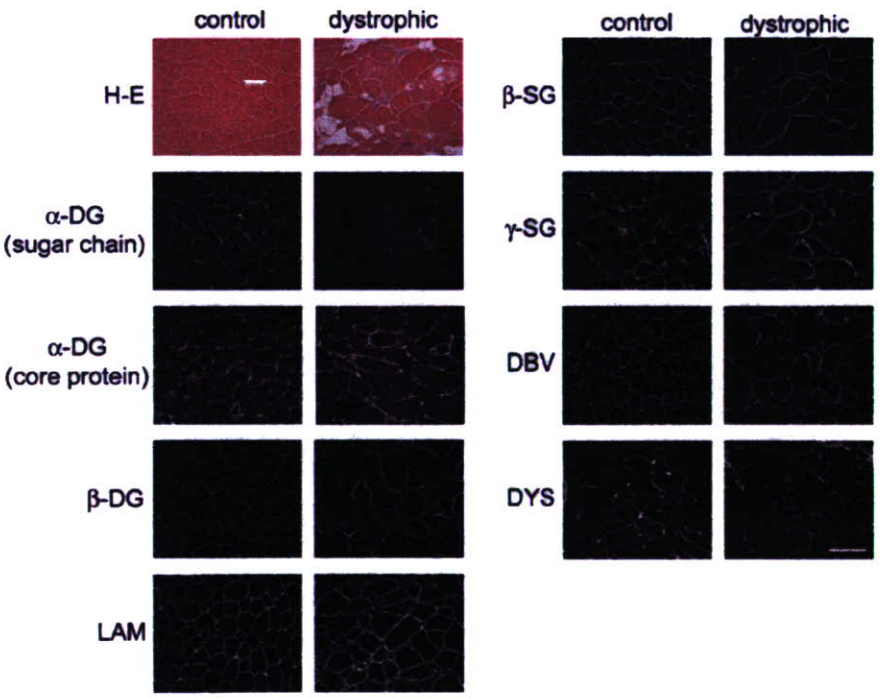

Fig. 1. Immunoreactivity of α-dystroglycan is reduced in the skeletal muscle of dystrophic chicken when probed by antibody against sugar chain moiety. Expression and localization of each component of the DGC were analyzed by immunofluorescent microscopy. The immunoreactivity of α-DG, as revealed by antibody against sugar chain moiety of α-DG (IIH6), is reduced in dystrophic chicken. However, the expression of α-DG core protein is not altered. DG, dystroglycan; LAM, laminin; SG, sarcoglycan; DBV, dystrobrevin; DYS, dystrophin. Bar indicates 100 μm.

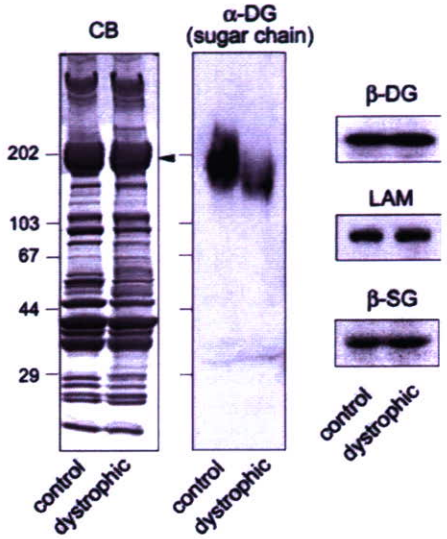

Fig. 2. The molecular mass of α-DG is decreased in the skeletal muscle of dystrophic chicken. Western blotting was performed to examine the expression of α-DG using whole skeletal muscle homogenates. The amount of protein loaded for each lane was normalized using myosin heavy chain as internal standard (arrowhead in the panel CB). α-DG in dystrophic chicken migrates faster than that in control and the immunoreactivity of α-DG is decreased in dystrophic chicken using antibody against sugar chain moiety of α-DG (IIH6). The expression of other components of the DGC is not altered. CB, Coomassie blue staining; DG, dystroglycan; LAM, laminin; SG, sarcoglycan.

downward shift in the molecular mass of α-DG in cardiac muscle, but not in other tissues including brain, peripheral nerve, kidney, spleen and liver (data not shown), indicating

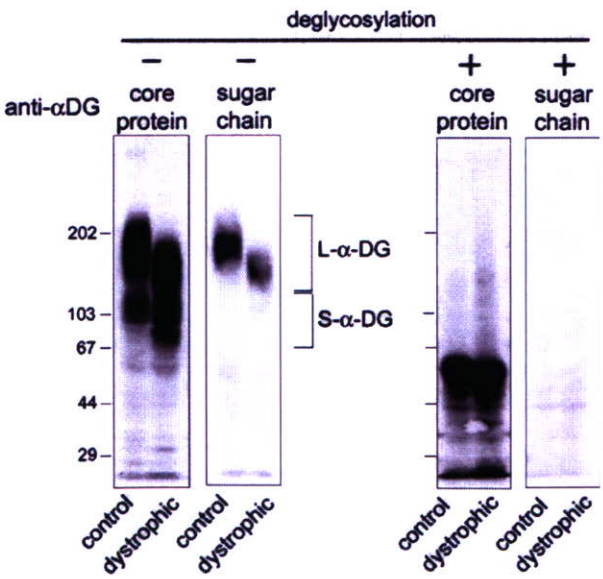

Fig. 3. Deglycosylation eliminates the difference in molecular mass of α-DG between control and dystrophic chicken. α-DG was enriched by WGA chromatography and chemically deglycosylated with trifluoromethanesulfonic acid. Antibody against core protein of α-DG recognizes α-DG species with higher molecular mass (L-α-DG), which are also detected by antibody against sugar chain moiety of α-DG (VIA4-1). In addition, the anti-core protein of α-DG recognizes α-DG species with lower molecular mass (S-α-DG). After deglycosylation, the molecular mass of α-DG decreases to 55 kD in both control and dystrophic chicken equally (deglycosylation +).

that glycosylation of α-DG was also altered in the cardiac muscle of dystrophic chicken.

3.3. Laminin-binding activity of α-DG is decreased in the skeletal muscle of dystrophic chicken

Blot overlay assays demonstrated that the binding of laminin 1 and 2 to α-DG was greatly reduced in dystrophic chicken (Fig. 4A). Notably, both laminin 1 and 2 bound to L-α-DG, but not S-α-DG (Fig. 4A). The band intensity of S-α-DG and L-α-DG was measured and the ratio of S-α-DG against total α-DG (intensity of S-α-DG/intensity of S-α-DG + L-α-DG) was calculated. The ratio of S-α-DG was 16.8 ± 4.5% in control versus 40.9 ± 4.1% in dystrophic chicken (Fig. 4B), indicating that many more α-DG molecules in dystrophic chicken lack the laminin-binding activity than control. Next, we performed quantitative solid-phase assay. The total laminin-binding activity was significantly decreased in the skeletal muscle of dystrophic chicken (Fig. 4C).

3.4. Glycosylation defects of dystrophic chicken α-DG analyzed by lectin chromatography

To investigate the change in glycan structure of α-DG in dystrophic chicken, we performed a set of lectin chromatographies.

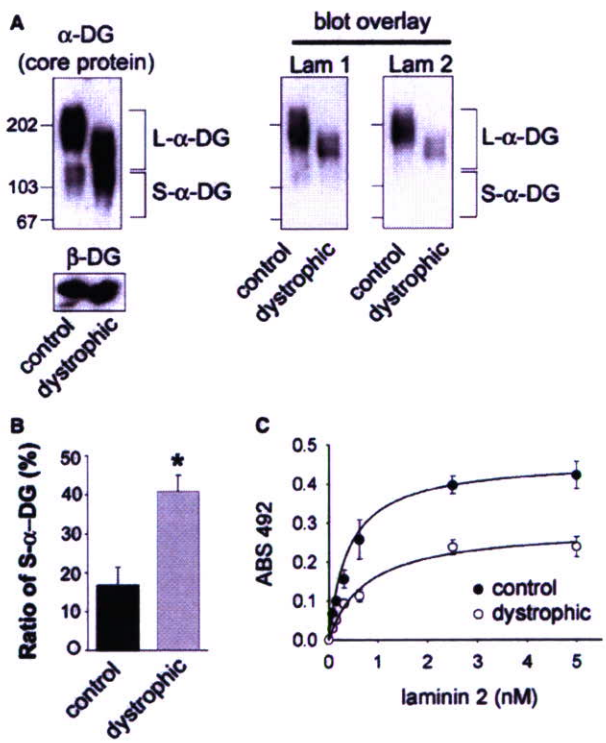

Fig. 4. Laminin-binding activity of α-DG is decreased in the skeletal muscle of dystrophic chicken. (A) Equal amount of DG was transferred to PVDF membranes as revealed by Western blotting for α-DG and β-DG. Blot overlay assays demonstrate that the binding of both laminin 1 and 2 to α-DG is substantially decreased in dystrophic chicken. Both laminin 1 and 2 bind to L-α-DG, but not S-α-DG. Lam 1, laminin 1; Lam 2, laminin 2. (B) The band intensity of L-α-DG and S-α-DG was measured and the ratio of S-α-DG against total α-DG was calculated. The ratio of S-α-DG is significantly higher in dystrophic chicken. $^{*}P < 0.003$. (C) Solid-phase assay reveals that laminin-binding activity is significantly reduced in the skeletal muscle of dystrophic chicken.

As shown in Fig. 5A, Con A bound most of the α-DG species, whereas LCA had no significant interaction with any α-DG species (Fig. 5A). In sharp contrast, MAM bound L-α-DG in control, while it interacted only weakly with α-DG in dystrophic chicken (Fig. 5A), indicating that Siaα2-3Gal moieties are profoundly reduced in α-DG of dystrophic chicken. Interestingly, PNA bound to a fraction of S-α-DG in dystrophic chicken, while no binding to α-DG occurred in control (Fig. 5B, sialidase −). VVA-B_4 bound weakly to S-α-DG in control, whereas it strongly interacted with L-α-DG and S-α-DG in dystrophic chicken. Because the reactivity of these lectins are known to be severely decreased when sialic acids are attached to non-reducing termini of their binding sugar chain moieties [18], we enzymatically removed sialic acids by sialidase and repeated the experiments. After sialidase digestion, both S-α-DG and L-α-DG extensively interacted with PNA in dystrophic chicken, whereas only a small amount of S-α-DG was recovered in control. These results indicate that Galβ1-3GalNAc moieties are much more abundant on α-DG in dystrophic chicken than that in control (Fig. 5B, sialidase +). Similar result was obtained with VVA-B_4, indicating that GalNAc structures are much more abundant on α-DG of dystrophic chicken (Fig. 5B, sialidase +). The amount of glycosidically bound sialic acids quantified by periodate–resorcinol method was substantially reduced in dystrophic chicken (Fig. 5C), which is consistent with the result of MAM lectin chromatography.

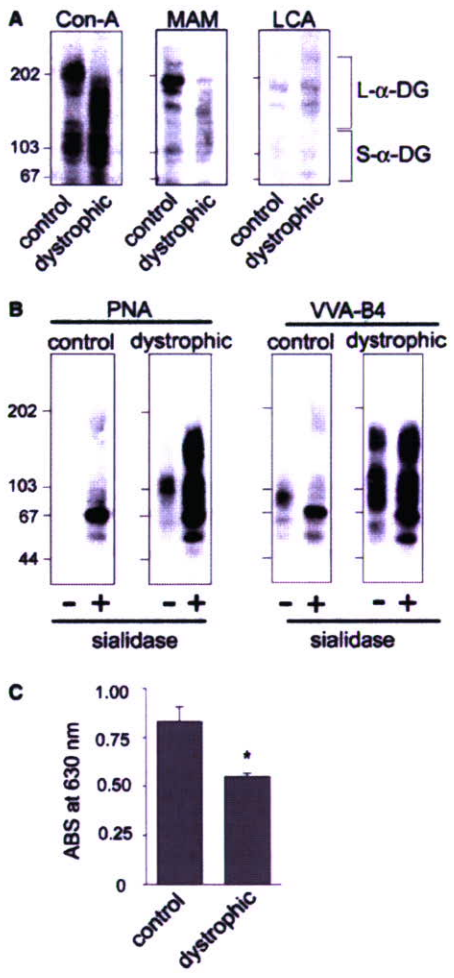

Fig. 5. Glycosylation of α-DG is altered in the skeletal muscle of dystrophic chicken. (A) α-DG was extracted using Triton X-100 and applied to lectin chromatography. The bound α-DG was visualized by Western blotting using antibody against core protein of α-DG. Con-A binds most of the α-DG species, while LCA does not interact with any α-DG species significantly. MAM strongly binds only to L-α-DG in control. (B) The Triton X-100 extracts were applied to PNA or VVA-B_4 chromatography with or without prior digestion by sialidase. Without sialidase treatment, PNA binds S-α-DG in dystrophic chicken, while it does not interact with α-DG in control (sialidase −). VVA-B_4 binds S-α-DG in control only weakly, while it interacts strongly with both L- and S-α-DG in dystrophic chicken (sialidase −). With sialidase digestion, both PNA and VVA-B_4 bind extensively to L-α-DG and S-α-DG in dystrophic chicken compared to control (sialidase +). (C) Quantification of sialic acid by periodate–resorcinol method reveals that the amount of glycosidically bound sialic acids in the skeletal muscle of dystrophic chicken is significantly less than that of control chicken. $^*P < 0.001$.

4. Discussion

The mucin-like domain of α-DG is heavily glycosylated by *O*-linked glycans [19], with the sugar chain moieties constituting up to two-thirds of its total molecular mass [1,2]. The antibody against sugar chain moiety of α-DG detected only L-α-DG, while anti-α-DG core protein detected both L-α-DG and S-α-DG (Figs. 2 and 3), indicating diverse glycosylation of α-DG in vivo. Notably, laminin bound to L-α-DG, but not to S-α-DG, in both control and dystrophic chicken (Fig. 4), indicating that the interaction of laminin with α-DG is strictly regulated through glycosylation of α-DG and that a fraction of α-DG does not possess the sugar chain moieties necessary for the binding of laminin in vivo. Furthermore, the ratio of non-laminin-binding α-DG (S-α-DG) is greatly increased in dystrophic chicken compared to control (Fig. 4). It would be intriguing to postulate that the increase of non-laminin-binding α-DG may contribute to the dystrophic phenotype by exerting a dominant negative effect in dystrophic chicken, where non-laminin-binding α-DG competes with laminin-binding α-DG for the cytoskeletal linkage via dystrophin. Consistent with this hypothesis, we have observed that adenovirus mediated gene transfer of non-laminin-binding α-DG constructs results in the degeneration of skeletal muscle in mice (Saito and Campbell, unpublished observation).

The results of lectin chromatography indicate that, compared to control chicken, the amount of Galβ1-3GalNAc and GalNAc residues are increased significantly while Siaα2-3Gal structure is severely decreased in α-DG of dystrophic chicken (Fig. 5). The reduction in the amount of sialic acids was confirmed by periodate–resorcinol sialic acid assay (Fig. 5). However, α-DG appears to be hyposialylated rather than asialylated (Fig. 5C). We have reported recently that hyposialylation of α-DG alone is not enough to abolish its laminin-binding activity in vivo [20]. It remains to be determined if hyposialylation in dystrophic chicken reflects the reduction of the sialyl *O*-mannosyl glycan, Sia2-3Galβ1-4GlcNAcβ1-2Man-Ser/Thr, implicated in the binding of laminin [21,22].

Pavoni et al. [23] reported recently that antibody against C-terminal portion of α-DG core protein detected α-DG with molecular mass of 109 kD in the skeletal muscle of normal chicken. Our S-α-DG may correspond to this small α-DG, as judged by molecular mass. Pavoni et al. further postulated that this 109 kD α-DG might be a partially glycosylated form of

α-DG. In the present study, we provided clear evidence of actual alteration of glycosylation of this small α-DG molecule (Figs. 3 and 5). The molecular mass of α-DG in the skeletal muscle of normal chicken was reported to change during development [24]. It would be thus interesting to see if the molecular mass of α-DG in the skeletal muscle of dystrophic chicken also changes during development by future studies.

In conclusion, we have demonstrated altered glycosylation and decreased laminin-binding activity of α-DG in chicken muscular dystrophy. Furthermore, we have demonstrated that Siaα2-3Gal structure is reduced, while Galβ1-3GalNAc and GalNAc moieties are increased on α-DG of this animal model of muscular dystrophy. These data would contribute to further understand the molecular mechanism of muscular degeneration caused by disturbed glycosylation of α-DG in human muscular dystrophies.

Acknowledgments: We thank Hiroko F. Ohi, Miki Yamanaka and Yuka Sasayama for their expert technical assistance. This work was supported by [1] Research Grants 14B-4 and 16B-1 for Nervous and Mental Disorders (Ministry of Health, Labor and Welfare), [2] Research on Psychiatric and Neurological Diseases and Mental Health (Ministry of Health, Labor and Welfare), [3] Research Grant 16390256 and "High-Tech Research Center" Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), 2004–2008, and [4] a research grant from the Ichiro Kanahara Foundation.

References

[1] Ibraghimov-Beskrovnaya, O., Ervasti, J.M., Leveille, C.J., Slaughter, C.A., Sernett, S.W. and Campbell, K.P. (1992) Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature 355, 696–702.

[2] Ervasti, J.M. and Campbell, K.P. (1993) A role for the dystrophin–glycoprotein complex as a transmembrane linker between laminin and actin. J. Cell. Biol. 122, 809–823.

[3] Bowe, M.A., Deyst, K.A., Leszyk, J.D. and Fallon, J.R. (1994) Identification and purification of an agrin receptor from Torpedo postsynaptic membranes: a heteromeric complex related to the dystroglycans. Neuron 12, 1173–1180.

[4] Peng, H.B., Ali, A.A., Daggett, D.F., Rauvala, H., Hassell, J.R. and Smalheiser, N.R. (1998) The relationship between perlecan and dystroglycan and its implication in the formation of the neuromuscular junction. Cell Adhes. Commun. 5, 475–489.

[5] Jung, D., Yang, B., Meyer, J., Chamberlain, J.S. and Campbell, K.P. (1995) Identification and characterization of the dystrophin anchoring site on β-dystroglycan. J. Biol. Chem. 270, 27305–27310.

[6] Kobayashi, K., Nakahori, Y., Miyake, M., Matsumura, K., Kondo-Iida, E., Nomura, Y., Segawa, M., Yoshioka, M., Saito, K., Osawa, M., Hamano, K., Sakakihara, Y., Nonaka, I., Nakagome, Y., Kanazawa, I., Nakamura, Y., Tokunaga, K. and Toda, T. (1998) An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. Nature 394, 388–392.

[7] Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M., Herrmann, R., Straub, V., Talim, B., Voit, T., Topaloglu, H., Toda, T. and Endo, T. (2001) Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. Dev. Cell 1, 717–724.

[8] Beltran-Valero De Bernabe, D., Currier, S., Steinbrecher, A., Celli, J., Van Beusekom, E., Van Der Zwaag, B., Kayserili, H., Merlini, L., Chitayat, D., Dobyns, W.B., Cormand, B., Lehesjoki, A.E., Cruces, J., Voit, T., Walsh, C.A., van Bokhoven, H. and Brunner, H.G. (2002) Mutations in the *O*-mannosyltransferase gene POMT1 give rise to the severe neuronal migration disorder Walker–Warburg syndrome. Am. J. Hum. Genet. 71, 1033–1043.

[9] Brockington, M., Blake, D.J., Prandini, P., Brown, S.C., Torelli, S., Benson, M.A., Ponting, C.P., Estournet, B., Romero, N.B., Mercuri, E., Voit, T., Sewry, C.A., Guicheney, P. and Muntoni, F. (2001) Mutations in the fukutin-related protein gene (FKRP) cause a form of congenital muscular dystrophy with secondary laminin α2 deficiency and abnormal glycosylation of α-dystroglycan. Am. J. Hum. Genet. 69, 1198–1209.

[10] Longman, C., Brockington, M., Torelli, S., Jimenez-Mallebrera, C., Kennedy, C., Khalil, N., Feng, L., Saran, R.K., Voit, T., Merlini, L., Sewry, C.A., Brown, S.C. and Muntoni, F. (2003) Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of α-dystroglycan. Hum. Mol. Genet. 12, 2853–2861.

[11] Michele, D.E., Barresi, R., Kanagawa, M., Saito, F., Cohn, R.D., Satz, J.S., Dollar, J., Nishino, I., Kelley, R.I., Somer, H., Straub, V., Mathews, K.D., Moore, S.A. and Campbell, K.P. (2002) Posttranslational disruption of dystroglycan–ligand interactions in congenital muscular dystrophies. Nature 418, 417–422.

[12] Michele, D.E. and Campbell, K.P. (2003) Dystrophin–glycoprotein complex: post-translational processing and dystroglycan function. J. Biol. Chem. 278, 15457–15460.

[13] Asmundson, V.S. and Julian, L.M. (1956) Inherited muscle abnormalityin the domestic fowl. J. Hered. 47, 248–252.

[14] Lee, E.J., Yoshizawa, K., Mannen, H., Kikuchi, H., Kikuchi, T., Mizutani, M. and Tsuji, S. (2002) Localization of the muscular dystrophy AM locus using a chicken linkage map constructed with the Kobe University resource family. Anim. Genet. 33, 42–48.

[15] Herrmann, R., Straub, V., Blank, M., Kutzick, C., Franke, N., Jacob, E.N., Lenard, H.G., Kroger, S. and Voit, T. (2000) Dissociation of the dystroglycan complex in caveolin-3-deficient limb girdlemuscular dystrophy. Hum. Mol. Genet. 9, 2335–2340.

[16] Yamada, H., Chiba, A., Endo, T., Kobata, A., Anderson, L.V., Hori, H., Fukuta-Ohi, H., Kanazawa, I., Campbell, K.P., Shimizu, T. and Matsumura, K. (1996) Characterization of dystroglycan–laminin interaction in peripheral nerve. J. Neurochem. 66, 1518–1524.

[17] Jourdian, G.W., Dean, L. and Roseman, S. (1971) The sialic acids. XI. A periodate–resorcinol method for the quantitative estimation of free sialic acids and their glycosides. J. Biol. Chem. 246, 430–435.

[18] Ervasti, J.M., Burwell, A.L. and Geissler, A.L. (1997) Tissue-specific heterogeneity in α-dystroglycan sialoglycosylation. Skeletal muscle α-dystroglycan is a latent receptor for *Vicia villosa* agglutinin B4 masked by sialic acid modification. J. Biol. Chem. 272, 22315–22321.

[19] Brancaccio, A., Schulthess, T., Gesemann, M. and Engel, J. (1995) Electron microscopic evidence for a mucin-like region in chick muscle α-dystroglycan. FEBS Lett. 368, 139–142.

[20] Saito, F., Tomimitsu, H., Arai, K., Nakai, S., Kanda, T., Shimizu, T., Mizusawa, H. and Matsumura, K. (2004) A Japanese patient with distal myopathy with rimmed vacuoles: Missense mutations in the epimerase domain of the UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE) gene accompanied by hyposialylation of skeletal muscle glycoproteins. Neuromuscul. Disord. 14, 158–161.

[21] Chiba, A., Matsumura, K., Yamada, H., Inazu, T., Shimizu, T., Kusunoki, S., Kanazawa, I., Kobata, A. and Endo, T. (1997) Structures of sialylated *O*-linked oligosaccharides of bovine peripheral nerve α-dystroglycan. J. Biol. Chem. 272, 2156–2162.

[22] Sasaki, T., Yamada, H., Matsumura, K., Shimizu, T., Kobata, A. and Endo, T. (1998) Detection of *O*-mannosyl glycans in rabbit skeletal muscle α-dystroglycan. Biochim. Biophys. Acta 1425, 599–606.

[23] Pavoni, E., Sciandra, F., Barca, S., Giardina, B., Petrucci, T.C. and Brancaccio, A. (2005) Immunodetection of partially glycosylated isoforms of α-dystroglycan by a new monoclonal antibody against its β-dystroglycan-binding epitope. FEBS Lett. 579, 493–499.

[24] Leschziner, A., Moukhles, H., Lindenbaum, M., Gee, S.H., Butterworth, J., Campbell, K.P. and Carbonetto, S. (2000) Neural regulation of α-dystroglycan biosynthesis and glycosylation in skeletal muscle. J. Neurochem. 74, 70–80.

Acta Myologica • 2005; XXIV: p. 60-63

Fukutin and α-dystroglycanopahties

T. Toda[1], T. Chiyonobu[1], H. Xiong[1], M. Tachikawa[1], K. Kobayashi[1], H. Manya[2], S. Takeda[3], M. Taniguchi[1, 4], H. Kurahashi[4], T. Endo[2]

[1]*Division of Clinical Genetics, Department of Medical Genetics, Osaka University Graduate School of Medicine, Osaka,*
[2]*Glycobiology Research Group, Tokyo Metropolitan Institute of Gerontology, Tokyo,*
[3]*Otsuka GEN Research Institute, Otsuka Pharmaceutical Co. Ltd., Tokushima,*
[4]*Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, Aichi, Japan*

Fukuyama-type congenital muscular dystrophy (FCMD), Walker-Warburg syndrome (WWS), and muscle-eye-brain (MEB) disease are clinically similar autosomal recessive disorders characterized by congenital muscular dystrophy, lissencephaly, and eye anomalies. We identified the gene for FCMD and MEB, which encodes the fukutin protein and the protein *O*-linked mannose β1, 2-N-acetylglucosaminyltransferase (POMGnT1), respectively. Recent studies have revealed that posttranslational modification of α-dystroglycan is associated with these congenital muscular dystrophies with brain malformations. All are characterized by hypoglycosylated α-dystroglycan. Fukutin's function and the relation with other α-dystroglycanopathies are discussed.

Key words: Fukuyama congenital muscular dystrophy (FCMD), fukutin, α-dystroglycanopathy

FCMD

Fukuyama congenital muscular dystrophy (FCMD: MIM 253800) is the second most common muscular dystrophy and one of the most prevalent autosomal recessive disorders in Japan. Its clinical symptoms include congenital muscular dystrophy associated with brain malformation and eye disorders (1). FCMD is also typified by increased serum creatine kinase levels and prominent necrosis and regeneration in muscle tissue. MRI examination reveals pachygyria and transient T2-weighted high intensity.

Fukutin gene

We previously identified on chromosome 9q31 the gene responsible for FCMD, which encodes a novel 461-amino-acid protein we have named fukutin (2-4). The gene spans more than ~100 kb genomic DNA region. It is composed of 10 exons (5). Most FCMD-bearing chromosomes (87%) have been derived from a single ancestral founder, who lived 2,000-2,500 years ago (6) and whose mutation consisted of a 3kb SVA retrotransposal insertion in the 3' non-coding region of the fukutin gene (7). This insertion results in the reduction of mRNA. FCMD is the first known human disease to be caused by an ancient retrotransposal integration (4).

Point mutations have been seen to render the FCMD phenotype rather severe. Only two Turkish WWS-like FCMD patients (8, 9) have been identified with non-founder (point) mutations on both alleles, suggesting that such patients are embryonic-lethal and that fukutin is essential for normal development. This may explain why FCMD could occur almost only in the Japanese patients who have a milder retrotransposon mutation (10). Fukutin is a 461-amino-acid protein with a predicted molecular weight of 53.7 kDa (4). It is a type II membrane protein, but its precise function currently is unknown.

α-dystroglycanopathies

FCMD is one of several autosomal recessive congenital muscular dystrophies (CMDs) that show similar symptoms. Others include muscle-eye-brain disease (MEB: MIM 253280), Walker-Warburg syndrome (WWS: MIM 236670), congenital muscular dystrophy 1C (MDC1C: MIM 606612), and congenital muscular dystrophy 1D (MDC1D: MIM 608840). Common characteristics include severe muscular dystrophy, neuronal migration defects including lissencephaly type II (cobblestone complex), pachygyria, cerebellar and brainstem abnor-

Address for correspondence: Prof. Tatsushi Toda, Division of Clinical Genetics, Department of Medical Genetics, Osaka University Graduate School of Medicine, 2-2-B9 Yamadaoka, Suita, Osaka 565-0871, Japan, fax. +81-6-6879-3389, Email toda@clgene.med.osaka-u.ac.jp.

malities, and various ocular anomalies. MEB was first described in Finland, where it is most prevalent. It has since been demonstrated that MEB exists outside Finland and that the clinical spectrum of MEB is broader than was recognized previously (11). MEB presents with more severe ocular abnormalities including severe congenital myopia, congenital glaucoma, pallor of the optic discs, and retinal hypoplasia. WWS generally presents with the most severe brain involvement and is lethal either prenatally or within the first year of life.

MEB, WWS, MDC1C, and MDC1D result respectively from mutations in protein *O*-mannose β1,2-*N*-acetylglucosaminyltlansferase 1 (POMGnT1) (12), protein *O*-mannosyltransferase 1 (POMT1/2) (13, 14), fukutin-related protein (FKRP) (15), and like-glycosyltransferase (LARGE) (16). With identification of the responsible genes came the recognition that the phenotypic spectrum and the regional distribution for individual genetic defects are likely wider than previously assumed, thus blurring the boundaries between these clinically defined entities.

Abnormal glycosylation of α-dystroglycan and fukutin's function

Common to all four disorders is the hypoglycosylation of α-dystroglycan (*α-DG*). *Yet glycosyl*transferase enzymatic activity has been demonstrated only for POMGnT1 (12) and POMT1 (17). POMGnT1, a 660-amino-acid protein with a predicted molecular weight of 75 kDa, adds *N*-acetylglucosamine to *O*-mannose protein (12,18). A recent study revealed that LARGE can functionally bypass α-DG glycosylation defects in many CMDs, including FCMD, MEB and WWS. LARGE may also affect an alternative glycosylation pathway for α-DG (19).

No glycosyltransferase activity has been reported for fukutin; however, like MEB, WWS and Large^{myd} mice, FCMD shows a ~60 kDa reduction in the relative molecular weight of in α-DG (20). Since POMT1 localizes to the endoplasmic reticulum (ER), whereas fukutin is reported to localize in the Golgi apparatus (21), we suspect a relationship between fukutin and another Golgi-resident glycosyltransferase, such as POMGnT1, that would ultimately result in the transfer of sugars to α-DG. We demonstrated an interaction between fukutin and POMGnT1. We also showed that the transmembrane domain of fukutin participates in the interaction with POMGnT1 and modulates its enzymatic activity (unpublished data). We suggest that fukutin forms a complex with POMGnT1 and modulates its enzymatic activity. These findings might reveal new pathways for understanding the function of these proteins and the pathomechanism of CMDs.

Mouse model and fukutin's function

There are no reported naturally occurring mice carrying mutations in the *fukutin* gene. Through targeted disruption of the orthologous mouse *fukutin* gene (22), we showed that the fukutin protein is essential, as homozygous null embryos die by embryonic day 9.5 (E9.5) of gestation. *Fukutin*-null embryos show phenotypic diversity, features of which include growth retardation, folding of the egg cylinder, leakage of maternal red blood cells into the yolk sac cavity, and an increased number of apoptotic cells in the ectoderm. Loss of immunoreactivity against sugar moieties in α-dystroglycan suggests a reduced laminin-binding capacity. Ultrastructural analysis shows thin and breached basement membranes (BMs). BM fragility may underlie all of these abnormal phenotypes, and maintenance of BM function may require fukutin-mediated glycosylation of α-dystroglycan early in embryonic development (23).

Chimeric mice generated using embryonic stem cells targeted for both *fukutin* alleles develop severe muscular dystrophy, with the selective deficiency of α-dystroglycan and its laminin-binding

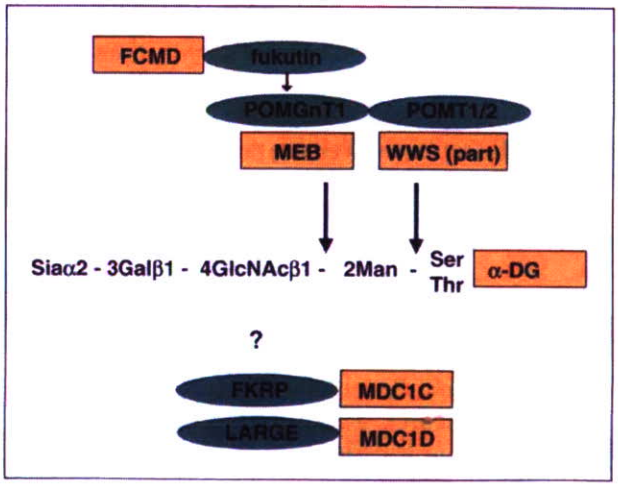

Figure 1. *O*-mannosyl glycan found in α-dystroglycan, its synthesis, and α-dystroglycanopathy-responsible proteins. Only POMGnT1 for MEB and POMT1/2 for WWS have demonstrated enzymatic activities. Fukutin may form a complex with POMGnT1 and modulate its enzymatic activity.

activity. In addition, these mice showed laminar disorganization of the cortical structures in the brain with impaired laminin assembly, focal interhemispheric fusion, and hippocampal and cerebellar dysgenesis. Further, chimeric mice showed anomaly of the lens, loss of laminar structure in the retina, and retinal detachment. Injection of fukutin by electroporation resulted in restoration of α-dystroglycan. These results indicate that fukutin is necessary for the maintenance of muscle integrity, cortical histogenesis, and normal ocular development and suggest the functional linkage between fukutin and α-dystroglycan (24).

The characteristic brain malformation in FCMD is polymicrogyria, which is caused by neurons migrating out of the developing brain through breaches in the pial basement membrane (25). It remains undetermined, however, whether the defect causing this abnormal migration resides in the migrating neurons or in the basement membrane. To elucidate the pathogenesis of brain abnormalities in FCMD, we histologically and immunohistochemically analyzed the developing forebrain in fukutin-deficient chimeric mice. In chimeric embryos, ectopias became apparent as early as E14, and laminar organization became progressively distorted. The pial basement membrane in chimeras showed defects at E14, coinciding with the earliest time point at which ectopias were detected. Immunohistochemical analysis of glycosylated α-dystroglycan showed progressive defects coincidental with the disruption of the pial basement membrane. Neuronal migration was not affected in chimeras, as determined by detection of bromodeoxyuridine-labeled neurons. Extension of radial glial fibers was intact in chimeras. Taken together, disruption of the pial basement membrane, caused by the loss of interaction between hypoglycosylated α-dystroglycan and its ligands, plays a key role in the pathogenesis of cortical dysplasia in FCMD (26).

Acknowledgements

This work was supported by a Health Science Research Grant, Research on Psychiatric and Neurological Diseases and Mental Health and by a Research Grant for Nervous and Mental Disorders (14B-4 and 17A-10), both from the ministry of Health, Labor, and Welfare of Japan; and by the 21st Century COE program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

1. Fukuyama Y, Osawa M, Suzuki H. Congenital muscular dystrophy of the Fukuyama type - clinical, genetic and pathological considerations. Brain Dev 1981;3:1-29.
2. Toda T, Segawa M, Nomura Y, et al. Localization of a gene for Fukuyama type congenital muscular dystrophy to chromosome 9q31-33. Nat Genet 1993;5:283-6.
3. Toda T, Miyake M, Kobayashi K, et al. Linkage-disequilibrium mapping narrows the Fukuyama-type congenital muscular dystrophy (FCMD) candidate region to <100 kb. Am J Hum Genet 1996;59:1313-20.
4. Kobayashi K, Nakahori Y, Miyake M, et al. An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. Nature 1998;394:388-92.
5. Kobayashi K, Sasaki J, Kondo-Iida E, et al. Structural organization, complete genomic sequences, and mutational analyses of the Fukuyama-type congenital muscular dystrophy gene, fukutin. FEBS Lett 2001;489:192-6.
6. Colombo R, Bignamini AA, Carobene A, et al. Age and origin of the FCMD 3'-untranslated-region retrotransposal insertion mutation causing Fukuyama-type congenital muscular dystrophy in Japanese population. Hum Genet 2000;107:559-67.
7. Watanabe M, Kobayashi K, Jin F, et al. Founder SVA retrotransposal insertion in Fukuyama-type congenital muscular dystrophy and its origin in Japanese and Northeast Asian populations. Am J Med Genet 2005;138:344-8.
8. Silan F, Yoshioka M, Kobayashi K, et al. A new mutation of the *fukutin* gene in a non-Japanese patient. Ann Neurol 2003;53:392-6.
9. de Bernabe DB, van Bokhoven H, van Beusekom E, et al. A homozygous nonsense mutation in the fukutin gene causes a Walker-Warburg syndrome phenotype. J Med Genet 2003;40:845-8.
10. Kondo-Iida E, Kobayashi K, Watanabe M, et al. Novel mutations and genotype-phenotype relationships in 107 families with Fukuyama-type congenital muscular dystrophy (FCMD). Hum Mol Genet 1999;8:2303-9.
11. Taniguchi K, Kobayashi K, Saito K, et al. Worldwide distribution and broader clinical spectrum of muscle-eye-brain disease. Hum Mol Genet 2003;12:527-34.
12. Yoshida A, Kobayashi K, Manya H, et al. Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. Dev Cell 2001;1:717-24.
13. de Bernabe DB, Currier S, Steinbrecher A, et al. Mutations in the O-mannosyltransferase gene POMT1 give rise to the severe neuronal migration disorder Walker-Warburg syndrome. Am J Hum Genet 2002;71:1033-43.
14. van Reeuwijk J, Janssen M, van den Elzen C, et al. POMT2 mutations cause α-dystroglycan hypoglycosylation and Walker Warburg syndrome. J Med Genet 2005 May 13; [Epub ahead of print]
15. Brockington M, Blake DJ, Prandini P, et al. Mutations in the fukutin-related protein gene (FKRP) cause a form of congenital muscular dystrophy with secondary laminin α2 deficiency and abnormal glycosylation of a-dystroglycan. Am J Hum Genet 2001;69:1198-1209.

16. Longman C, Brockington M, Torelli S, et al. Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of α-dystroglycan. Hum Mol Genet 2003;12:2853-61.
17. Manya H, Chiba A, Yoshida A, et al. Demonstration of mammalian protein O-mannosyltransferase activity: coexpression of POMT1 and POMT2 required for enzymatic activity. Proc Natl Acad Sci USA 2004;101:500-5.
18. Takahashi S, Sasaki T, Manya H, et al. A new β-1,2-N-acetylglucosaminyltransferase that may play a role in the biosynthesis of mammalian O-mannosyl glycans. Glycobiology 2001;11:37-45.
19. Barresi R, Michele DE, Kanagawa M, et al. LARGE can functionally bypass alpha-dystroglycan glycosylation defects in distinct congenital muscular dystrophies. Nat Med 2004;10:696-703.
20. Michele D, Barresi R, Kanagawa M, et al. Post-translational disruption of dytroglycan-ligand interactions in congenital muscular dystrophies. Nature 2002;418:417-22.
21. Matsumoto H, Noguchi S, Sugie K, et al. Subcellular localization of fukutin and fukutin-related protein in muscle cells. J Biochem 2004;135:709-12.
22. Horie M, Kobayashi K, Takeda S, et al. Isolation and characterization of the mouse ortholog of the Fukuyama-type congenital muscular dystrophy gene. Genomics 2002;80:482-6.
23. Kurahashi H, Taniguchi M, Meno C, et al. Basement membrane fragility underlies embryonic lethality in fukutin-null mice. Neurobiol Dis 2005;19:208-17.
24. Takeda S, Kondo M, Sasaki J, et al. Fukutin is required for maintenance of muscle integrity, cortical histiogenesis, and normal eye development. Hum Mol Genet 2003;12:1449-59.
25. Nakano I, Funahashi M, Takada K, et al. Are breaches in the glia limitans the primary cause of the micropolygyria in Fukuyama-type congenital muscular dystrophy (FCMD)? - Pathological study of the cerebral cortex of an FCMD fetus. Acta Neuropathol 1996;91:313-21.
26. Chiyonobu T, Sasaki J, Nagai Y, et al. Effects of fukutin deficiency in the developing mouse brain. Neuromuscul Disord 2005;15:416-26.

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 350 (2006) 935–941
BBRC
www.elsevier.com/locate/ybbrc

Molecular interaction between fukutin and POMGnT1 in the glycosylation pathway of α-dystroglycan

Hui Xiong [a,1], Kazuhiro Kobayashi [a,1], Masaji Tachikawa [a,1], Hiroshi Manya [b,1], Satoshi Takeda [c], Tomohiro Chiyonobu [a], Nobuhiro Fujikake [a], Fan Wang [a], Akemi Nishimoto [a], Glenn E. Morris [d], Yoshitaka Nagai [a], Motoi Kanagawa [a], Tamao Endo [b], Tatsushi Toda [a,*]

[a] *Division of Clinical Genetics, Department of Medical Genetics, Osaka University Graduate School of Medicine, 2-2-B9 Yamadaoka, Suita, Osaka 565-0871, Japan*
[b] *Glycobiology Research Group, Tokyo Metropolitan Institute of Gerontology, Itabashi, Tokyo 173-0015, Japan*
[c] *Otsuka GEN Research Institute, Otsuka Pharmaceutical Co. Ltd., Tokushima, Tokushima 771-0192, Japan*
[d] *Centre for Inherited Neuromuscular Disease (CIND), RJAH Orthopaedic Hospital, Oswestry, Shropshire SY10 7AG, UK*

Received 21 September 2006
Available online 2 October 2006

Abstract

The recent identification of mutations in genes encoding demonstrated or putative glycosyltransferases has revealed a novel mechanism for congenital muscular dystrophy. Hypoglycosylated α-dystroglycan (α-DG) is commonly seen in Fukuyama-type congenital muscular dystrophy (FCMD), muscle–eye–brain disease (MEB), Walker–Warburg syndrome (WWS), and Large^{myd} mice. POMGnT1 and POMTs, the gene products responsible for MEB and WWS, respectively, synthesize unique *O*-mannose sugar chains on α-DG. The function of fukutin, the gene product responsible for FCMD, remains undetermined. Here we show that fukutin co-localizes with POMGnT1 in the Golgi apparatus. Direct interaction between fukutin and POMGnT1 was confirmed by co-immunoprecipitation and two-hybrid analyses. The transmembrane region of fukutin mediates its localization to the Golgi and participates in the interaction with POMGnT1. Y371C, a missense mutation found in FCMD, retains fukutin in the ER and also redirects POMGnT1 to the ER. Finally, we demonstrate reduced POMGnT1 enzymatic activity in transgenic knock-in mice carrying the retrotransposal insertion in the *fukutin* gene, the prevalent mutation in FCMD. From these findings, we propose that fukutin forms a complex with POMGnT1 and may modulate its enzymatic activity.

Keywords: Fukutin; POMGnT1; Fukuyama-type congenital muscular dystrophy; Muscle–eye–brain disease; α-Dystroglycan

Fukuyama-type congenital muscular dystrophy (FCMD: MIM 253800) is the second most common muscular dystrophy and is among the most prevalent autosomal recessive disorders in Japan. Its clinical symptoms include congenital muscular dystrophy associated with brain malformation and eye disorders [1]. FCMD also is characterized by increased serum creatine kinase levels and prominent necrosis and regeneration in muscle tissue. Magnetic resonance imaging examination reveals pachygyria and transient T2-weighted high intensity. The responsible gene for FCMD, *fukutin*, was identified at 9q31 by linkage analysis and positional cloning [2,3]. Most FCMD patients have a 3-kb retrotransposal insertion in the 3′ noncoding region of *fukutin*. Point mutations in *fukutin* also have been identified, and the severe phenotype generated by two point mutations precludes survival [4]. Fukutin is a 461-amino-acid protein with a predicted molecular weight of 53.7 kDa. It is a type II membrane protein, but its precise function is undetermined.

* Corresponding author. Fax: +81 6 6879 3389.
E-mail address: toda@clgene.med.osaka-u.ac.jp (T. Toda).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.09.129

Muscle–eye–brain disease (MEB: MIM 253280), Walker–Warburg syndrome (WWS: MIM 236670), congenital muscular dystrophy 1C (MDC1C: MIM 606612), and congenital muscular dystrophy 1D (MDC1D: MIM 608840) are autosomal recessive congenital muscular dystrophies (CMDs) that share similar symptoms to FCMD. Common characteristics include severe muscular dystrophy, neuronal migration defects including lissencephaly type II (cobblestone complex), pachygyria, cerebellar, and brainstem abnormalities, and various ocular anomalies. MEB was first described in Finland, where it is most prevalent. It has since been demonstrated that MEB exists outside of Finland and has a broader clinical spectrum than originally thought [5,6]. MEB presents with more severe ocular abnormalities, including severe congenital myopia, congenital glaucoma, pallor of the optic discs, and retinal hypoplasia. WWS generally presents with the most severe brain involvement and is lethal either prenatally or within the first year of life.

Responsible genes for these disorders have been identified: MEB is causally associated with mutations in protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1); WWS with protein *O*-mannosyltransferase 1 and 2 (POMT1 and POMT2); MDC1C with fukutin-related protein (FKRP); and MDC1D with like-glycosyltransferase (LARGE) [5,7]. Hypoglycosylation of α-dystroglycan (α-DG) is commonly seen in all four disorders. α-DG is a highly glycosylated protein that serves as the laminin-receptor unit in the dystrophin–glycoprotein complex (DGC), linking extracellular laminin to the actin cytoskeleton across the skeletal muscle plasma membrane [7]. Glycosyltransferase activity has been demonstrated only for POMGnT1 and POMT1/POMT2, however [5,8]. POMGnT1, a 660 amino-acid protein with a predicted molecular weight of 75 kDa, adds *N*-acetylglucosamine to *O*-mannose protein. The POMT1/POMT2 complex adds a mannose directly to the polypeptide backbone of α-DG [8,9]. A recent study revealed that LARGE can functionally bypass α-DG glycosylation defects in cells from FCMD, MEB, and WWS [10]. No glycosyltransferase activity has been reported for fukutin; however, as in MEB, WWS, and Large^{myd} mice, FCMD also shows a ~60 kDa reduction in the relative molecular weight of α-DG [11].

POMT1 localizes to the endoplasmic reticulum (ER) and requires the formation of a protein complex with POMT2 to show enzymatic activity [9]. Fukutin is reported to localize to the Golgi apparatus [3,12]; therefore we suspected an interaction between fukutin and Golgi-resident glycosyltransferases that ultimately results in the transfer of sugars to α-DG. Here we demonstrate an interaction between fukutin and POMGnT1 that is mediated by the transmembrane region of fukutin. Decreased POMGnT activity in fukutin-deficient tissues suggests a role for fukutin in the α-DG *O*-mannosylation pathway. These findings demonstrate a function for fukutin for the first time, contributing to a greater understanding of the α-DG modification pathway and the pathomechanism of glycosylation-defect congenital muscular disorders.

Materials and methods

Molecular constructs and mice. Expression vectors were constructed by cloning human *POMGnT1* or *LARGE* into pEF1/V5-HisA (Invitrogen, Carlisbad, CA). Human *fukutin* and its mutated versions with FLAG epitope were cloned into pcDNA3.1+ (Invitrogen), and the fukutin transmembrane region was cloned into pEGFP-N1 (Clonetech, Palo Alto, CA). The transgenic knock-in mice carrying the human 3-kb retrotransposal insertion in the 3′ noncoding region of the *fukutin* gene (fukutin knock-in mice) were generated by a site-directed DNA integration technique [13]. Briefly, lox71 and loxP sites were inserted 5′ and 3′ to exon 10 of mouse *fukutin*, and the exon 10 was excised by Cre expression in mouse embryonic stem (ES) cells. Subsequently, a targeting construct containing exon 10 of human *fukutin* with a retrotransposal insertion put between lox66 and loxP sites was transfected into the ES cells with Cre, and recombination was confirmed by Southern blotting. Mice were maintained in accordance with the animal care guidelines of Otsuka Pharmaceutical Co. Ltd. and Osaka University.

Antibodies. Antibodies used in this study were obtained elsewhere; monoclonal anti-V5 and anti-GFP (Invitrogen), polyclonal anti-V5 and anti-GFP (MBL, Nagoya, Japan), monoclonal and polyclonal anti-FLAG (Sigma, St. Louis, MO), monoclonal anti-β-DG clone 8D5 (Novocastra Laboratories, Newcastle, UK), monoclonal anti-GM130 (BD Biosciences, San Jose, CA), and monoclonal anti-KDEL antibodies (Stressgen, Victoria, Canada). Monoclonal anti-fukutin antibodies were produced as described previously [14]. Affinity-purified sheep anti-α-DG core protein antibody was kindly provided by Dr. Kevin P. Campbell [10].

Cell culture and transfection. Cos-7 and C2C12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfection of cell lines was carried out using Trans IT-LT1 transfection reagent (Mirus, Madison, WI). Transfected cells were grown at 37 °C and harvested 48 h after transfection.

Immunofluorescence analysis. Transfected cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. After blocking with 5% BSA, the cells were incubated with antibodies. Next, the cells were washed and incubated with fluorescent secondary antibodies. After the final wash, cells were observed using fluorescence microscopy.

Binding assay. Transfected cells were lysed with the lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 1.0% CHAPS) containing protease inhibitor mixture (Nacalai, Kyoto, Japan). Lysates were precleared with Protein G Sepharose (Amersham Bioscience, Piscataway, NJ), and the supernatants were incubated with antibodies and then mixed with Protein G Sepharose preblocked with 2% BSA. Immunocomplexes were pelleted and washed five times with lysis buffer. Cell lysates and immunocomplexes were analyzed by Western blotting.

Assay for POMGnT1 enzymatic activity. The enzymatic activity assay was performed as described previously [15].

Results

Golgi localization of fukutin and POMGnT1

The intracellular localization of POMGnT1, LARGE, and fukutin was examined in Cos-7, NIH3T3, and C2C12 cells. Cells were transfected with expression constructs encoding POMGnT1–V5, LARGE–V5, or fukutin–FLAG, then immunostained with a Golgi marker (GM130) and anti-V5 or anti-FLAG antibodies. Fukutin–FLAG co-localized with GM130, indicating localization of fukutin to the Golgi apparatus (Fig. 1A). Similar results were observed in Cos-7 and NIH3T3 cells (data not shown). Monoclonal antibodies specific for fukutin (3C7, 7A2, and 1B5) detected overexpressed fukutin with the same localization pattern in transfected cells (Fig. 1B,

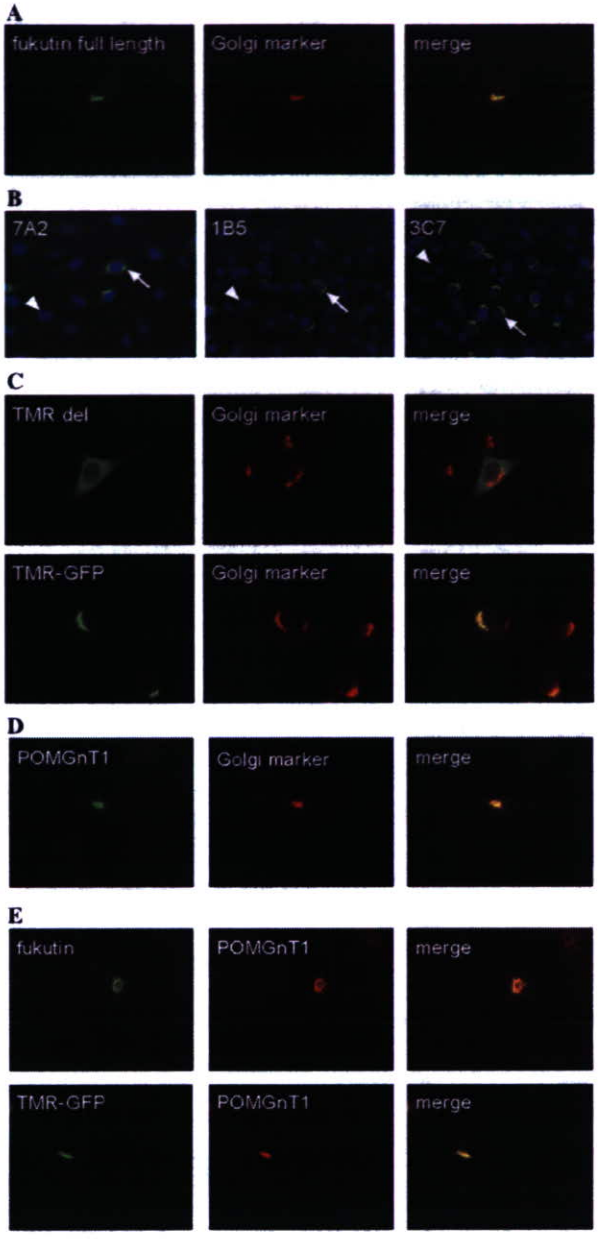

Fig. 1. Co-localization of fukutin and POMGnT1 in the Golgi apparatus. (A) Double labeling of fukutin–FLAG (left panel, green) and the Golgi marker GM130 (middle panel, red) in transfected C2C12 cells. Co-localization can be seen in the merged image (right panel). (B) Labeling of fukutin–FLAG in transfected C2C12 cells with anti-fukutin antibodies (green). The antibodies (7A2, 1B5, and 3C7) were able to detect only overexpressed fukutin in the Golgi (arrows) but could not detect endogenous fukutin (arrowheads). (C) Double labeling of fukutin lacking the transmembrane region (TMR del) or the fukutin transmembrane region (TMR–GFP) (left panels, green) and GM130 (middle panels, red) in transfected C2C12 cells. (D) Double labeling of POMGnT1–V5 (left panel, green) and GM130 (middle panel, red) in transfected Cos-7 cells. (E) Double labeling of fukutin–FLAG or TMR–GFP (left panels, green) and POMGnT1–V5 (middle panels, red) in transfected Cos-7 cells.

arrows) but failed to detect endogenous fukutin (Fig. 1B, arrowheads), suggesting that endogenous fukutin levels are below the range detectable by antibodies. Deletion of the fukutin transmembrane region (TMR del) shifted the localization of fukutin from the Golgi to the cytoplasm (Fig. 1C), whereas the fukutin transmembrane region fused to GFP (TMR–GFP) remained in the Golgi (Fig. 1C). These observations indicate that the transmembrane region targets fukutin to the Golgi apparatus. Double staining of Cos-7 cells transfected with POMGnT1–V5 shows co-localization of POMGnT1–V5 with GM130 (Fig. 1D). We found that LARGE–V5 also localized to the Golgi apparatus (data not shown; also seen in Fig. 3B). Co-transfection and double staining of fukutin–FLAG and POMGnT1–V5 demonstrated co-localization of POMGnT1–V5 with fukutin–FLAG (Fig. 1E). In addition, TMR–GFP also co-localizes with POMGnT1–V5 (Fig. 1E). These data suggest that both proteins are Golgi residents and localize together.

Interaction of fukutin with POMGnT1 through the transmembrane region of fukutin

We performed immunoprecipitation experiments to further investigate potential associations between fukutin and POMGnT1 or LARGE. Since endogenous expression of these proteins is undetectable, we co-transfected fukutin–FLAG with either POMGnT1–V5 or LARGE–V5 into Cos-7 cells and immunoprecipitated fukutin–FLAG from the cell lysates (Fig. 2A and B). As shown in Fig. 2A, POMGnT1–V5 co-precipitated with fukutin–FLAG by anti-FLAG antibody (upper panel, lane 2). In addition, anti-V5 antibody immunoprecipitated both POMGnT1–V5 (upper panel, lane 4) and fukutin–FLAG (lower panel, lane 2) (Fig. 2A). These results indicate an interaction between fukutin and POMGnT1. In contrast, immunoprecipitation did not reveal significant interaction between fukutin–FLAG and LARGE–V5 (Fig. 2B). *In vitro* translated fukutin–FLAG and POMGnT1–V5 also co-precipitated from a reticulocyte cell-free system, suggesting that the interaction is direct (data not shown). We further verified the interaction using bacteria two-hybrid system (data not shown). Together, these data support a direct interaction between fukutin and POMGnT1.

Fig. 1B shows that the transmembrane region of fukutin possesses a Golgi-localization signal. To identify the POMGnT1 binding region in fukutin, we performed immunoprecipitation experiments using Cos-7 cells co-transfected with POMGnT1–V5 and a series of epitope-tagged fukutin truncations containing the transmembrane region [fukutin$_{6-27}$–GFP (TMR); fukutin$_{1-143}$–FLAG (F2); fukutin$_{1-235}$–FLAG (F3); fukutin$_{1-328}$–FLAG (F4); and fukutin$_{1-422}$–FLAG (F5)] (Fig. 2C). Using immunofluorescence, we confirmed the localization of all truncation proteins to the Golgi apparatus (data not shown). POMGnT1–V5 co-immunoprecipitated with the entire series of proteins (Fig. 2D). These experiments also showed that TMR interacts with POMGnT1–V5 (Fig. 2E), indicating that the fukutin transmembrane region is sufficient to bind to POMGnT1.

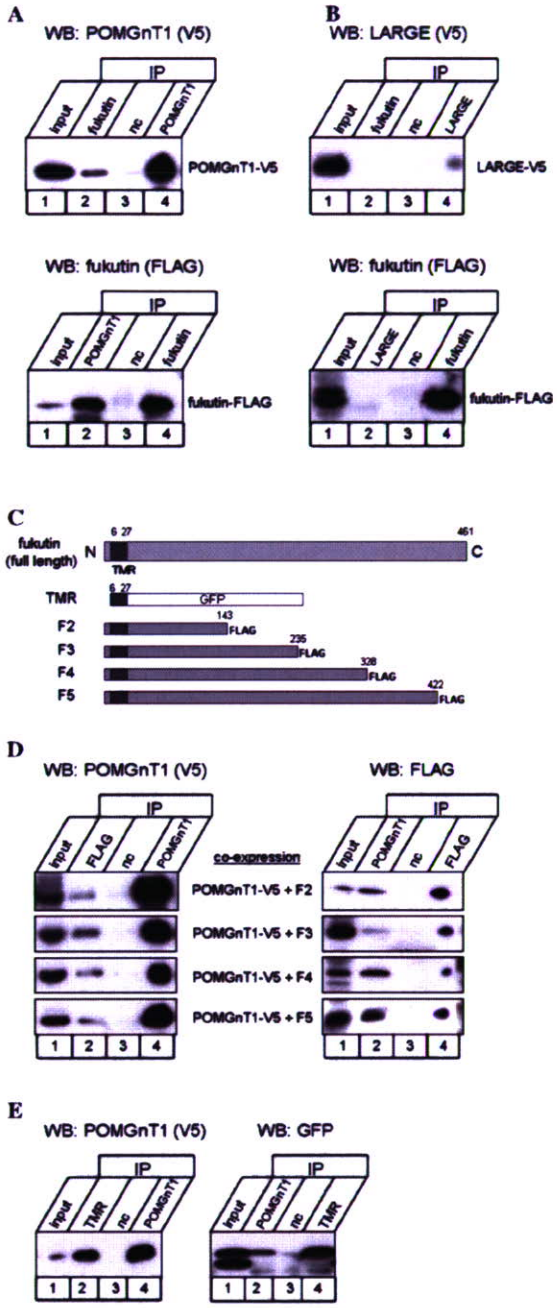

Fig. 2. Interaction of fukutin with POMGnT1 through the transmembrane region. (A) Immunoprecipitation from Cos-7 cells co-expressing fukutin–FLAG and POMGnT1–V5 using anti-FLAG (fukutin) or anti-V5 (POMGnT1) antibodies (IP) and Western blotting with anti-V5 or anti-FLAG antibodies (WB). Nc, non-immune mouse IgG as a negative control; Input, total lysate before immunoprecipitation. (B) Immunoprecipitation from Cos-7 cells co-expressing fukutin–FLAG and LARGE–V5 using anti-FLAG (fukutin) or anti-V5 (LARGE) antibodies (IP). (C) Schematic representation of full-length and truncated fukutin constructs (TMR, F2, F3, F4, and F5). TMR, transmembrane region. (D) Immunoprecipitation from Cos-7 cells co-expressing FLAG-tagged fukutin deletion mutants and POMGnT1–V5 using anti-FLAG (fukutin) or anti-V5 (POMGnT1) antibodies (IP) and Western blotting with anti-V5 or anti-FLAG antibodies (WB). (E) Immunoprecipitation from Cos-7 cells co-expressing fukutin TMR and POMGnT1–V5 using anti-GFP (TMR) or anti-V5 (POMGnT1) antibodies (IP) and Western blotting with anti-V5 or anti-GFP antibodies (WB).

A mutation in fukutin changes the localization of fukutin and POMGnT1

Although the prevalent mutation in FCMD patients is a 3-kb retrotransposal insertion, several missense mutations have been identified [4,16]. We examined the effects of known FCMD point mutations found in FCMD on the cellular localization of fukutin. One of these, Y371C fukutin–FLAG, localized to the ER instead of the Golgi (Fig. 3A). When co-expressed with Y371C, POMGnT1–V5 localization also shifted to the ER (Fig. 3B). The Golgi-specific localization of LARGE–V5 remained unchanged in cells co-expressing LARGE–V5 and Y371C (Fig. 3B). These data suggest that fukutin not only interacts with POMGnT1 but also influences its subcellular localization.

Fukutin-deficiency affects POMGnT1 enzymatic activity

We examined whether fukutin had enzymatic activity related to *O*-mannosylation of α-DG by measuring several sugar nucleotide transfer activities in microsome fractions prepared from cells overexpressing fukutin. These experiments detected no significant glycosyltransferase activity for fukutin (data not shown).

However, we have shown here that fukutin co-localizes and interacts with POMGnT1. Moreover, a similar reduction in the relative molecular weight of α-DG, about 60 kDa, occurs in both FCMD and MEB [11]. To investigate possible effects of fukutin deficiency on POMGnT activity, we measured the POMGnT activity levels (GlcNAc transfer to a mannosyl peptide) in brain microsome fractions prepared from wild type and fukutin knock-in mice that carry the retrotransposal insertion in *fukutin*. Western blotting analysis of wheat-germ agglutinin (WGA)-enriched fractions from wild type brain showed the α-DG core protein as a broad band with a molecular weight of 100–120 kDa, whereas fukutin knock-in mice had hypoglycosylated α-DG (~70 kDa) (Fig. 4A). POMGnT1 enzymatic activity in fukutin knock-in mice was reduced by approximately 30% compared to wild type (Fig. 4B). Real-time PCR analysis confirmed that POMGnT1 mRNA levels were not decreased in the presence of the retrotransposal insertion (data not shown). These data suggest that defects in fukutin may disrupt the *O*-mannosylation pathway of α-DG by affecting POMGnT1 activity.

Discussion

Recent studies have revealed that α-DG glycosylation is required for maintenance of muscle integrity, organization of neuromuscular junction, and neural cell migration in the central nervous system [7,17]. Disruption of DG-matrix linkage due to abnormal glycosylation of α-DG is thought to be the primary cause for several forms of CMDs. The *O*-mannose-linked glycan Siaα2,3-Galβ1,4-GlcNAcβ1,

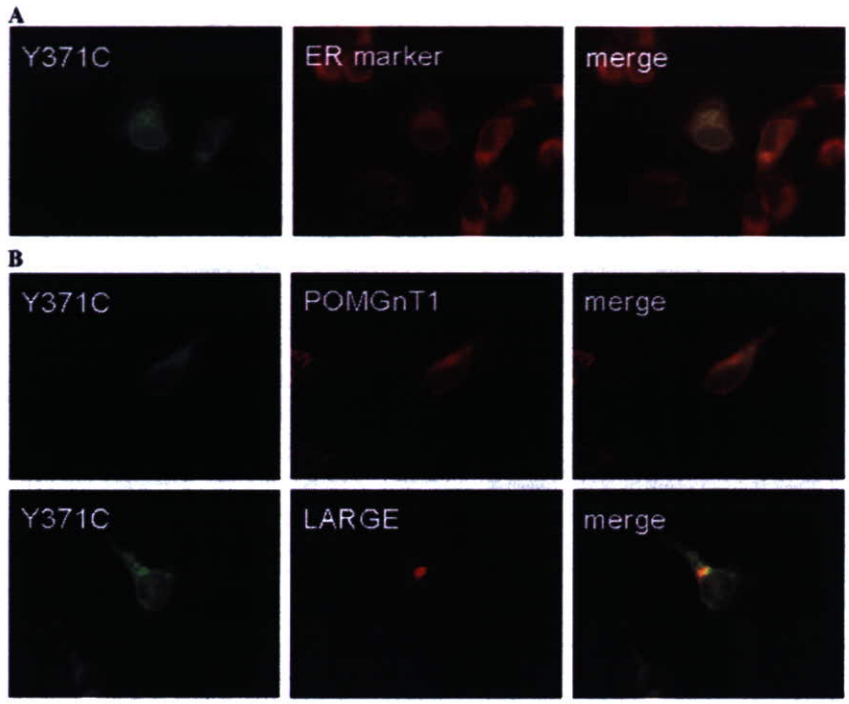

Fig. 3. Fukutin affects POMGnT1 localization. (A) Double labeling of mutant fukutin–FLAG (Y371C) (left panel, green) and the ER marker KDEL (middle panel, red) in transfected Cos-7 cells. Co-localization can be seen in the merged images (right panel). (B) Double labeling of Y371C fukutin–FLAG (left panels, green) and POMGnT1–V5 or LARGE–V5 (middle panels, red) in transfected Cos-7 cell cultures.

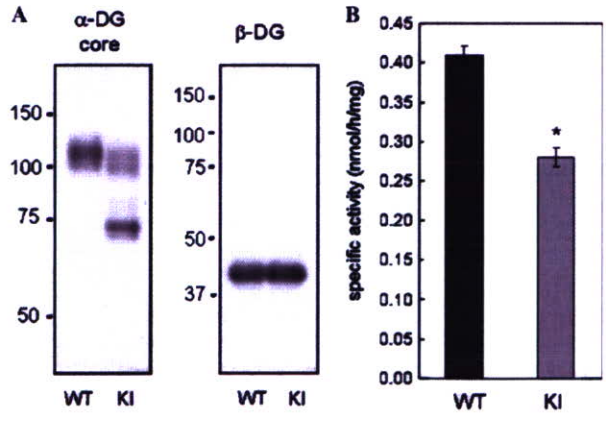

Fig. 4. Fukutin affects POMGnT1 enzymatic activity. (A) Western blotting analysis of WGA-enriched brain DG from wild type (WT) or fukutin knock-in (KI) mice with antibodies to α-DG core peptide (left) and β-DG (right). Staining of β-DG shows that equal amounts of DG were loaded on the gel. (B) POMGnT activities in brain microsome preparations from wild type (WT) and fukutin knock-in (KI) mice ($n = 3$, $p < 0.0001$).

2-Manα1-*O*-Ser/Thr has been identified in both brain and muscle tissue [18,19]. POMT1/POMT2 and POMGnT1 are glycosyltransferases known to be involved in the synthesis of this glycan [5,8]. It has been reported that similar decreases in the molecular weight of α-DG and loss of α-DG–laminin-binding activity occur in FCMD, MEB, WWS, and Large^{myd} mice [10,11]. Since mutations in POMTs and POMGnT1 result in abnormal glycosylation of α-DG in WWS and MEB, the molecular mass shift of α-DG can be attributed to abnormal *O*-mannosylation of α-DG [7]. Fukutin is predicted to belong to a family of enzymes involved in modifying cell surface molecules such as glycoproteins and glycolipids [7]. It is reasonable to hypothesize that fukutin possesses enzymatic activity that contributes to the synthesis of the *O*-mannosyl glycans in α-DG; however, our experiments detected no such activity. Alternatively, we suspected that fukutin might interact and function with other glycosyltransferases, participating indirectly in *O*-mannosylation of α-DG. Consistent with this hypothesis, we have demonstrated a direct interaction between fukutin and POMGnT1 that is mediated through the transmembrane region of fukutin.

This study demonstrates the Golgi-localization of POMGnT1 for the first time. Co-localization studies further support the fukutin–POMGnT1 interaction. Interestingly, our data indicate that a missense mutation in fukutin changes the subcellular localization of POMGnT1. The ~30% decrease in POMGnT1 enzymatic activity in the fukutin-defective (knock-in) mouse brain suggests that fukutin is associated with POMGnT1 activity *in vivo*. Barresi et al. found that overexpression of POMGnT1 cannot rescue the α-DG glycosylation defect in FCMD cells [10]. Thus, POMGnT1 might require fukutin to correct defects in α-DG glycosylation.

It has been reported that a unique molecular chaperone, Cosmc, is required for the activity of human core 1 β3-galactosyltransferase (C1β3Gal-T) that generates the core 1

O-glycan Galβ1-3GalNAcα1-Ser/Thr. Altered *O*-glycosylation caused by deficiency of C1β3Gal-T activity is associated with acquired human diseases such as IgA nephropathy and Tn syndrome, indicating roles for molecular chaperones in the pathogenesis of glycosylation-related human diseases [20]. Thus, proper *O*-mannosylation of α-DG may require chaperones as well as glycosyltransferases. Our results also suggest that fukutin may play a molecular chaperone-like role in *O*-glycosylation.

Only about 15 point mutations have been identified in FCMD patients to date. Recently, two non-Japanese individuals homozygous for truncating mutations in *fukutin* were reported to show more severe, WWS-like phenotypes [21,22]. It has been postulated that most individuals carrying two fukutin point mutations will be embryonic lethal [4]. Homozygous-null mouse embryos carrying targeted disruptions of fukutin die by E9.5 and show basement membrane fragility [23]. However, POMGnT1 mutations cause milder phenotypes even though the mutations deprive POMGnT1 of its enzymatic activity [5,6,24]. The fact suggests additional interaction of fukutin with other protein(s). Genetic studies have implicated POMTs, fukutin, and FKRP in WWS, but these data still only account for a minority of WWS cases [7]. It might be possible that unidentified enzymes and/or chaperones underlie α-DG glycosylation and the pathogenesis of α-dystroglycanopathies and that these proteins comprise a large multi-enzyme complex exerting their enzymatic functions together. Detailed analyses will be required for full understanding of the α-DG glycosylation pathway, which may lead to novel therapeutic strategies for α-dystroglycanopathies.

Acknowledgments

We thank Dr. Kevin P. Campbell for providing anti-core α-DG antibody and Dr. Jennifer Logan for comments on the manuscript. This work was supported by a Health Science Research Grant, Research on Psychiatric and Neurological Diseases and Mental Health, and by a Research Grant for Nervous and Mental Disorders (17A-10), both from the ministry of Health, Labor, and Welfare of Japan; and by the 21st Century COE program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This work was also supported by a grant from the Muscular Dystrophy Campaign (RA565) in UK.

References

[1] Y. Fukuyama, M. Osawa, H. Suzuki, Congenital progressive muscular dystrophy of the Fukuyama type—clinical, genetic and pathological considerations, Brain Dev. 3 (1981) 1–29.

[2] T. Toda, M. Segawa, Y. Nomura, I. Nonaka, K. Masuda, T. Ishihara, M. Sakai, I. Tomita, Y. Origuchi, K. Ohno, N. Misugi, Y. Sasaki, K. Takada, M. Kawai, K. Otani, T. Murakami, K. Saito, Y. Fukuyama, T. Shimizu, I. Kanazawa, Y. Nakamura, Localization of a gene for Fukuyama type congenital muscular dystrophy to chromosome 9q31-33, Nat. Genet. 5 (1993) 283–286.

[3] K. Kobayashi, Y. Nakahori, M. Miyake, K. Matsumura, E. Kondo-Iida, Y. Nomura, M. Segawa, M. Yoshioka, K. Saito, M. Osawa, K. Hamano, Y. Sakakihara, I. Nonaka, Y. Nakagome, I. Kanazawa, Y. Nakamura, K. Tokunaga, T. Toda, An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy, Nature 394 (1998) 388–392.

[4] E. Kondo-Iida, K. Kobayashi, M. Watanabe, J. Sasaki, T. Kumagai, H. Koide, K. Saito, M. Osawa, Y. Nakamura, T. Toda, Novel mutations and genotype-phenotype relationships in 107 families with Fukuyama-type congenital muscular dystrophy (FCMD), Hum. Mol. Genet. 8 (1999) 2303–2309.

[5] A. Yoshida, K. Kobayashi, H. Manya, K. Taniguchi, H. Kano, M. Mizuno, T. Inazu, H. Mitsuhashi, S. Takahashi, M. Takeuchi, R. Herrmann, V. Straub, B. Talim, T. Voit, H. Topaloglu, T. Toda, T. Endo, Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1, Dev. Cell 1 (2001) 717–724.

[6] K. Taniguchi, K. Kobayashi, K. Saito, H. Yamanouchi, A. Ohnuma, Y.K. Hayashi, H. Manya, D.K. Jin, M. Lee, E. Parano, R. Falsaperla, P. Pavone, R. Van Coster, B. Talim, A. Steinbrecher, V. Straub, I. Nishino, H. Topaloglu, T. Voit, T. Endo, T. Toda, Worldwide distribution and broader clinical spectrum of muscle-eye-brain disease, Hum. Mol. Genet. 12 (2003) 527–534.

[7] M. Kanagawa, T. Toda, The genetic and molecular basis of muscular dystrophy: roles of cell-matrix linkage in the pathogenesis, J. Hum. Genet. 51 (2006), doi:10.1007/s10038-006-0056-7.

[8] H. Manya, A. Chiba, A. Yoshida, X. Wang, Y. Chiba, Y. Jigami, R.U. Margolis, T. Endo, Demonstration of mammalian protein *O*-mannosyltransferase activity: coexpression of POMT1 and POMT2 required for enzymatic activity, Proc. Natl. Acad. Sci. USA 101 (2004) 500–505.

[9] K. Akasaka-Manya, H. Manya, A. Nakajima, M. Kawakita, T. Endo, Physical and functional association of human protein *O*-mannosyltransferases 1 and 2, J. Biol. Chem. 281 (2006) 19339–19345.

[10] R. Barresi, D.E. Michele, M. Kanagawa, H.A. Harper, S.A. Dovico, J.S. Satz, S.A. Moore, W. Zhang, H. Schachter, J.P. Dumanski, R.D. Cohn, I. Nishino, K.P. Campbell, LARGE can functionally bypass α-dystroglycan glycosylation defects in distinct congenital muscular dystrophies, Nat. Med. 10 (2004) 696–703.

[11] D.E. Michele, R. Barresi, M. Kanagawa, F. Saito, R.D. Cohn, J.S. Satz, J. Dollar, I. Nishino, R.I. Kelley, H. Somer, V. Straub, K.D. Mathews, S.A. Moore, K.P. Campbell, Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies, Nature 418 (2002) 417–422.

[12] H. Matsumoto, S. Noguchi, K. Sugie, M. Ogawa, K. Murayama, Y.K. Hayashi, I. Nishino, Subcellular localization of fukutin and fukutin-related protein in muscle cells, J. Biochem. 135 (2004) 709–712.

[13] K. Araki, M. Araki, K. Yamamura, Targeted integration of DNA using mutant lox sites in embryonic stem cells, Nucleic Acids Res. 25 (1997) 868–872.

[14] N.T. Man, G.E. Morris, Production of panels of monoclonal antibodies by the hybridoma method, in: G.E. Morris (Ed.), Methods in Molecular Biology, Epitope Mapping Protocols, vol. 66, Humana Press, Totowa, 1996, pp. 377–389.

[15] S. Takahashi, T. Sasaki, H. Manya, Y. Chiba, A. Yoshida, M. Mizuno, H. Ishida, F. Ito, T. Inazu, N. Kotani, S. Takasaki, M. Takeuchi, T. Endo, A new β-1,2-*N*-acetylglucosaminyltransferase that may play a role in the biosynthesis of mammalian *O*-mannosyl glycans, Glycobiology 11 (2001) 37–45.

[16] K. Kobayashi, J. Sasaki, E. Kondo-Iida, Y. Fukuda, M. Kinoshita, Y. Sunada, Y. Nakamura, T. Toda, Structural organization, complete genomic sequences and mutational analyses of the Fukuyama-type congenital muscular dystrophy gene, *fukutin*, FEBS Lett. 489 (2001) 192–196.

[17] M. Taniguchi, H. Kurahashi, S. Noguchi, T. Fukudome, T. Okinaga, T. Tsukahara, Y. Tajima, K. Ozono, I. Nishino, I. Nonaka, T. Toda,

Aberrant neuromuscular junctions and delayed terminal muscle fiber maturation in α-dystroglycanopathies, Hum. Mol. Genet. 15 (2006) 1279–1289.

[18] A. Chiba, K. Matsumura, H. Yamada, T. Inazu, T. Shimizu, S. Kusunoki, I. Kanazawa, A. Kobata, T. Endo, Structures of sialylated *O*-linked oligosaccharides of bovine peripheral nerve α-dystroglycan. The role of a novel *O*-mannosyl-type oligosaccharide in the binding of α-dystroglycan with laminin, J. Biol. Chem. 272 (1997) 2156–2162.

[19] T. Sasaki, H. Yamada, K. Matsumura, T. Shimizu, A. Kobata, T. Endo, Detection of *O*-mannosyl glycans in rabbit skeletal muscle α-dystroglycan, Biochim. Biophys. Acta 1425 (1998) 599–606.

[20] T. Ju, R.D. Cummings, Protein glycosylation: chaperone mutation in Tn syndrome, Nature 437 (2005) 1252.

[21] F. Silan, M. Yoshioka, K. Kobayashi, E. Simsek, M. Tunc, M. Alper, M. Cam, A. Guven, Y. Fukuda, M. Kinoshita, K. Kocabay, T. Toda, A new mutation of the *fukutin* gene in a non-Japanese patient, Ann. Neurol. 53 (2003) 392–396.

[22] D. Beltrán-Valero de Bernabé, H. van Bokhoven, E. van Beusekom, W. van den Akker, S. Kant, W.B. Dobyns, B. Cormand, S. Currier, B. Hamel, B. Talim, H. Topaloglu, H.G. Brunner, A homozygous nonsense mutation in the *fukutin* gene causes a Walker-Warburg syndrome phenotype, J. Med. Genet. 40 (2003) 845–848.

[23] H. Kurahashi, M. Taniguchi, C. Meno, Y. Taniguchi, S. Takeda, M. Horie, H. Otani, T. Toda, Basement membrane fragility underlies embryonic lethality in *fukutin*-null mice, Neurobiol. Dis. 19 (2005) 208–217.

[24] H. Manya, K. Sakai, K. Kobayashi, K. Taniguchi, M. Kawakita, T. Toda, T. Endo, Loss-of-function of an *N*-acetylglucosaminyltransferase, POMGnT1, in muscle-eye-brain disease, Biochem. Biophys. Res. Commun. 306 (2003) 93–97.