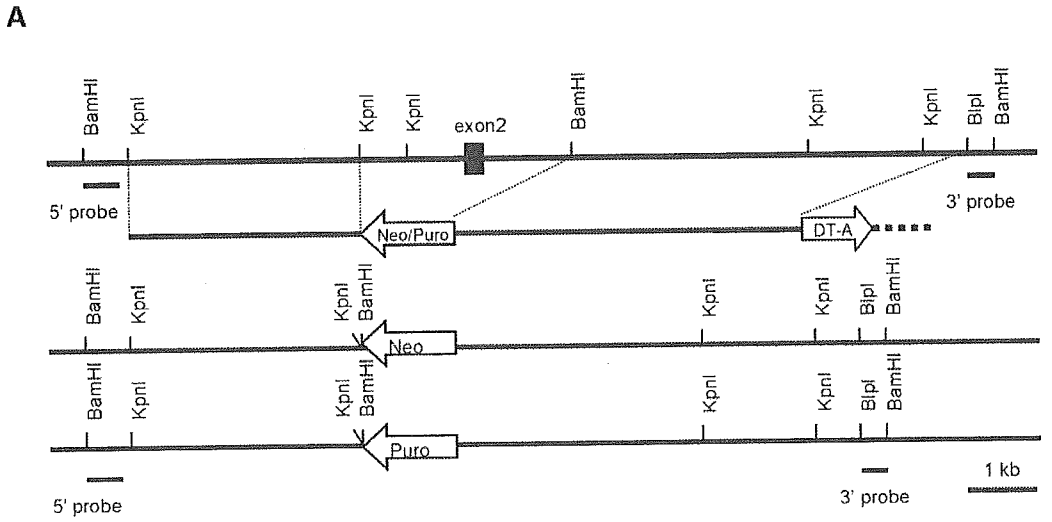
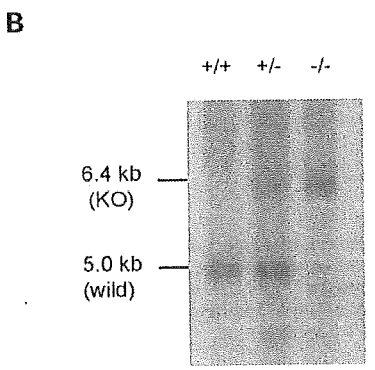
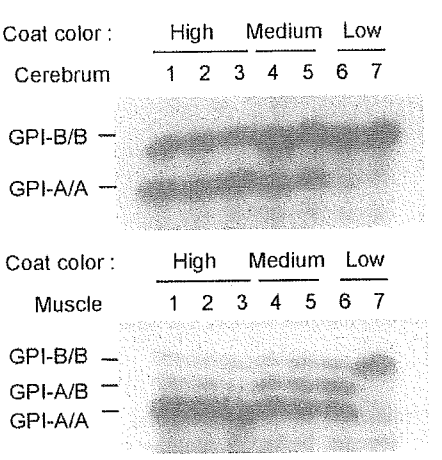
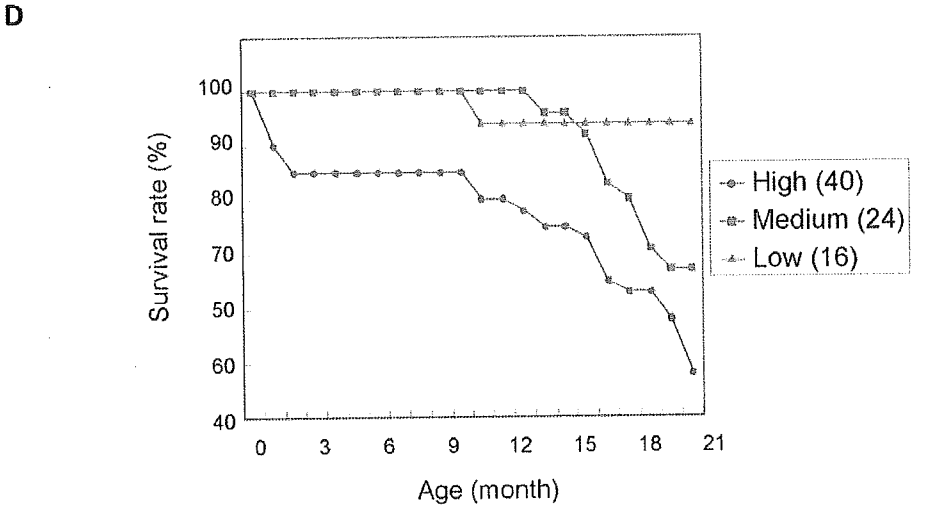

A
BamHI
KpnI
exon2
5' probe
Neo/Puro
DT-A
3' probe
Neo
Puro
1 kb


B
+/+ +/- -/-
6.4 kb (KO)
5.0 kb (wild)


C
Coat color : High Medium Low
Cerebrum
1 2 3 4 5 6 7
GPI-B/B
GPI-A/A
Muscle
GPI-A/B


D
Survival rate (%)
100
90
80
70
60
50
40
0 3 6 9 12 15 18 21
Age (month)
High (40)
Medium (24)
Low (16)

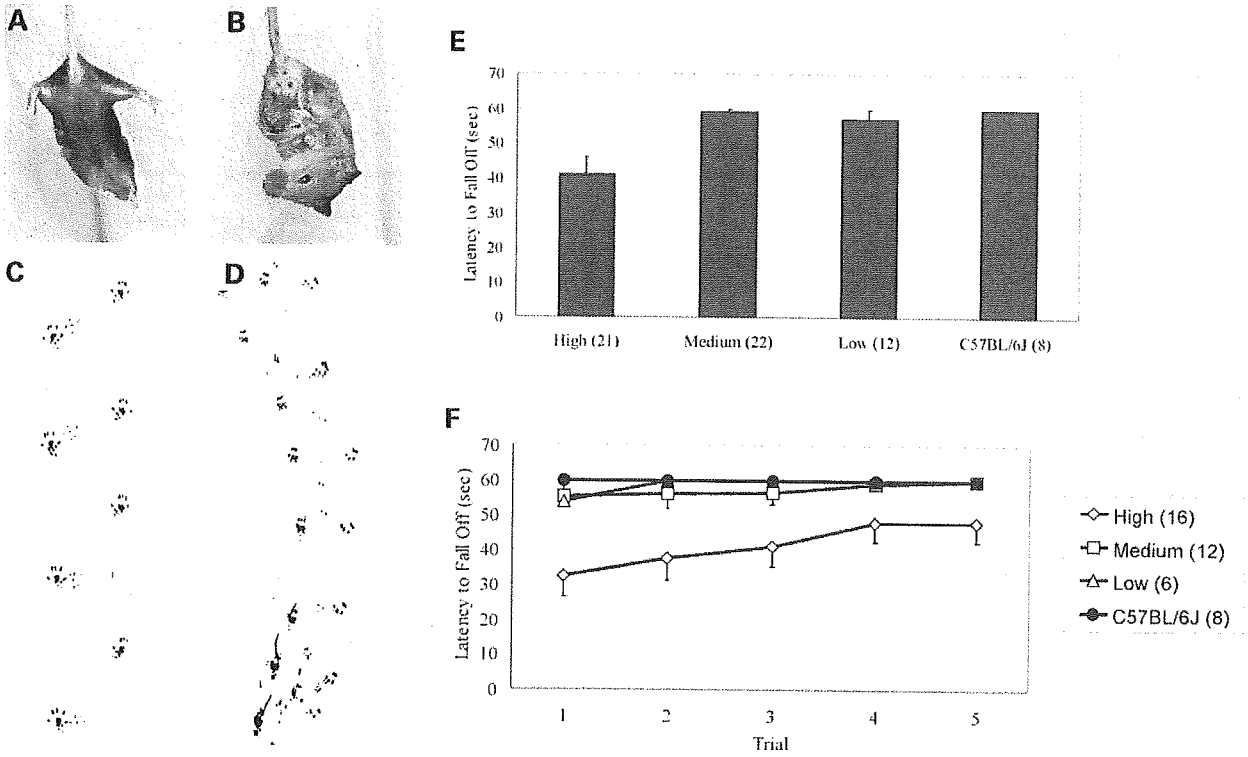


Figure 2. Behavioral abnormalities of fukutin-deficient chimeric mice. (**A** and **B**) When lifted by the tail, wild-type (C57BL/6J) mice extended their limbs (A), whereas chimeric mice folded their limbs towards the trunk (B). (**C** and **D**) Walking footprints. In contrast with wild-type mice (C), chimeric mice could not walk along a straight line and dragged their feet (D). (**E** and **F**) Hanging wire grip test (E) and Rotor-rod test (F). In both tests, the chimeric mice with coat colors indicating a high contribution of fukutin-deficient cells (high) fell with a shorter latency than either wild-type mice or chimeric mice with a lower contribution of fukutin-deficient cells (medium and low). The number of mice examined is shown in parentheses. $*P < 0.05$.

weakness of facial and limb muscles, and general hypotonia which usually appears before 9 months of age. Functional disability is more severe in FCMD patients than in DMD patients; usually the maximum level of motor function achieved is sliding while sitting on the buttocks, and most FCMD patients are never able to walk. Patients usually become bedridden before 10 years of age due to generalized muscle atrophy and joint contracture, and most of them die by 20 years of age. Another manifestation observed in all cases is severe mental retardation; IQ scores in most FCMD patients lie between 30 and 50. Seizures occur in nearly half of the cases, in association with abnormal electroencephalograms (7).

Through positional cloning, we previously identified the gene responsible for FCMD, which encodes the fukutin protein (8–10). Most FCMD patients carry an ancestral mutation (11), which arose as a consequence of the integration of a 3 kb retrotransposon element into the 3′ untranslated region of the *fukutin* gene (10). FCMD is the first known human disease to be caused by an ancient retrotransposal integration. No FCMD patients 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 (12). There are no reported naturally occurring mice carrying mutations in the *fukutin* gene. Targeted homozygous mutation of this gene in mice leads to lethality at embryonic day 6.5–7.5, prior to development of skeletal muscle, cardiac muscle or mature neurons, suggesting that fukutin is essential for early embryonic development

Figure 1. Targeted disruption of both copies of *fukutin* in ES cells. (**A**) Physical map of the *fukutin* wild-type locus containing exon 2, targeting vector harboring the neomycin (neo^r) or puromycin ($puro^r$) resistant gene, and the disrupted *fukutin* locus. The arrowhead indicates the orientation of the drug-resistance genes. (**B**) Southern blot of *BamH*I-digested ES cell genomic DNA with the 3′ probe depicted in (A). A 5.0 kb wild-type fragment is detected in the single copy neo^r targeted clone (lane 2) as in parental ES cells (lane 1). In the double-copy targeted clone (lane 3), only a 6.4 kb band corresponding to the mutant allele was detected. (**C**) Contribution of *fukutin*-disrupted ES cells to brain and skeletal muscle. 129/SvEv and C57BL/6J mouse strains from which the *fukutin*-disrupted and wild-type tissues were derived have distinct electrophoretic variants of the two subunits of the glucose phosphate isomerase (129/SvEv, Gpi-1a; C57BL/6J, Gpi-1b). In correspondence with the coat color of the chimeric mice, the contribution of *fukutin*-deficient cells was high (lanes 1–3), medium (lanes 4 and 5), and low (lanes 6 and 7). (**D**) Survival rate of chimeric mice. The number of mice examined is shown in parentheses.

(Kurahashi *et al.*, unpublished data). To test the hypothesis that fukutin is necessary for maintenance of muscle integrity or histiogenesis of cerebral and cerebellar cortices, we here generated fukutin-deficient chimeric mice using embryonic stem (ES) cells targeted for both *fukutin* alleles. Interestingly, they also showed anomaly of the lens, loss of laminar structure in the retina, and retinal detachment. Our results indicate that fukutin is necessary for the maintenance of muscle integrity, cortical histiogenesis and normal ocular development, and suggest functional linkage between fukutin and α-dystroglycan.

RESULTS

Generation of fukutin-deficient chimeric mice

We used targeting vectors to generate ES cells in which both *fukutin* alleles have been disrupted by homologous recombination. To design a targeting vector for the generation of fukutin-deficient chimeric mice, we characterized a cosmid clone spanning exons 1–5 of the mouse *fukutin* gene (13) isolated from a 129/SvEv mouse library. The 2.3 kb *KpnI–BamHI* fragment containing the first coding exon (exon 2) was replaced with either a neomycin or a puromycin resistance gene (Fig. 1A), thereby removing the coding sequence corresponding to amino acids 1–35, as well as the splicing donor and acceptor sites. Targeted *fukutin* gene disruption was confirmed by Southern blot analysis of genomic DNA from ES cells (Fig. 1B).

After seven rounds of blastocyst injections, a total of 62 mice were born that were determined to be highly chimeric because of their coat color (agouti). Glucose phosphate isomerase isozyme (Gpi) assay revealed that the extent of chimerism in various tissues corresponded well with that determined by coat color (Fig. 1C). Therefore, we classified chimeric mice into three groups depending on chimerism of coat color (high, over 70% contribution of fukutin$^{-/-}$ cells; medium, 70–20%; and low, under 20%). Body weight was lower in the agouti mice (highly chimeric), and some died within one month. From 12 months of age, survival rates of high and medium chimeras gradually decreased. At 21 months survival rates of high, medium and low chimeras were 48, 67 and 94%, respectively (Fig. 1D).

Behavioral abnormalities in fukutin-deficient chimeric mice

Chimeric mice were dystrophic, although those with 50% or greater contribution from heterozygous ES cells showed no obvious phenotype, consistent with the lack of phenotype in fukutin$^{+/-}$ mice (Kurahashi *et al.*, unpublished data). Agouti mice (high) developed clasping when suspended by the tail (Fig. 2A and B). Analysis of hind footprints showed that they were unable to walk in a straight line and dragged their feet (Fig. 2C and D). Chimeras also showed muscle weakness in the hanging wire grip test (Fig. 2E) and positional instability in the rotor-rod test (Fig. 2F). These features first appeared at about 1 month but were not progressive.

Muscular dystrophic changes in fukutin-deficient chimeric mice

Figure 3A shows the histology of skeletal muscle in the hindlimbs of fukutin-deficient chimeric mice (high, lanes 1–3 in Fig. 1C). Massive necrosis with phagocytosis, mononuclear cell infiltration, basophilic regenerating muscle fibers, and increase of interstitial connective tissue were present at 1 month after birth. At later stages (7–9 months), a large number of small-sized muscle fibers contained central nuclei, indicating active regeneration. A small number of muscle fibers were undergoing degeneration and phagocytosis by macrophages. Connective tissue hyperplasia and fat cell deposition were also present.

α-Dystroglycan, a component of the DGC, is a heavily glycosylated mucin-type glycoprotein on the surface of muscle cells (3–5). It is the key component of the DGC, providing a tight linkage between the cell and basement membranes by binding laminin via its carbohydrate residues (3–5). α-Dystroglycan plays an active role in the basement membrane assembly itself (6). It was demonstrated recently that α-dystroglycan was selectively deficient in skeletal muscle from FCMD patients (14). We thus investigated the expression of α-dystroglycan, as well as the other components of the DGC, in skeletal muscle from fukutin-deficient chimeric mice by immunohistochemistry. α-Dystroglycan was greatly reduced in the sarcolemma of most muscle fibers in chimeric mice, while the other proteins, including β-dystroglycan and laminin α2 chain, were preserved (Fig. 3B). Interestingly, all regenerating muscle fibers were deficient in α-dystroglycan, suggesting that the fukutin deficiency did not significantly interfere with muscle fiber regeneration. Anti-fukutin antibody reacts with Golgi only in normal cell lines transfected with fukutin, not in non-transfected normal cells or normal human muscle, suggesting that the expression level of endogenous fukutin may be low (Kobayashi *et al.*, unpublished data). As muscle fibers are multi-nucleated, *in situ* hybridization for the targeting vector neo^r did not show complete correspondence between neo^r-positive and α-dystroglycan-negative fibers, although most neo^r-positive fibers were α-dystroglycan-negative (data not shown).

Immunoblot analysis confirmed the reduction of α-dystroglycan, especially in the antibody against the laminin-binding sugar chain of α-dystroglycan (IIH6; Fig. 3C). In addition, laminin blot overlay analysis revealed a deficiency in the laminin-binding activity of α-dystroglycan in chimeric mice (Fig. 3C). These results indicate that the linkage between laminin-2 and α-dystroglycan on the sarcolemma is disrupted in chimeric mice.

Brain anomalies in fukutin-deficient chimeric mice

We found markedly disorganized laminar structures in the cerebral and cerebellar cortices and hippocampus of fukutin-deficient chimeric mice. In the cerebral cortex, the normal six-layered structure was not clearly discernible (Fig. 4A and B). In some areas, cortical neurons had overmigrated, and the molecular layer (layer I) of the cerebral cortex had disappeared. The midline interhemispheric fissure was partially deficient, with fusion of medial surfaces of the cerebral cortex (Fig. 4C

and D). A small number of pyramidal cells in the CA3 sector of the hippocampus were not laminated, although the majority of pyramidal cells were aggregated in a compact lamina. Granule cells in the dentate gyrus were aggregated in a distorted, wavy distribution (Fig. 4E and F). In the cerebellum, the development of folia was defective, and the cerebellar fissure between the adjoining folia were partially fused. The granular layer was disorganized along the fusion lines of adjacent folia or at the pia matter, and Purkinje cells were sporadically malpositioned. Fusions between the caudal surface of the inferior colliculus and the rostral surface of the cerebellum were also observed (Fig. 4G and H).

After the injection of horseradish peroxidase (HRP) into the lumbar cord, corticospinal neurons with pyramidal somata were retrogradely labeled. While the somata of HRP-labeled corticospinal neurons were situated exclusively in layer V in the motor-sensory cortex of control mice (Fig. 4I and K), they were not distributed in any specific zone but were scattered diffusely throughout all depths of the cortex in chimeric mice (Fig. 4J and L).

Immunostaining with anti-laminin antibody revealed irregular interruptions of the meningeal basement membrane and granular deposits of laminin in the disorganized cerebral cortex (Fig. 4M and N). The meningeal basement membrane was also deficient over the fused medial cortex along the midline interhemispheric fissure or the fused cerebellar folia along the cerebellar sulci (data not shown). In contrast, the basement membrane surrounding blood vessels in the brain parenchyma was preserved. These findings indicate that fukutin is required for the assembly and/or remodeling of the meningeal basement membrane during the developmental period of brain cortical structures.

Immunoblot analysis revealed that α-dystroglycan was greatly reduced in the cerebral surface in chimeric mice and laminin blot overlay analysis revealed a deficiency in the laminin-binding activity of α-dystroglycan in chimeric mice (Fig. 4O). These results were similar to those in skeletal muscle and suggest that abnormal laminin–dystroglycan complex possibly cause brain anomalies in chimeric mice.

Eye anomalies in fukutin-deficient chimeric mice

Eye findings were quite remarkable. Fukutin-deficient chimeric mice showed corneal opacification with vascular infiltration (Fig. 5A and B). Microscopic analysis revealed a number of anomalies, including anomalous formation of the eyeball and lens (Fig. 5C and D); loss of laminar structure of the retina and retinal detachment (Fig. 5E and F); extensive folding of the retina (Fig. 5G); and thickened cornea with granular tissue formation, adhesion of the lens and cornea, and corneal inflammation and degeneration (Fig. 5H). Electroretinography (ERG) revealed extinction of the b-wave (Fig. 5I).

DISCUSSION

Here we have generated chimeric mice deficient in fukutin and shown that, similar to FCMD patients, the mutant mice develop neuronal migration disorder and ocular abnormality in addition to severe muscular dystrophy. At present, the function of fukutin remains unknown, and the mechanism by which its

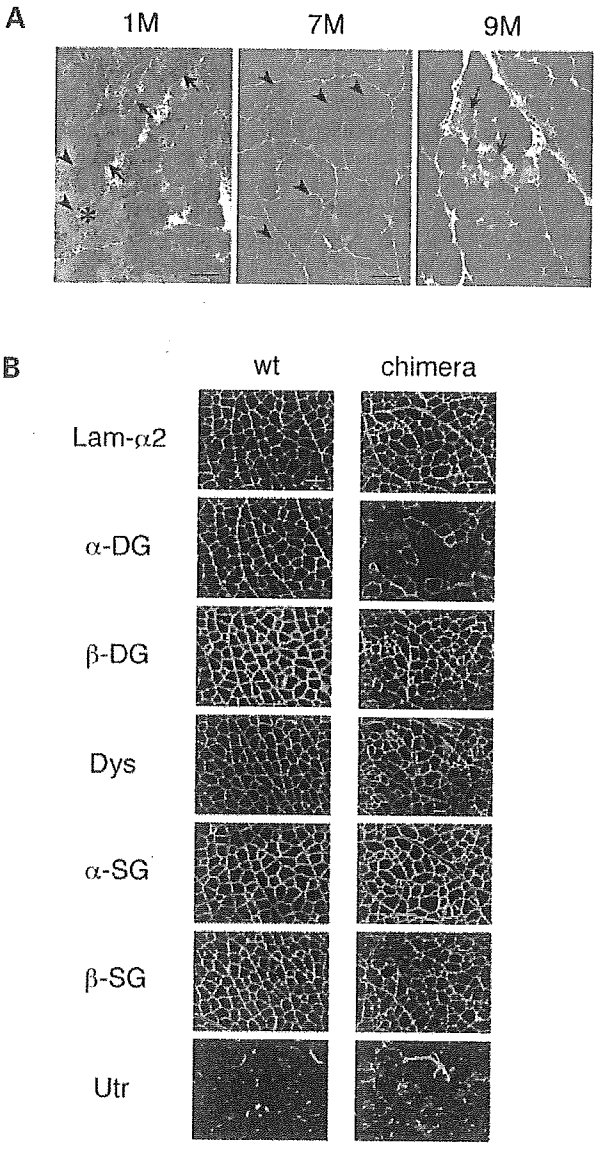


Figure 3. Muscular dystrophic changes in fukutin-deficient chimeric mice. (**A**) HE stained quadriceps muscle of chimeric mice. Massive necrosis with phagocytosis (asterisk), mononuclear cell infiltration (arrowhead), basophilic regenerating fibers (arrow), and an increase in connective tissue mass was present at 1 month of age. At the late stages of 7–9 months, a large number of small-sized fibers were found to have central nuclei (arrowhead), while a small number of fibers were undergoing active degeneration (arrow). Scale bar, 100 μm. (**B**) Immunohistochemical analysis of sarcolemmal proteins in quadriceps muscle from normal (wt) and chimeric mice. Lam-α2, laminin α2 chain; DG, dystroglycan; Dys, dystrophin; SG, sarcoglycan; Utr, utrophin. Selective and scattered deficiency of α-dystroglycan was observed in chimeric mice, while the other proteins were preserved. Scale bar, 100 μm. (**C**) Immunoblot and laminin blot overlay analyses of quadriceps muscle from the two normal (wt) and three chimeric mice. α-Dystroglycan immunoreactivity, as well as its laminin-binding activity, was greatly reduced in chimeric mice, while β-dystroglycan and laminin α2 chain were preserved. The deficiency of α-dystroglycan revealed by laminin blot overlay paralleled that revealed by monoclonal antibody IIH6C4 and was more prominent than that revealed by monoclonal antibody VIA4-1

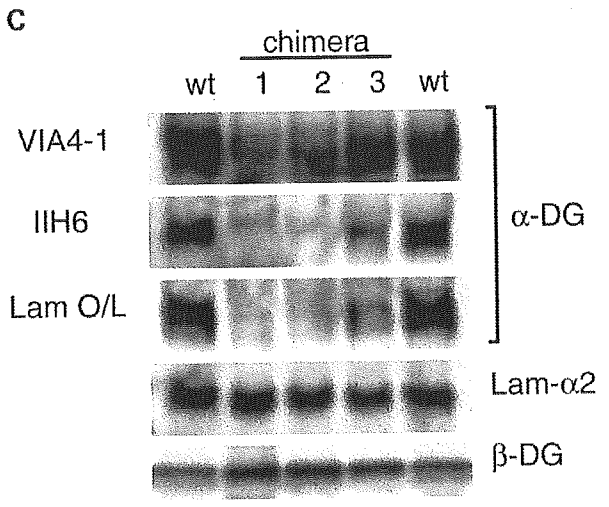


Figure 3 *continued.*

deficiency causes defects in multiple organs has not been clarified. In this respect, it should be noted that sequence analysis predicts fukutin to be an enzyme that modifies cell-surface glycoproteins or glycolipids (15). There are several lines of indirect but significant evidence to support this. First, it was reported that highly glycosylated α-dystroglycan was selectively deficient in the skeletal muscle of FCMD patients (14). Second, we have reported that muscle–eye–brain disease (MEB), an autosomal-recessive disorder having skeletal muscle, eye and brain defects similar to FCMD (16), is caused by mutations in the gene encoding the protein *O*-linked mannose β1, 2-*N*-acetylglucosaminyltransferase (POMGnT1), which cause the loss of the enzyme activity (17). Moreover, we have found the selective deficiency of α-dystroglycan in the skeletal muscle of MEB patients (18). Finally, defective glycosylation of α-dystroglycan has also been reported in another form of congenital muscular dystrophy, MDC1C, caused by mutations in the gene encoding the putative glycosyltransferase named FKRP (fukutin-related protein) (19) and in *myd* mice, an animal model of congenital muscular dystrophy, caused by the mutation in the gene encoding a putative glycosyltransferase named Large (20), although brain and eye anomalies are not the hallmarks of MDC1C. Quite recently 20% of Walker–Warburg syndrome patients have been found to have mutations in POMT1, a putative human counterpart of yeast O-mannosyltransferase (21). Moreover, Michele *et al.* (22) showed, in MEB, FCMD and myodystrophy mouse, that α-dystroglycan is expressed at the muscle membrane, but similar hypoglycosylation in the diseases directly abolishes binding activity of dystroglycan for the ligands laminin, neurexin and agrin. Together with the present results, these findings suggest that defective glycosylation of α-dystroglycan due to the primary genetic defects of glycosyltransferases may be the common denominator causing muscle cell degeneration in these diseases.

Strikingly, the deficiency of α-dystroglycan revealed by laminin blot overlay paralleled that revealed by the monoclonal antibody IIH6C4, which recognizes the laminin-binding carbohydrate residues of α-dystroglycan, and was more prominent than that revealed by the monoclonal antibody VIA4-1, which recognizes carbohydrate residues unrelated to the binding of laminin (4,23) (Figs 3C and 4O). It is of course possible that α-dystroglycan is still present but in a hypoglycosylated form, as was suggested to occur in FCMD by Michele *et al.* (22). This raises the possibility that fukutin may be involved in the modification of laminin-binding carbohydrate residues in α-dystroglycan and, in the absence of fukutin, the linkage between laminin and α-dystroglycan may never be established on the muscle cell surface. This scenario is also consistent with the report that chimeric mice lacking skeletal muscle dystroglycan developed muscular dystrophy similar to the mice described here (24).

Finally, the role of dystroglycan in the pathogenesis of brain and eye defects in FCMD remains unclear. Brain and/or eye defects similar to those reported here have recently been observed in mice lacking dystroglycan in the brain via Cre/*loxP*-mediated gene inactivation (25) and in *myd* mice, although the retina was apparently morphologically normal (26). Interestingly, brain and/or eye defects similar to those reported here have also been observed in mice lacking integrin β1 subunit in brain via Cre/*loxP*-mediated gene inactivation (27) and in mice carrying a targeted mutation in the integrin α6 subunit gene (28), respectively. We can envisage that the defects in dystroglycan have similar consequences as those caused by integrin deficiency, because these two receptors are thought to work in concert. Alternatively, fukutin might be involved in the modification of the carbohydrate residues of integrin subunits in brain and eye, and integrin might not function normally in the absence of fukutin, although immunostaining for integrin α7 on skeletal muscle cryosections showed no difference (data not shown).

Our data indicates that fukutin is essential for maintenance of muscle integrity, cortical histiogenesis, and normal eye development and suggest the functional linkage between fukutin and α-dystroglycan. Fukutin-deficient chimeric mice are suitable models for studying not only the biological function of fukutin but also the molecular pathogenesis of and therapeutic approaches to complex disorders exhibiting the simultaneous occurrence of central nervous, ocular and muscular abnormalities seen in FCMD and its related diseases.

MATERIALS AND METHODS

Targeted disruption of *fukutin*

A cosmid clone spanning exons 1–5 of the mouse *fukutin* gene was isolated from a 129/SvEv mouse library. Two targeting vectors were constructed with a 12 kb *Kpn*I–*Blp*I fragment containing the first coding exon (exon 2) and flanking introns. The 2.3 kb *Kpn*I–*Bam*HI fragment containing exon 2 was replaced with either a neomycin or a puromycin resistance gene (pKO-Select Neo or pKO-Select Puro, Lexicon), thereby removing the coding sequence corresponding to amino acids 1–35, as well as the splicing donor and acceptor sites. Targeting vectors were linearized using *Not*I, and AB2.2 prime ES cells (Lexicon) were first electroporated with the neo^r vector. We

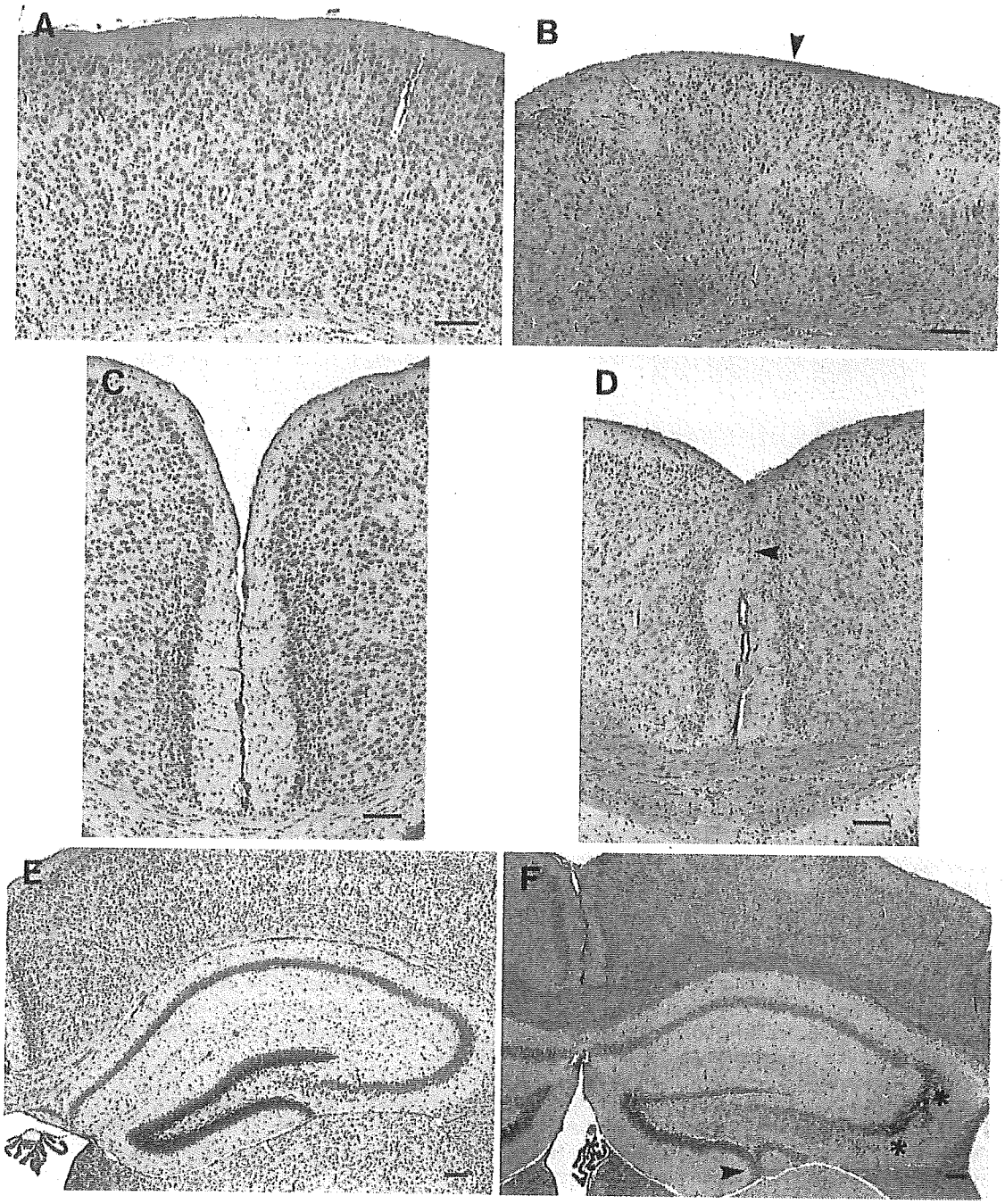


Figure 4. Brain anomalies in fukutin-deficient chimeric mice. (**A–H**) HE-stained sections of brain from normal (A, C, E and G) and chimeric (B, D, F and H) mice. (A and B) cerebral cortex; (C and D) interhemispheric fissure; (E and F) hippocampus and dentate gyms; (G and H) cerebellum. In chimeric mice, cerebral cortical neurons overmigrated, and the molecular layer was absent (arrowhead) (B). The midline interhemispheric fissure was absent due to the fusion of the adjoining medial surfaces of the cerebral cortex (arrowhead) (D). A single laminar distribution of pyramidal neurons was disorganized in the CA3 sector of the hippocampus (asterisk), and dentate granular cells were aggregated in a distorted wavy distribution (arrowhead) (F). The granular cell layer in the cerebellar cortex was disorganized along the fused cerebellar folia (asterisk) (H). (**I–L**) HRP retrograde labeling of cerebral cortex from normal (I and K) and chimeric (J and L) mice. In chimeric mice, HRP-labeled corticospinal neurons were not localized in layer V but scattered throughout all the depths of the motor cortex. (**M** and N) Laminin immunostaining of cerebral surface from normal (M) and chimeric (N) mice. In chimeric mice, the basement membrane was interrupted (arrowhead), and laminin was deposited in sporadic granules (asterisk) in the cerebral cortex. Scale bar, 100 μm. (**O**) Immunoblot and laminin blot overlay analyses of cerebral surface from the three normal (wt) and four chimeric mice. α-dystroglycan immunoreactivity, as well as its laminin-binding activity, was greatly reduced in chimeric mice. The results were similar to those in skeletal muscle (Fig. 3C).

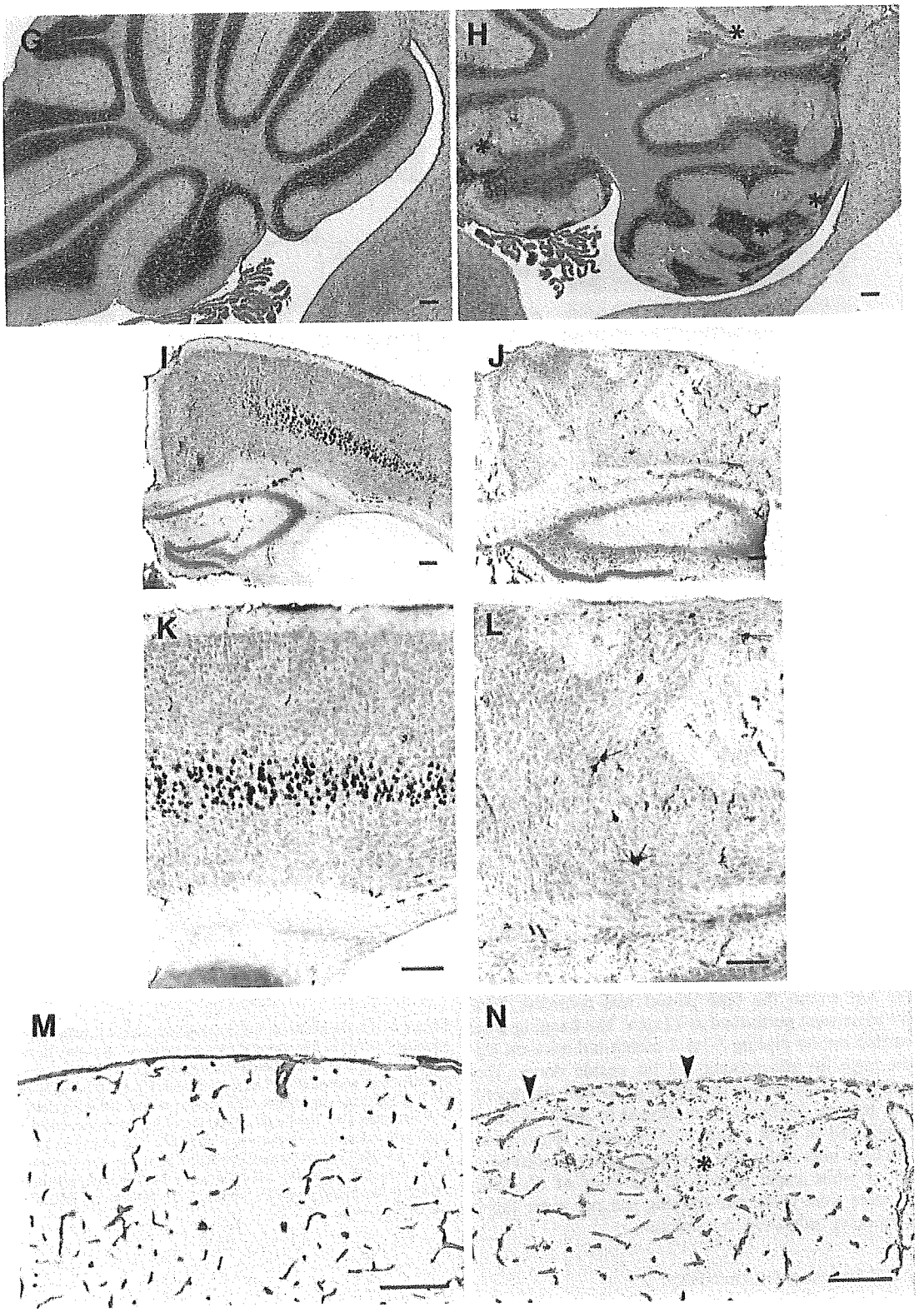


Figure 4 *continued.*

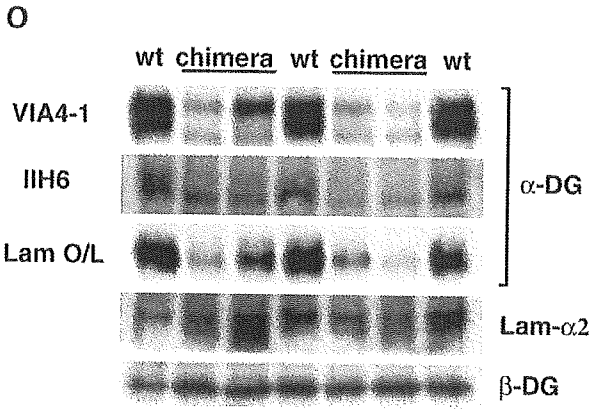


Figure 4 *continued.*

picked, expanded and screened Geneticin-resistant (Sigma) clones for homologous recombination. One clone (no. 8) was chosen and electroporated with the puro^r vector. Puromycin-resistant clones were screened to identify homologous recombinant clones. The double copy-targeted ES cell clone P182 was injected into E3.5 blastocysts of the C57BL/6 mouse strain using standard methods. Mice were maintained in accordance with the Animal Care guidelines of Otsuka Pharmaceutical Co. Ltd.

Evaluation of fukutin-null ES cell contribution to chimeric mice

Mice were sacrificed between 1 and 9 months of age. Protein extracts from tissue samples were subjected to Gpi electrophoresis, and the Gpi variants were stained on the cellulose acetate gels (Helena).

Behavioral phenotyping

Mice were tested during the light phase of their light-dark cycle between 13:00 and 17:00. Mice 7–9 months old were placed on the rotor-rod treadmill apparatus (rod diameter, 3 cm, MK-600, Muromachi Kikai) for a maximum of 60 s, and the latency to fall off the rod within the time period was recorded. Five consecutive trials were performed at 12 rpm. The hanging wire test was carried out by placing 5- to 7-month-old mice on top of the wire cage lid and turning the lid upside down. The latency to fall off the wire was measured up to 60 s. To record the footprint pattern, hindpaws were dipped in India ink, and mice were allowed to walk along a 35 cm long, 10 cm wide runway with 6 cm high walls. The footprints were recorded on a clean sheet of white paper placed on the floor of the dark tunnel. All mice were given one training run per week for 3 weeks before being subjected to a test run.

Histology and immunohistochemistry

Animals were anesthetized with ether and exsanguinated. Brains and eyes were removed and fixed with 10% formalin in 10 mM phosphate buffer (pH 7.2) for 4 days. After paraffin embedding, cerebrums were sectioned serially to 8 μm thickness at coronale. Cerebellums and eyeballs were sectioned to

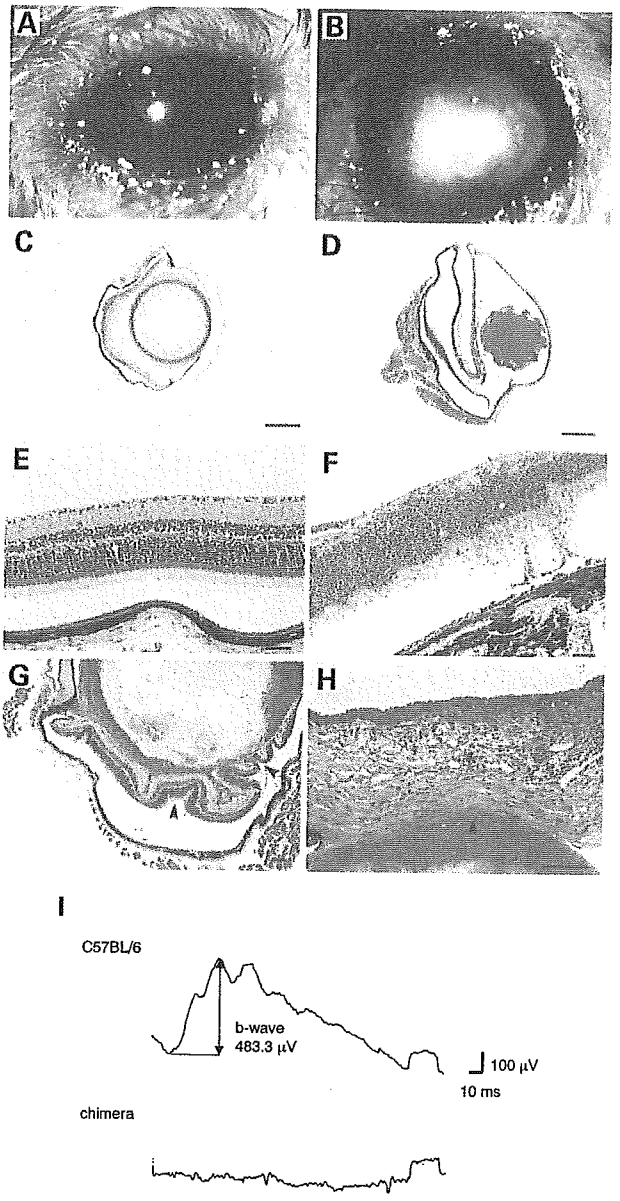


Figure 5. Eye anomalies in fukutin-deficient chimeric mice. (**A** and **B**) Gross appearance of eyes from normal (A) and chimeric (B) mice. In chimeric mice, corneal and/or lens opacification was present (B). (**C–H**) HE-stained sections of eyeballs from normal (C and E) and chimeric (D, F–H) mice. In chimeric mice, the lens had a peculiar shape, with the sclera bent and intercalated between the lens and retina (D). The laminar structure of the retina was completely disorganized and retinal detachment was present (F). The retina was misfolded extensively in a wavy pattern (arrowhead) (G). The cornea was thickened by granular tissue formation (asterisk) and adhered to the lens with inflammation (arrowhead) (H). Scale bar, C and D, 1.3 mm; E–H, 100 μm. (**I**) ERG revealed extinction of the b-wave.

8 μm thickness at sagittal. Every tenth section was counterstained with hematoxylin and eosin (HE) or by Klüver–Barrera's (KB) methods. Several adjoining sections were immunostained with a polyclonal anti-laminin antibody (Harber Bio-products). For skeletal muscle, serial frozen cryosections (8 μm) were stained with HE or immunostained with the monoclonal α-dystroglycan antibody VIA4-1 (Upstate) or antisera against laminin α2 chain (29), β-dystroglycan (30), dystrophin (31), α-sarcoglycan (29), β-sarcoglycan (32), and utrophin (30). After incubation with Alexa Fluor 488-labeled secondary antibodies (Molecular Probes), sections were examined under a fluorescent microscope.

Immunoblot and laminin blot overlay

SDS–PAGE, 3–12%, and immunoblotting of skeletal muscle and cerebral surface cryosections were performed as described previously (33), using the monoclonal antibodies IIH6C4 and VIA4-1 against α-dystroglycan (Upstate), the antiserum against β-dystroglycan, and the monoclonal antibody 2D9 against the laminin α2 chain (34). The laminin blot overlay was performed using 3 nM laminin-1 (Koken), and the laminin bound to α-dystroglycan on the polyvinylidene difluoride membrane was detected using a polyclonal anti-laminin antibody (Sigma).

HRP labeling

One microliter of 10% aqueous solution of HRP (type VI, Sigma) was injected into both sides of the upper lumbar cord. After 48 h, the animals were transcardially perfused with 1.25% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain and spinal cord were immersed in 30% phosphate-buffered sucrose overnight at 4°C, sectioned coronally at 40 μm thickness on a freezing microtome, and reacted for the presence of HRP using the chromagen tetramethylbenzidine (TMB) (35).

Electroretinography

Following 15 min of dark adaptation, ERGs were recorded from the corneal surface electrode after mydriasis. Stimulation with white flash light (10 J) was used (Photostimulator 3G21, NEC-Sanei).

ACKNOWLEDGEMENTS

We thank Drs Masato Horie, Ei-ichi Takahashi, Kenji Araishi, Yukiko K. Hayashi and Eva Engvall for discussion and antibodies; Sawako Muroi, Takashi Wadatsu, Noritaka Koseki, Norihiro Miyazawa, Yuji Fujimori, Tomoyuki Iwanaga, Mai Okano and Kuniko Ohmori for assistance; and Dr Jennifer Logan for editing the manuscript. This study was supported by a Health Science Research Grant, 'Research on Brain Science' (H12-Brain-017) and by a Research Grants for Nervous and Mental Disorders (14B-4), both from the Ministry of Health, Labor and Welfare of Japan.

REFERENCES

1. Hoffman, E.P., Brown, R.H. Jr. and Kunkel, L.M. (1987) Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell*, **51**, 919–928.
2. Campbell, K.P. (1995) Three muscular dystrophies: loss of cytoskeleton–extracellular matrix linkage. *Cell*, **80**, 675–679.
3. 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.
4. 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.
5. 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. The role of a novel *O*-mannosyl-type oligosaccharide in the binding of α-dystroglycan with laminin. *J. Biol. Chem.*, **272**, 2156–2162.
6. Henry, M.D. and Campbell, K.P. (1998) A role for dystroglycan in basement membrane assembly. *Cell*, **95**, 859–870.
7. Fukuyama, Y., Osawa, M. and Suzuki, H. (1981) Congenital progressive muscular dystrophy of the Fukuyama types—clinical, genetic and pathological considerations. *Brain Dev.*, **3**, 1–29.
8. Toda, T., Segawa, M., Nomura, Y., Nonaka, I., Masuda, K., Ishihara, T., Sakai, M., Tomita, I., Origuchi, Y., Suzuki, M. *et al.* (1993) Localization of a gene for Fukuyama type congenital muscular dystrophy to chromosome 9q31-33. *Nat. Genet.*, **5**, 283–286.
9. Toda, T., Miyake, M., Kobayashi, K., Mizuno, K., Saito, K., Osawa, M., Nakamura, Y., Kanazawa, I., Nakagome, Y., Tokunaga, K. and Nakahori, Y. (1996) Linkage-disequilibrium mapping narrows the Fukuyama-type congenital muscular dystrophy (FCMD) candidate region to <100 kb. *Am. J. Hum. Genet.*, **59**, 1313–1320.
10. Kobayashi, K., Nakahori, Y., Miyake, M., Matsumura, K., Kondo-Iida, E., Nomura, Y., Segawa, M., Yoshioka, M., Saito, K., Osawa, M. *et al.* (1998) An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. *Nature*, **394**, 388–392.
11. Colombo, R., Bignamini, A.A., Carobene, A., Sasaki, J., Tachikawa, M., Kobayashi, K. and Toda, T. (2000) Age and origin of the FCMD 3′-untranslated-region retrotransposal insertion mutation causing Fukuyama-type congenital muscular dystrophy in the Japanese population. *Hum. Genet.*, **107**, 559–567.
12. Kondo-Iida, E., Kobayashi, K., Watanabe, M., Sasaki, J., Kumagai, T., Koide, H., Saito, K., Osawa, M., Nakamura, Y. and Toda, T. (1999) Novel mutations and genotype-phenotype relationships in 107 families with Fukuyama-type congenital muscular dystrophy (FCMD). *Hum. Mol. Genet.*, **8**, 2303–2309.
13. Horie, M., Kobayashi, K., Takeda, S., Nakamura, Y., Lyons, G.E. and Toda, T. (2002) Isolation and characterization of the mouse ortholog of the Fukuyama-type congenital muscular dystrophy gene. *Genomics*, **80**, 482–486.
14. Hayashi, Y.K., Ogawa, M., Tagawa, K., Noguchi, S., Ishihara, T., Nonaka, I. and Arahata, K. (2001) Selective deficiency of α-dystroglycan in Fukuyama-type congenital muscular dystrophy. *Neurology*, **57**, 115–121.
15. Aravind, L. and Koonin, E.V. (1999) The fukutin protein family—predicted enzymes modifying cell-surface molecules. *Curr. Biol.*, **9**, R836–837.
16. Santavuori, P., Somer, H., Sainio, K., Rapola, J., Kruus, S., Nikitin, T., Ketonen, L. and Leisti, J. (1989) Muscle–eye–brain disease (MEB). *Brain Dev.*, **11**, 147–153.
17. Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M. *et al.* (2001) Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. *Dev. Cell*, **1**, 717–724.
18. Kano, H., Kobayashi, K., Herrmann, R., Tachikawa, M., Manya, H., Nishino, I., Nonaka, I., Straub, V., Talim, B., Voit, T. *et al.* (2002) Deficiency of α-dystroglycan in muscle-eye-brain disease. *Biochem. Biophys. Res. Commun.*, **291**, 1283–1286.
19. 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. *et al.* (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.

20. Grewal, P.K., Holzfeind, P.J., Bittner, R.E. and Hewitt, J.E. (2001) Mutant glycosyltransferase and altered glycosylation of α-dystroglycan in the myodystrophy mouse. *Nat. Genet.*, **28**, 151–154.
21. Beltrán-Valero de Bernabé, D., Currier, S., Steinbrecher, A., Celli, J., van Beusekom, E., van der Zwaag, B., Kayserili, H., Merlini, L., Chitayat, D., Dobyns, W.B. *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.
22. Michele, D., Barresi, R., Kanagawa, M., Saito, F., Cohn, R.D., Satz, J.S., Dollar, J., Nishino, I., Kelley, R.I., Somer, H. *et al.* (2002) Post-translational disruption of dytroglycan–ligand interactions in congenital muscular dystrophies. *Nature*, **418**, 417–422.
23. Matsumura, K., Chiba, A., Yamada, H., Fukuta-Ohi, H., Fujita, S., Endo, T., Kobata, A., Anderson, L.V., Kanazawa, I., Campbell, K.P. and Shimizu, T. (1997) A role of dystroglycan in schwannoma cell adhesion to laminin. *J. Biol. Chem.*, **272**, 13904–13910.
24. Cote, P.D., Moukhles, H., Lindenbaum, M. and Carbonetto, S. (1999) Chimaeric mice deficient in dystroglycans develop muscular dystrophy and have disrupted myoneural synapses. *Nat. Genet.*, **23**, 338–342.
25. Moore, S.A., Saito, F., Chen, J., Michele, D.E., Henry, M.D., Messing, A., Cohn, R.D., Ross-Barta, S.E., Westra, S., Williamson, R.A. *et al.* (2002) Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy. *Nature*, **418**, 422–425.
26. Holzfeind, P.J., Grewal, P.K., Reitsamer, H.A., Kechvar, J., Lassmann, H., Hoeger, H., Hewitt, J.E. and Bittner, R.E. (2002) Skeletal, cardiac and tongue muscle pathology, defective retinal transmission, and neuronal migration defects in the Large^{myd} mouse defines a natural model for glycosylation-deficient muscle–eye–brain disorders. *Hum. Mol. Genet.*, **11**, 2673–2687.
27. Graus-Porta, D., Blaess, S., Senften, M., Littlewood-Evans, A., Damsky, C., Huang, Z., Orban, P., Klein, R., Schittny, J.C. and Muller, U. (2001) β1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex. *Neuron*, **31**, 367–379.
28. Georges-Labouesse, E., Mark, M., Messaddeq, N. and Gansmuller, A. (1998) Essential role of α6 integrins in cortical and retinal lamination. *Curr. Biol.*, **8**, 983–986.
29. Araishi, K., Sasaoka, T., Imamura, M., Noguchi, S., Hama, H., Wakabayashi, E., Yoshida, M., Hori, T. and Ozawa, E. (1999) Loss of the sarcoglycan complex and sarcospan leads to muscular dystrophy in β-sarcoglycan-deficient mice. *Hum. Mol. Genet.*, **8**, 1589–1598.
30. Imamura, M., Araishi, K., Noguchi, S. and Ozawa, E. (2000) A sarcoglycan–dystroglycan complex anchors Dp116 and utrophin in the peripheral nervous system. *Hum. Mol. Genet.*, **9**, 3091–3100.
31. Hagiwara, Y., Sasaoka, T., Araishi, K., Imamura, M., Yorifuji, H., Nonaka, I., Ozawa, E. and Kikuchi, T. (2000) Caveolin-3 deficiency causes muscle degeneration in mice. *Hum. Mol. Genet.*, **9**, 3047–3054.
32. Noguchi, S., Wakabayashi, E., Imamura, M., Yoshida, M. and Ozawa, E. (2000) Formation of sarcoglycan complex with differentiation in cultured myocytes. *Eur. J. Biochem.*, **267**, 640–648.
33. Matsumura, K., Tome, F.M., Collin, H., Azibi, K., Chaouch, M., Kaplan, J.C., Fardeau, M. and Campbell, K.P. (1992) Deficiency of the 50K dystrophin-associated glycoprotein in severe childhood autosomal recessive muscular dystrophy. *Nature*, **359**, 320–322.
34. Hori, H., Kanamori, T., Mizuta, T., Yamaguchi, N., Liu, Y. and Nagai, Y. (1994) Human laminin M chain: epitope analysis of its monoclonal antibodies by immunoscreening of cDNA clones and tissue expression. *J. Biochem.*, **116**, 1212–1219.
35. Mesulam, M.M. (1978) Tetramethyl benzidine for horseradish peroxidase neurohistochemistry: a non-carcinogenic blue reaction product with superior sensitivity for visualizing neural afferents and efferents. *J. Histochem. Cytochem.*, **26**, 106–117.

ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Biochemical and Biophysical Research Communications 306 (2003) 93–97

BBRC

www.elsevier.com/locate/ybbrc

Loss-of-function of an *N*-acetylglucosaminyltransferase, POMGnT1, in muscle–eye–brain disease

Hiroshi Manya,[a] Keiwa Sakai,[b] Kazuhiro Kobayashi,[c] Kiyomi Taniguchi,[c] Masao Kawakita,[b] Tatsushi Toda,[c] and Tamao Endo[a,*]

[a] *Glycobiology Research Group, Tokyo Metropolitan Institute of Gerontology, Foundation for Research on Aging and Promotion of Human Welfare, 35-2 Sakaecho, Itabashi-ku, Tokyo 173-0015, Japan*

[b] *Department of Applied Chemistry, Kogakuin University, Tokyo 163-8677, Japan*

[c] *Division of Functional Genomics, Department of Post-Genomics and Diseases, Osaka University of Graduate School of Medicine, Suita, Osaka 565-0871, Japan*

Received 8 May 2003

Abstract

Muscle–eye–brain disease (MEB), an autosomal recessive disorder, is characterized by congenital muscular dystrophy, brain malformation, and ocular abnormalities. Previously, we found that MEB is caused by mutations in the gene encoding the protein O-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1), which is responsible for the formation of the GlcNAcβ1-2Man linkage of *O*-mannosyl glycan. Although 13 mutations have been identified in patients with MEB, only the protein with the most frequently observed splicing site mutation has been studied. This protein was found to have no activity. Here, we expressed the remaining mutant POMGnT1s and found that none of them had any activity. These results clearly demonstrate that MEB is inherited as a loss-of-function of POMGnT1.

Keywords: Muscle–eye–brain disease; Muscular dystrophy; Dystroglycan; Glycosylation; POMGnT1; Neuronal migration disorder

Muscular dystrophies are genetic diseases that cause progressive muscle weakness [1]. The best known is that described by Duchenne which results from mutations in the gene encoding a protein called dystrophin. Another subclass is congenital muscular dystrophies, where muscle weakness is apparent at birth or shortly afterwards. One of them is muscle–eye–brain disease (MEB: OMIM 253280). MEB is an autosomal recessive disorder characterized by congenital muscular dystrophy, ocular abnormalities, and brain malformation (type II lissencephaly) [2]. Patients with MEB show congenital muscular dystrophy, severe congenital myopia, congenital glaucoma, pallor of the optic discs, retinal hypoplasia, mental retardation, hydrocephalus, abnormal electroencephalograms, and myoclonic jerks. All infants with MEB are floppy with generalized muscle weakness, including facial and neck muscles, from birth. Muscle biopsies show dystrophic changes and brain MRIs reveal pachygyria-type cortical neuronal migration disorder, flat brainstem, and cerebellar hypoplasia.

α-Dystroglycan is an extracellular peripheral membrane glycoprotein anchored to the cell membrane by binding to a transmembrane glycoprotein, β-dystroglycan. The α-dystroglycan–β-dystroglycan complex is widely expressed in a broad array of tissues and is thought to act as a transmembrane linker between the extracellular matrix and intracellular cytoskeleton. This is because α-dystroglycan binds to laminin and the intracellular domain of β-dystroglycan interacts with dystrophin in skeletal muscle [3]. α-Dystroglycan is heavily glycosylated and its sugars have a role in binding to laminin, neurexin, and agrin [3,4]. We previously found that the sugar moiety of α-dystroglycan includes *O*-mannosyl glycan, which is a rare type of glycan in mammals [5]. We also found that a sialyl *O*-mannosyl glycan, Siaα2-3Galβ1-4GlcNAcβ1-2Man, is a laminin-binding ligand of α-dystroglycan [6].

* Corresponding author. Fax: +81-3-3579-4776.
E-mail address: endo@tmig.or.jp (T. Endo).

0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)00924-0

Recently, we identified a glycosyltransferase, protein O-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1), which catalyzes the transfer of GlcNAc to *O*-mannose of glycoproteins, and showed that the *POMGnT1* gene is responsible for MEB [7]. We identified 13 independent disease-causing mutations in *POMGnT1* in MEB patients [7,8]. To confirm that the mutations observed in patients with MEB are responsible for the defects in the synthesis of *O*-mannosyl glycan, we expressed the protein with the most frequent mutation and found that it had lost enzymatic activity. It is important to determine whether the remaining mutations similarly cause loss of function of POMGnT1 because the 13 mutations are dispersed throughout the entire *POMGnT1* gene and some mutations seem to be located outside the catalytic domain. In the current study, we examined the enzymatic activity of mutant POMGnT1 proteins observed in patients with MEB.

Materials and methods

Construction of POMGnT1 mutants. An expression vector encoding each mutant of *POMGnT1* was prepared by site-directed mutagenesis. Template cDNA for site-directed mutagenesis encoding full-length *POMGnT1* tagged with the His-tag and Xpress epitope was cloned into pcDNA 3.1 Zeo(+) (Invitrogen), as described previously [7]. Site-directed mutagenesis for missense and frameshift mutants (E223K, C269Y, P493R, H573fs, L611fs, and V626fs) was performed using a QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The primers used to make the different mutants were: E223K, 5′-GGAGGTCCTGTCTTCGGGAAGAAA CATTCTAAATC-3′ and 5′-GATTTAGAATGTTTCTTCCCGAAG ACAGGACCTCC-3′; C269Y, 5′-GCCGGCGCTTCTACAGCAAA GTTGAGGG-3′ and 5′-CCCTCAACTTTGCTGTAGAAGCGCCG GC-3′; P493R, 5′-CCGAGAGTGCATCATCCGTGACGTTTCCC G-3′ and 5′-CGGGAAACGTCACGGATGATGCACTC-3′; H573fs, 5′-CAGACACAGAGGGCCAACCTACGTGGCC-3′ and 5′-GGCC ACGTAGGTTGGCCCTCTGTGTCTG-3′; L611fs, 5′-GGCAACC ATCGGGGCCGTGGAGATTGTTTC-3′ and 5′-GAAACAATCTC CACGGCCCCGATGGTTGCC-3′; and V626fs, 5′-CTTCCTGGTG GTGGGGTCCCGGCTTCCC-3′ and 5′-GGGAAGCCGGGACCC CACCACCAGGAAG-3′. Site-directed mutagenesis for splicing site mutant (Δ536–550) was constructed by joining two PCR products. Two fragments were ligated at a *Sal*I site and introduced into the *Pst*I and *Xba*I sites of template plasmid. The primers used to make the mutant were: *Pst*I–*Sal*I, 5′-GCTTCTGCAGCAAAGTTGAGG-3′ and 5′-CTGTCGACATTCCTGAGCTGGACACC-3′; and *Sal*I–*Xba*I, 5′-GGGTCGACAGTGAGGCTGAGGTTCTGGACC-3′ and 5′-AACGGGCCCTCTAGACTCGAGG-3′. All mutant clones were sequenced to confirm the presence of the mutations.

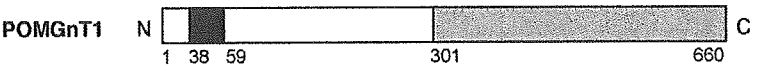
Cell culture and expression of POMGnT1 mutants. Human embryonic kidney 293T (HEK293T) cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO_2. The expression plasmids of POMGnT1 mutants were transfected into HEK293T cells using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. The transfected cells were harvested and homogenized after being cultured for 2 days in complete medium.

Western blot analysis. The cells were homogenized in 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 250 mM sucrose. After centrifugation at 900*g* for 10 min, the supernatant was subjected to ultracentrifugation at 100,000*g* for 1 h. The precipitate was used as the microsomal membrane fraction. Protein concentration was determined by BCA assay. The proteins in the microsomal fraction (20 μg) were separated by SDS–PAGE (10% gel) and transferred to a PVDF membrane. The membrane was blocked in PBS containing 5% skim milk and 0.5% Tween 20, incubated with anti-Xpress monoclonal antibody (Invitrogen), and treated with anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Amersham Biosciences). Proteins that bound to the antibody were visualized with an ECL Plus kit (Amersham Biosciences). To determine the amount of each mutant POMGnT1, we used Positope protein (Invitrogen) as a mass standard of Xpress-tagged protein and the intensities of bands in Western blotting. The band intensities were measured with a densitometer and NIH Image 1.61/ppc software.

Assay for POMGnT1 activity. POMGnT1 activity was based on the amount of [^{3}H]GlcNAc transferred to a mannosylpeptide [9]. Briefly, a reaction mixture containing 140 mM Mes buffer (pH 7.0), 200 μM UDP-[^{3}H]GlcNAc (~228,000 dpm/mol), 400 μM mannosylpeptide (Ac-Ala–Ala–Pro–Thr(Man)–Pro–Val–Ala–Ala–Pro-NH_2), 10 mM $MnCl_2$, 2% Triton X-100, 5 mM AMP, 200 mM GlcNAc, 10% glycerol, and enzyme solution was incubated at 37 °C for 1 h. After boiling for 3 min, the mixture was analyzed by reversed phase HPLC with a Wakopak 5C18-200 column (4.6 × 250 mm). The gradient solvents were aqueous 0.1% trifluoroacetic acid (solvent A) and acetonitrile containing 0.1% trifluoroacetic acid (solvent B). The mobile phase consisted of: (1) 100% A for 10 min, (2) a linear gradient to 75% A, 25% B over 25 min, (3) a linear gradient to 100% B over 5 min, and (4) 100% B for 5 min. The peptide separation was monitored at 214 nm and the radioactivity of each fraction (1 ml) was measured by a liquid scintillation counter.

Results and discussion

Previously, we identified 13 mutations in the *POMGnT1* gene in patients with MEB [7,8]. These mutations were all simple point mutations that caused nonsense, missense, frameshift and premature termination, and splicing-site mutation (read-through of intronic sequences and/or skipping of the upstream exon) (Fig. 1). They are dispersed through the entire *POMGnT1* gene. MEB patients have either homozygous or compound heterozygous mutations in the gene. Among these mutated enzymes, only two were examined and showed a loss of activity [7]. These are the splicing mutants IVS17+1 G > A and IVS17+1 G > T (mutations 8 and 9 in Fig. 1). Their products are characterized by a read-through at residue 514 (E514rt) and a deletion of amino acid residues 472–513 (Δ472–513), respectively. As predicted by computer analysis, the 660-amino acid POMGnT1 protein is divided into four domains: a cytoplasmic tail (Met1–Arg37), a transmembrane domain (Phe38–Ile58), a stem domain (Leu59–Leu300), and the catalytic domain (Asn301–Thr660) [7,10]. Nonsense or frameshift mutations near the 5′-terminus (281 C > T, 541 del T, 1077 ins G, and 1106 ins T in Fig. 1) shorten the POMGnT1 protein significantly. Because these products (R63X, F149fs, V328fs, and D338fs) probably result in a loss-of-function, we did not perform further studies. The remaining mutants were constructed and



	Mutations	Effects
1	281 C > T	Arg 63 Stop Nonsense (R63X)
2	541 del T	Phe 149 frameshift 167 Stop (F149fs)
3	761 G > A	Glu 223 Lys missense (E223K)
4	900 G > A	Cys 269Tyr missense (C269Y)
5	1077 ins G	Val 328 frameshift 338 Stop (V328fs)
6	1106 ins T	Asp 338 frameshift 338 Stop (D338fs)
7	1572 C > G	Pro 493 Arg missense (P493R)
8	IVS17+1 G > A	Glu 514 read-through 526 Stop (E514rt)
9	IVS17+1 G > T	or Leu 472 – His 513 del (Δ472-513)
10	1743 G > A	Leu 536 – Ser 550 del (Δ536-550)
11	1813 del C	His 573 frameshift 633 Stop (H573fs)
12	1926 del T	Leu 611 frameshift 633 Stop (L611fs)
13	1970 del G	Val 626 frameshift 633 Stop (V626fs)

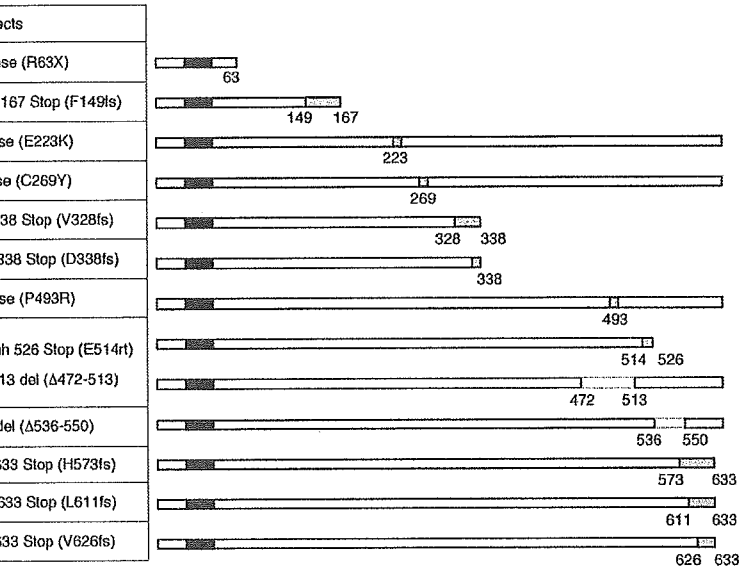


Fig. 1. Schematic representation of POMGnT1 and the predicted products corresponding to each mutation and a summary of mutations of MEB patients. Each protein is represented by a box with its N-terminus to the left. Each box from the left indicates a cytoplasmic tail (white box), a transmembrane domain (black box), a stem domain (white box), and the catalytic domain (gray box). The hatched boxes and dotted-line boxes represent mutated amino acids and deletion regions, respectively. The numbers below the boxes indicate the amino acid residue numbers of POMGnT1 and each mutant. rt, read-through; Δ, deletion; and fs, frameshift.

expressed in HEK293T cells and then we examined the enzyme activity of each product.

The 1743 G > A mutation (G-to-A base substitution at position 1743; mutation 10 in Fig. 1) caused skipping of exon 19, resulting in the deletion of 15 amino acids. The product (Δ536–550) did not show any enzymatic activity (Fig. 2). Three mutants with deletions near the 3′-terminus of the *POMGnT1* coding region (1813 del C, 1926 del T, and 1970 del G in Fig. 1) result in a frameshift and premature termination codon 633. These products (H573fs, L611fs, and V626fs) did not show any enzymatic activity (Fig. 2). They were thought to retain some ability to transfer a GlcNAc residue because they have the sequence of the normal POMGnT1 protein

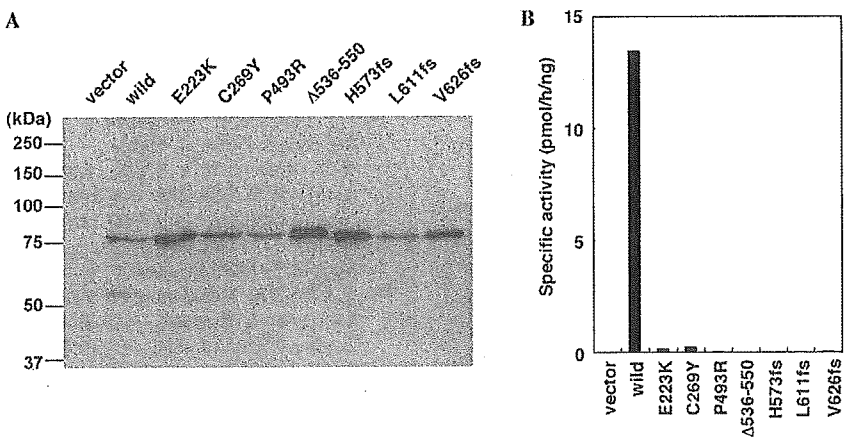


Fig. 2. Expression and activity of POMGnT1 mutant proteins. (A) Western blot analysis of Xpress-tagged POMGnT1 mutants expressed in HEK293T cells. The proteins (20 μg each) were subjected to SDS–PAGE (10% gel) and the separated proteins were transferred to a PVDF membrane. The PVDF membrane was stained with anti-Xpress monoclonal antibody. Molecular weight standards are shown on the left. (B) POMGnT1 activity was based on the rate of GlcNAc transfer to a mannosylpeptide using each of the membrane fractions from each transfectant. The product was separated by HPLC.

until positions 573, 611, and 626, respectively. However, this was not the case. The results suggest that the C-terminal portion of the POMGnT1 protein is necessary for enzymatic activity. The remaining three mutants with base substitutions (761 G > A, 900 G > A, and 1572 C > G in Fig. 1) result in amino acid substitutions, Glu223Lys, Cys269Tyr, and Pro493Arg, respectively. These products did not show any enzymatic activity (Fig. 2). Because position 493 is located in the probable catalytic domain of the protein [7], the amino acid substitution P493R may have an effect on enzymatic activity. On the other hand, positions 223 and 269 are located in the stem domain. Although the function of the stem region is not clear, it may contain a targeting signal for the Golgi apparatus [11]. Because we used a crude microsomal membrane fraction as an enzyme source in this study, we cannot exclude the possibility that, in addition to a loss of enzymatic activity, the enzyme was not translocated to the Golgi apparatus correctly. In any case, we conclude that an amino acid substitution of 223 or 269 leads to a loss-of-function. In summary, none of the mutated POMGnT1 in MEB patients have any enzyme activity. This suggests that MEB patients have a defect of *O*-mannosyl glycosylation. However, measurement of the POMGnT1 activity or a structural analysis of the sugars of α-dystroglycan in each patient tissue will be necessary to reach this conclusion, although it is very hard to perform it at this stage due to the limited amount of the sample and the ethical problem.

In a previous study of MEB patients with mutations in *POMGnT1* [8], we observed that patients with mutations near the 5′-terminus of the coding region had relatively severe brain symptoms, especially hydrocephalus, while patients with mutations near the 3′-terminus had milder phenotypes. The fact that all mutant POMGnT1s lost all activity suggests that additional factors play a role in determining disease severity in the brain.

Like MEB, Fukuyama-type congenital muscular dystrophy (FCMD) and Walker–Warburg syndrome (WWS) are autosomal recessive disorders that are characterized by congenital muscular dystrophy, lissencephaly, and eye anomalies [12,13]. FCMD is caused by mutations in the gene encoding fukutin, a protein of unknown function [14]. A sequence analysis predicts it to be an enzyme that modifies cell-surface glycoproteins or glycolipids. Recently, 20% of WWS patients have been found to have mutations in *POMT1*, a putative *O*-mannosyltransferase that catalyzes the transfer of Man to a Ser or Thr residue [15]. However, it is unclear whether the POMT1 protein actually catalyzes the reaction. Interestingly, in each of these diseases, a highly glycosylated α-dystroglycan was selectively deficient in skeletal muscle [3,15–18]. Additionally, defective glycosylation of α-dystroglycan has been implicated in congenital muscular dystrophy type 1C (MDC1C). The defective glycosylation is caused by mutations in a gene encoding a putative glycosyltransferase (FKRP, fukutin-related protein) [19]. The gene *large*, which is mutated in the myodystrophy (*myd*) mouse, encodes a putative glycosyltransferase [20]. Moreover, hypoglycosylated α-dystroglycan in the muscle membrane of MEB, FCMD, and the *myd* mouse has greatly reduced affinities for laminin, neurexin, and agrin [4]. In other words, interference in O-mannosylation of α-dystroglycan may lead to a combination of muscle, eye, and brain abnormalities and is a new pathomechanism for muscular dystrophy as well as neuronal migration disorder. Some forms of muscular dystrophy may be due to defects of glycosyltransferases, but the substrates of these enzymes, with the exception of POMGnT1, are largely unknown. Identification and characterization of each enzyme will help to reveal the molecular pathomechanisms of congenital muscular dystrophies with brain malformation.

Acknowledgments

We thank Dr. James Raymond for editing the manuscript. This study was supported by a Research Grant for Nervous and Mental Disorders (14B-4) from the Ministry of Health, Labour and Welfare of Japan, by a Grant-in-Aid for Scientific Research on Priority Area (14082209) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and grants from The Naito Foundation and Yamanouchi Foundation for Research on Metabolic Disorders.

References

[1] A.E. Emery, The muscular dystrophies, Lancet 359 (2002) 687–695.
[2] P. Santavuori, H. Somer, K. Sainio, J. Rapola, S. Kruus, T. Nikitin, L. Ketonen, J. Leisti, Muscle–eye–brain disease (MEB), Brain Dev. 11 (1989) 147–153.
[3] F. Montanaro, S. Carbonetto, Targeting dystroglycan in the brain, Neuron 37 (2003) 193–196.
[4] 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.
[5] T. Endo, *O*-Mannosyl glycans in mammals, Biochim. Biophys. Acta 1473 (1999) 237–246.
[6] 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.
[7] 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.

[8] 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.

[9] 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.

[10] U.M. Unligil, S. Zhou, S. Yuwaraj, M. Sarkar, H. Schachter, J.M. Rini, X-ray crystal structure of rabbit *N*-acetylglucosaminyltransferase I: catalytic mechanism and a new protein superfamily, EMBO J. 19 (2000) 5269–5280.

[11] P.A. Gleeson, Targeting of proteins to the Golgi apparatus, Histochem. Cell Biol. 109 (1998) 517–532.

[12] 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.

[13] W.B. Dobyns, R.A. Pagon, D. Armstrong, C.J. Curry, F. Greenberg, A. Grix, L.B. Holmes, R. Laxova, V.V. Michels, M. Robinow, et al., Diagnostic criteria for Walker–Warburg syndrome, Am. J. Med. Genet. 32 (1989) 195–210.

[14] 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.

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

[16] H. Kano, K. Kobayashi, R. Herrmann, M. Tachikawa, H. Manya, I. Nishino, I. Nonaka, V. Straub, B. Talim, T. Voit, H. Topaloglu, T. Endo, H. Yoshikawa, T. Toda, Deficiency of α-dystroglycan in muscle–eye–brain disease, Biochem. Biophys. Res. Commun. 291 (2002) 1283–1286.

[17] Y.K. Hayashi, M. Ogawa, K. Tagawa, S. Noguchi, T. Ishihara, I. Nonaka, K. Arahata, Selective deficiency of α-dystroglycan in Fukuyama-type congenital muscular dystrophy, Neurology 57 (2001) 115–121.

[18] F. Muntoni, M. Brockington, D.J. Blake, S. Torelli, S.C. Brown, Defective glycosylation in muscular dystrophy, Lancet 360 (2002) 1419–1421.

[19] M. Brockington, D.J. Blake, P. Prandini, S.C. Brown, S. Torelli, M.A. Benson, C.P. Ponting, B. Estournet, N.B. Romero, E. Mercuri, T. Voit, C.A. Sewry, P. Guicheney, F. Muntoni, 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 (2001) 1198–1209.

[20] P.K. Grewal, P.J. Holzfeind, R.E. Bittner, J.E. Hewitt, Mutant glycosyltransferase and altered glycosylation of α-dystroglycan in the myodystrophy mouse, Nat. Genet. 28 (2001) 151–154.

ELSEVIER

Clinical Biochemistry 36 (2003) 339–344

CLINICAL BIOCHEMISTRY

Enzymatic diagnostic test for Muscle-Eye-Brain type congenital muscular dystrophy using commercially available reagents

Wenli Zhang[a], Jiri Vajsar[b,c], Pinjiang Cao[a], Galen Breningstall[d], Charlotta Diesen[e], William Dobyns[f], Ralph Herrmann[g], Anna-Elina Lehesjoki[e], Alice Steinbrecher[g], Beril Talim[h], Tatsushi Toda[i], Haluk Topaloglu[j], Thomas Voit[g], Harry Schachter[a,c,*]

[a]*Department of Structural Biology and Biochemistry, The Hospital for Sick Children, 555 University Avenue, Toronto, Ont. M5G 1X8, Canada*
[b]*Division of Neurology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ont. M5G 1X8, Canada*
[c]*University of Toronto, 1 King's College Circle, Toronto, Ont. M5S 1A8 Canada*
[d]*Department of Neurology, University of Minnesota, 420 Delaware Street SE, Minneapolis, MN 55455, and the Noran Neurological Clinic, 910 East 26th Street, Minneapolis, Minnesota 55404*
[e]*Folkhälsan Institute of Genetics and Department of Medical Genetics, University of Helsinki, FIN-00014 Helsinki, Finland*
[f]*The University of Chicago, Departments of Human Genetics, Neurology and Pediatrics, 920 E. 58th Street, Chicago, IL 60637*
[g]*Department of Pediatrics and Pediatric Neurology, University of Essen, D-45122 Essen, Germany*
[h]*Department of Pediatric Pathology, Hacettepe Children's Hospital, Ankara 06100, Turkey*
[i]*Division of Functional Genomics, Department of Post-Genomics and Diseases, Osaka University Graduate School of Medicine, 2-2-B9 Yamadaoka, Suita, Osaka 565-0871, Japan*
[j]*Department of Pediatric Neurology, Hacettepe Children's Hospital, Ankara 06100, Turkey*

Objectives: Mutations disrupting the interaction of extra-cellular ligands and α-dystroglycan are responsible for an etiologically heterogeneous group of autosomal recessive congenital muscular dystrophies (CMD) that can have associated brain and eye abnormalities. The objective is to develop a diagnostic test for one of these CMDs, Muscle-Eye-Brain disease (MEB), due to mutations in the gene encoding Protein *O*-Mannosyl β-1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1).
Design and Methods: POMGnT1 enzyme activity was determined in extracts of muscle biopsies from four MEB patients and various controls using commercially available reagents.
Results: All four MEB muscle samples showed a highly significant decrease in POMGnT1 activity relative to controls.
Conclusions: The assay of POMGnT1 activity in MEB muscle provides a rapid and relatively simple diagnostic test for this disease. CMDs associated with brain malformations such as MEB, WWS and FCMD are heterogenous in clinical presentation and on radiologic examination, suggesting that POMGnT1 assays of muscle biopsies should be used as a screening procedure for MEB in all CMD patients associated with brain malformations.

Keywords: Diagnosis; Neuromuscular; Dystrophy; Muscle; Eye; Brain; Glycosyltransferase; *N*-acetylglucosaminyltransferase; Enzyme Assay

1. Introduction

Dystroglycan (dystrophin-associated glycoprotein) is a central component of a multimeric protein assembly called the dystrophin glycoprotein complex (DGC), which is present in skeletal muscle and is comprised of dystrophin and several other proteins [1,2]. Defects in the DGC appear to play critical roles in several muscular dystrophies due to disruption of basement membrane organization [2,3]. Dystroglycan is expressed in many cell types and composed of α- and β-subunits encoded by a single mRNA [4]. The protein is synthesized as a precursor propeptide that is posttranslationally cleaved and differentially glycosylated to yield α- and β-dystroglycans. α-Dystroglycan is an extracellular protein which binds both β-dystroglycan, a transmembrane protein, and various extra-cellular ligands such as the laminin α2 chain of merosin [5–8]. Dystrophin binds both β-dystroglycan and the intra-cellular contractile protein F-actin. Dystroglycan therefore acts as a link between the extra-cellular matrix and intra-cellular actin.

Mutations that disrupt the interaction between dystrophin and actin lead to a severe Becker muscular dystrophy phe-

* Corresponding author. Tel.: +1-416-813-5915; fax: +1-416-813-5022.
E-mail address: harry@sickkids.ca (H. Schachter).

doi:10.1016/S0009-9120(03)00036-5

Nomenclature

CMD congenital muscular dystrophy
DGC dystrophin glycoprotein complex
FCMD Fukuyama congenital muscular dystrophy
MDC1C Congenital Muscular Dystrophy 1C
MEB Muscle-Eye-Brain disease
OMIM Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/)
POMGnT1 Protein *O*-Mannosyl β-1,2-*N*-acetylglucosaminyltransferase 1
POMT1 Protein *O*-Mannosyltransferase 1
WWS Walker-Warburg syndrome

notype whereas disruption of the β-dystroglycan-dystrophin interaction leads to a Duchenne muscular dystrophy phenotype [1,9]. Mutations disrupting the interaction of extracellular ligands and α-dystroglycan are responsible for an etiologically heterogeneous group of autosomal recessive congenital muscular dystrophies (CMD) that can have associated brain and eye abnormalities [10–14]. The diagnostic criteria for CMD include onset of muscle weakness at birth or first few months of life and dystrophic muscle changes [9–11,15,16]. In an epidemiologic study involving 2,586,830 inhabitants of north-east Italy, the recorded incidence rate of CMD for the period 1979 to 1993 was 4.65 in 100,000 [17]. The data indicate that this myopathy is among the most frequent neuromuscular diseases with autosomal recessive transmission.

Several CMD types are now known to be associated with abnormal glycosylation of α-dystroglycan. Protein *O*-mannosylation has been described in mammalian brain, in a ratio of 1:3 relative to the more common protein-bound GalNAc-1-*O*-Ser/Thr *O*-glycans [18]. However, only a limited number of glycoproteins of brain, peripheral nerve and skeletal muscle are known to be *O*-mannosylated [19]. Fig. 1 shows the major sialylated *O*-glycosidically linked oligosaccharide in α-dystroglycan from bovine peripheral nerve [19,20] and rabbit skeletal muscle [21]. The sialylα2,3Galβ1,4GlcNAc moiety of this sugar chain is required for interaction of α-dystroglycan with laminin [6,19,20]. *O*-mannosyl glycans are present in other mammalian glycoproteins and several different structures have been described (see [19] for references).

UDP-GlcNAc:α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase 1 (GnT1, EC 2.4.1.101) [22,23] is the entry point for the conversion of oligomannose to hybrid and complex *N*-glycans. A TBLASTN screen of the Expressed Sequence Tag (EST) database for genes with significant sequence similarities to GnT1 showed the presence of human (Hs.183860) and mouse (Mm.2069) Unigenes encoding a protein similar to human GnT1 [24]. The human

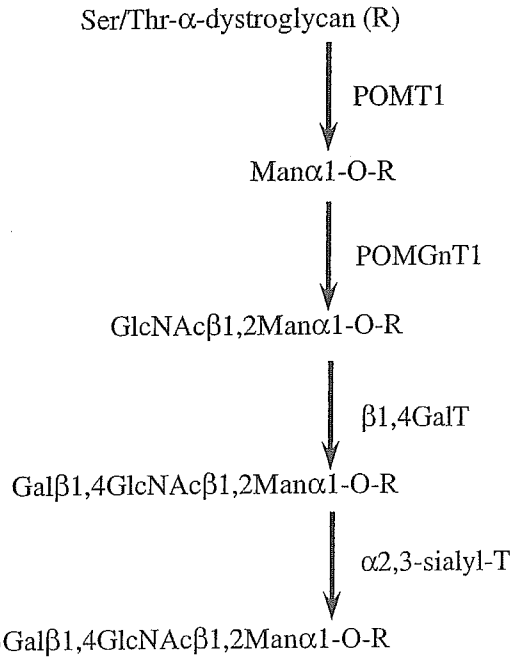


Fig. 1. The synthesis of the *O*-glycosidically linked tetrasaccharide of α-dystroglycan from bovine peripheral nerve and rabbit skeletal muscle. The first enzyme to act on the protein is POMT1 (Protein *O*-Mannosyltransferase 1) which adds a mannose residue (Man) to Ser and Thr residues of the protein. The gene encoding POMT1 is mutated in some cases of WWS. The next enzyme in the pathway is POMGnT1 (Protein *O*-Mannosyl β-1,2-*N*-acetylglucosaminyltransferase 1) which adds an *N*-acetylglucosamine residue (GlcNAc) in β1,2 linkage to the Man. The gene encoding POMGnT1 is defective in MEB disease. The next two enzymes, β1,4-galactosyltransferase (β1,4GalT) and α2,3-sialyltransferase (α2,3-sialyl-T), respectively add galactose (Gal) and sialic acid (sialyl) residues as indicated. No human disease has as yet been associated with these enzymes.

gene was cloned and expressed, and the recombinant protein was shown to incorporate GlcNAc from UDP-GlcNAc into several Manα1-O-R acceptors. The product formed with Manα1-O-benzyl was shown to be GlcNAcβ1,2 Manα1-O-benzyl and the enzyme was therefore named UDP-GlcNAc: α-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase 1.2 (GnT1.2; gene *MGAT1.2*) [24]. GnT1.2 was appreciably more active with the *O*-mannosyl glycopeptide CYA{Manα1-O-T}AV (Km = ~ 12 mM) than with Manα1-O-benzyl (Km > 30 mM). Yoshida *et al.* [25] cloned and expressed the same human gene and showed that the recombinant enzyme incorporated GlcNAc into the *O*-mannosyl glycopeptide *N*-acetyl-AAP(Man-T)PVAAP-NH_2. However, they did not detect any enzyme activity with Manα1-O-R acceptors in which R was not a peptide, at least under their assay conditions. They named the enzyme Protein *O*-Mannosyl β-1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) (Fig. 1).

Muscle-Eye-Brain disease (MEB, OMIM 253280; OMIM, Online Mendelian Inheritance in Man) was first described in 1978 [26]. MEB is an autosomal recessive disorder characterized by congenital muscular dystrophy, ocular abnormalities and cobblestone type brain malforma-

tion (previously called type II lissencephaly) of intermediate severity [14,27]. The MEB gene was mapped to 1p32-p34 by linkage analysis and homozygosity mapping [28]. Thirteen independent disease-causing mutations of the POMGnT1 gene have been reported in twelve patients with MEB [25,29]. Taniguchi *et al.* [29] recently reported a slight correlation between the location of the mutation on the POMGnT1 gene and clinical severity in the brain. Patients with mutations near the 5′ terminus of the POMGnT1 coding region show relatively severe brain symptoms such as hydrocephalus, while patients with mutations near the 3′ terminus have milder phenotypes. A selective deficiency [30] and hypoglycosylation [31] of α-dystroglycan has been reported in MEB patients suggesting that α-dystroglycan is a potential target of POMGnT1 and that altered glycosylation of α-dystroglycan may play a critical role in the etiology of MEB and other forms of CMD.

Yoshida *et al.* [25] established that their MEB patients had abnormalities in the POMGnT1 gene by DNA analysis. We now report an appreciably more rapid and inexpensive diagnostic test for MEB based on POMGnT1 enzyme assays using commercially available reagents and ~20 to 30 mg wet weight of muscle biopsy tissue.

2. Materials and methods

2.1. Materials

UDP-{^{3}H}GlcNAc (New England Nuclear) was diluted with nonradioactive UDP-GlcNAc (Sigma) to a specific activity of 34 dpm/pmole (GnT1 assays) or 600 dpm/pmole (POMGnT1 assays). Manα1,6(Manα1,3)Manβ1-O-octyl and Manα1-O-benzyl were purchased from Toronto Research Chemicals, Toronto, Canada. Sep-Pak C18 reverse phase cartridges were obtained from Waters.

2.2. Muscle biopsies

We obtained frozen muscle biopsies from four MEB patients who presented either at birth or after the first few months of life with developmental delay, mental retardation and muscle weakness. All patients showed elevated serum creatine kinase activity and brain malformations (neuronal migrational abnormalities, dilated ventricles, hypoplastic corpus callosum, brain hypomyelination, hypoplastic pons and cerebellum) and a variable degree of eye abnormalities (microphthalmia, buphthalmos, anterior chamber defects, optic nerve atrophy, retinal dysplasia and myopia). Skeletal muscle biopsies from all patients showed evidence of dystrophic changes. Immunohistochemical staining of the tissues with a mouse monoclonal anti-α-dystroglycan antibody prepared with a rabbit skeletal muscle membrane preparation as antigen [32] (clone VIA4-1, Upstate Biotechnology) showed deficient α-dystroglycan.

Patient D [25,30] is of Turkish origin with a homozygous G1743A transversion in exon 19 (Ser550Asn). Patient A [25,30] is a compound heterozygote of French origin with a C1572G transversion in exon 17 (Pro493Arg) in one allele and a single base pair deletion 1970delG in exon 21 in the other allele (frameshift at Val626 and a premature termination codon at codon 633). Patient B, a 7-yr-old boy (unpublished), is a compound heterozygote with an IVS17 + 1G>A mutation in one allele which causes a splicing error, read-through of intronic sequences resulting in the introduction of a premature termination codon, and skipping of upstream exon 17 resulting in a deletion of 42 amino acids (Leu472 to His513); the mutation in the other allele has not as yet been determined. Patient C, a 2-yr-old girl (unpublished), is a compound heterozygote with a G1908A 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). Age-matched control muscle biopsy samples were obtained from the Tissue Bank at the Hospital for Sick Children. We also obtained a frozen control muscle biopsy sample that had been shipped together with the sample from MEB patient A. Muscle biopsies from a 5-yr old boy with Becker and a 3-yr old boy with Duchenne muscular dystrophy were also analyzed.

2.3. Assay of POMGnT1 enzyme activity

The frozen muscle biopsy sample (10–60 mg wet weight) was gently homogenized with about 20 strokes of a hand-held glass Potter-Elvehjem tissue homogenizer at 4°C in 0.1 to 0.2 mL homogenizing buffer (1.8% Triton X-100, 0.2 mol/L NaCl in phosphate-buffered saline) containing 1/4 tablet of protease inhibitor cocktail (Boehringer Mannheim). The homogenate was kept on ice for 1 h, centrifuged at 4°C for 10 min at 3000 rpm with a microcentrifuge, and the supernatant was used for enzyme assays. The assay incubations contained, in a total volume of 0.020 mL, 0.017 mL muscle biopsy extract, 1 mM UDP-{^{3}H}GlcNAc (34 or 600 dpm/pmole for GnT1 or POMGnT1 respectively), 10 mM $MnCl_2$, 0.2% bovine serum albumin, 75 mM MES buffer (pH 6.5 or 6.0 for GnT1 or POMGnT1 respectively), 5 mM AMP, 0.2 mol/L GlcNAc, 0.5% Triton X-100, and either 1.25 mM Manα1,6(Manα1,3)Manβ1-O-octyl (GnT1 [33]) or 62.5 mM Manα1-O-benzyl (POMGnT1 [24]). The activity of POMGnT1 with 1.25 mM Manα1,6(Manα1,3) Manβ1-O-octyl is negligible and GnT1 does not act on Manα1-O-benzyl [24]. Incubations were carried out at 37°C for 2 h. SepPak C18 cartridges were used to determine the amount of radioactive product as previously described [24,33,34]. Controls were routinely carried out in the absence of acceptor and the value obtained was subtracted. All assays were carried out in duplicate or triplicate (as indicated in Table 1) and results are reported as averages of these determinations.

Table 1
GnT1 and POMGnT1 assays on muscle biopsy extracts

Muscle Sample	GnT1 pmoles/h/mg (SD, n)	POMGnT1 pmoles/h/mg (SD, n)	Ratio of POMGnT1 to GnT1
Controls			
1	22 (5.0, 2)	7.4 (0.8, 2)	0.34
2	15 (0.8, 2)	10 (0.8, 2)	0.67
3	33 (0.5, 2)	12 (0.8, 3)	0.36
4	13 (0.2, 2)	11 (<0.1, 2)	0.85
5	20 (0.2, 2)	10 (1.1, 3)	0.50
6		8.0 (0.2, 2)	
Mean ± SD	20.6 ± 7.0	9.73 ± 1.60	
Becker	33 (0.6, 2)	9.1 (0.1, 3)	0.28
Duchenne	45 (1.9, 2)	8.2 (0.3, 3)	0.18
MEB patients			
A	41 (0.4, 2)	0.8 (0.4, 3)	0.02
B	42 (0.4, 2)	0.7 (0.3, 3)	0.02
C	29 (0.1, 2)	0.5 (0.3, 3)	0.02
Mean ± SD	37.3 ± 5.9	0.67 ± 0.12	
D	5 (0.1, 2)	<0.1 (<0.1, 2)	

Enzyme assays are averages of duplicate or triplicate determinations; SD = Standard Deviation; n = number of samples.

3. Results

Table 1 shows the results of GnT1 and POMGnT1 enzyme assays on MEB and control muscle samples. Since the amounts of MEB muscle tissue available to us were small, the protein contents were not determined. POMGnT1 activity was expressed in two ways, relative to wet weight of muscle and to GnT1 activity. We have shown that GnT1 activity can serve as an effective base-line in our previous work on Congenital Disorder of Glycosylation type IIa [35–37]. GnT1 also has the advantage over protein analysis in that it gives an indication of the quality of the muscle biopsy tissue for enzyme analysis.

The POMGnT1 activity in six samples of control muscle varied from 7.4 to 12 pmoles/h/mg (average 9.7 pmoles/h/mg). The POMGnT1 to GnT1 ratios in these samples varied from 0.34 to 0.85. The Becker and Duchenne muscle samples showed the same levels of POMGnT1 as the other controls but slightly higher levels of GnT1. MEB patients A and B also showed somewhat higher GnT1 values. Further work is required to determine whether these very small differences in GnT1 activity are statistically significant.

MEB patients A, B and C showed very similar POMGnT1 levels (0.5–0.8 pmoles/h/mg) and POMGnT1/GnT1 ratios (0.02). The differences in POMGnT1 enzyme activities between these three MEB patients and the controls range from 5 to 10 times the sums of the standard deviations and are therefore highly significant. We could not detect significant POMGnT1 activity in patient D's muscle. The GnT1 activity in the D extract was significantly lower (5 pmoles/h/mg) than the activities in all our other samples, both MEB and controls (13–45 pmoles/h/mg), suggesting that the quality of the D muscle sample was not as good as samples from the other three MEB patients. However, the D data indicate that a reliable diagnosis of MEB can be made even with partially degraded muscle samples. The data also illustrate the value of GnT1 assays as a measure of tissue quality.

The low but significant levels of POMGnT1 detected in MEB patients A, B and C (Table 1) are consistent with the suggestion by Michele *et al.* [31] based on immunologic studies "that a small amount of proper glycosylation occurs in the presence of mutated POMGnT1, due either to residual enzymatic activity or to a secondary, partially compensatory enzyme".

4. Discussion

MEB [31], Walker-Warburg syndrome (WWS, OMIM 236670) [38], Fukuyama congenital muscular dystrophy (FCMD; OMIM 253800) [31,39–41] and Congenital Muscular Dystrophy 1C (OMIM 606612; MDC1C) [42] are all CMDs with deficient α-dystroglycan in the basal lamina of skeletal muscles as demonstrated by immunohistochemical techniques using antibodies against α-dystroglycan such as VIA4-1. However, α-dystroglycan staining can be normal in muscle from MEB and FCMD patients using a polyclonal antibody which can detect hypoglycosylated full-length α-dystroglycan [31] suggesting that VIA4-1 is directed against the glycans on α-dystroglycan and that α-dystroglycan is underglycosylated in muscle from these patients. Although the exact functions of α-dystroglycan and the dystrophin glycoprotein complex (DGC) are not known, they appear to provide structural support to the sarcolemma and may play a role in signaling, cell adhesion and the regulation of the intracellular calcium concentration [15,43,44]. Several cell surface and transmembrane molecules (laminins, integrins and lectins) have been proposed as ligands for dystroglycan [1,43]. Disruption of the binding of α-dystroglycan to its ligands could lead to weakened anchorage of muscle fibers to the extracellular matrix with very early (*i.e.*, embryonic) and rapid muscle dysfunction and necrosis as a result.

MEB, WWS and FCMD are complex disorders associated with a wide spectrum of brain malformations described as the "cobblestone complex". The specific abnormalities consist of simplified or absent brain convolutions, inappropriate migration of neurons and glia into the subarachnoid space where they intermix with fibroblasts and blood vessels, cystic degeneration of white matter, hydrocephalus, and a small dysplastic brainstem and cerebellum [14,27,45]. The brain changes are least severe in FCMD, intermediate in MEB and most severe in WWS. All three syndromes also have abnormalties of the eyes that consist of microphthalmia, buphthalmos, anterior chamber defects, congenital cataracts, optic nerve atrophy, retinal dysplasia, myopia and coloboma. In contrast, MDC1C presents only with muscular dystrophy [14,46–48]. The combination of brain, eye and muscle abnormalities leads to differing degrees of motor

developmental delay, physical disability, muscle pathology, elevation of serum creatine kinase, mental retardation, and structural brain and eye defects. The most severe and often lethal phenotype is generally associated with WWS followed in decreasing severity by MEB, FCMD and MDC1C.

Mutations in the gene encoding the enzyme which attaches mannose in *O*-glycosidic linkage to the Ser/Thr residues of proteins like α-dystroglycan (Protein *O*-Mannosyltransferase 1; POMT1; Figure 1) were found in 6 of 30 unrelated WWS patients [38]. The POMT1 gene maps to 9q34. FCMD is associated with mutations in a protein called fukutin which maps to 9q31 [40,41,49]. The function of fukutin is unknown but it is predicted to be a secreted protein with an amino acid sequence suggestive of a glycosyltransferase [50,51]. The structure of a recently cloned gene encoding a protein highly homologous to fukutin, fukutin-related protein (FKRP), is also suggestive of a putative glycosyltransferase [42,47,52]. The FKRP gene has been mapped to 19q13.3 Mutations in the gene lead to either severe CMD (MDC1C) [42,52], or to a later onset and milder allelic form of CMD called limb-girdle muscular dystrophy 2I (LGMD2I; OMIM 607155) [47].

The enzymatic diagnostic test we have demonstrated in this report will allow a rapid and accurate diagnosis of MEB no matter where the mutation in the POMGnT1 gene is located. We have not as yet tested leukocytes or fibroblasts to determine whether there is sufficient POMGnT1 expression in these tissues to allow enzyme assays. We have not had access to parental muscle biopsy material. If it proves possible to use either leukocytes or fibroblasts for the POMGnT1 assay, it may be possible to carry out the assay on parental tissues and to determine heterozygosity. CMDs associated with brain malformations such as MEB, WWS and FCMD are heterogenous in clinical presentation and on radiologic examination, suggesting that the simple enzymatic test described in this paper should be used as a screening procedure for MEB in these cases. Since the mutations in WWS, FCMD and MDC1C are also in genes encoding either established or putative glycosyltransferases, it is probable that similar diagnostic tests will eventually be developed for these diseases and possibly for other CMDs.

Acknowledgments

This research was supported by grants from the Canadian Institutes for Health Research (CIHR) and the Canadian Protein Engineering Network Centers of Excellence (PENCE) to HS, and from the Deutsche Forschungsgemeinschaft (STR 392/2–2) and by the EU (QLG1-1999–00870) to TV.

References

[1] Winder SJ. The complexities of dystroglycan. Trends Biochem Sci 2001;26(2):118–24.

[2] Williamson RA, Henry MD, Daniels KJ, Hrstka RF, Lee JC, Sunada Y, Ibraghimov-Beskrovnaya O, Campbell KP. Dystroglycan is essential for early embryonic development: disruption of Reichert's membrane in Dag1-null mice. Hum Mol Genet 1997;6(6):831–41.

[3] Henry MD, Campbell KP. A role for dystroglycan in basement membrane assembly. Cell 1998;95(6):859–70.

[4] Holt KH, Crosbie RH, Venzke DP, Campbell KP. Biosynthesis of dystroglycan: processing of a precursor propeptide. FEBS Lett 2000; 468(1):79–83.

[5] Ibraghimov-Beskrovnaya O, Ervasti JM, Leveille CJ, Slaughter CA, Sernett SW, Campbell KP. Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature 1992;355(6362):696–702.

[6] Yamada H, Chiba A, Endo T, Kobata A, Anderson LV, Hori H, Fukuta-Ohi H, Kanazawa I, Campbell KP, Shimizu T, Matsumura K. Characterization of dystroglycan-laminin interaction in peripheral nerve. J Neurochem 1996;66(4):1518–24.

[7] Matsumura K, Chiba A, Yamada H, Fukuta-Ohi H, Fujita S, Endo T, Kobata A, Anderson LV, Kanazawa I, Campbell KP, Shimizu T. A role of dystroglycan in schwannoma cell adhesion to laminin. J Biol Chem 1997;272(21):13904–10.

[8] Montanaro F, Lindenbaum M, Carbonetto S. alpha-Dystroglycan is a laminin receptor involved in extracellular matrix assembly on myotubes and muscle cell viability. J Cell Biol 1999;145(6):1325–40.

[9] Culligan KG, Mackey AJ, Finn DM, Maguire PB, Ohlendieck K. Role of dystrophin isoforms and associated proteins in muscular dystrophy (review). Int J Mol Med 1998;2(6):639–48.

[10] Dubowitz V, Fardeau M. Proceedings of the 27th ENMC sponsored workshop on congenital muscular dystrophy. 22-24 April 1994, The Netherlands. Neuromuscul Disord 1995;5(3):253–8.

[11] Dubowitz V. 68th ENMC international workshop (5th international workshop): On congenital muscular dystrophy, 9-11 April 1999, Naarden, The Netherlands. Neuromuscul Disord 1999;9(6-7):446–54.

[12] Santavuori P, Somer H, Sainio K, Rapola J, Kruus S, Nikitin T, Ketonen L, Leisti J. Muscle-eye-brain disease (MEB). Brain Dev 1989;11(3):147–53.

[13] Vajsar J, Chitayat D, Becker LE, Ho M, Ben-Zeev B, Jay V. Severe classical congenital muscular dystrophy and merosin expression. Clin Genet 1998;54(3):193–8.

[14] Cormand B, Pihko H, Bayes M, Valanne L, Santavuori P, Talim B, Gershoni-Baruch R, Ahmad A, van Bokhoven H, Brunner HG, Voit T, Topaloglu H, Dobyns WB, Lehesjoki AE. Clinical and genetic distinction between Walker-Warburg syndrome and muscle-eye-brain disease. Neurology 2001;56(8):1059–69.

[15] Campbell KP. Three muscular dystrophies: loss of cytoskeleton-extracellular matrix linkage. Cell 1995;80(5):675–9.

[16] Muntoni F, Brockington M, Blake DJ, Torelli S, Brown SC. Defective glycosylation in muscular dystrophy. Lancet 2002;360(9343): 1419–21.

[17] Mostacciuolo ML, Miorin M, Martinello F, Angelini C, Perini P, Trevisan CP. Genetic epidemiology of congenital muscular dystrophy in a sample from north-east Italy. Hum Genet 1996;97(3):277–9.

[18] Chai W, Yuen CT, Kogelberg H, Carruthers RA, Margolis RU, Feizi T, Lawson AM. High prevalence of 2-mono- and 2,6-di-substituted manol-terminating sequences among O-glycans released from brain glycopeptides by reductive alkaline hydrolysis. European Journal Of Biochemistry 1999;263(3):879–88.

[19] Endo T. O-mannosyl glycans in mammals. Biochim Biophys Acta 1999;1473(1):237–46.

[20] Chiba A, Matsumura K, Yamada H, Inazu T, Shimizu T, Kusunoki S, Kanazawa I, Kobata A, Endo T. Structures of sialylated O-linked oligosaccharides of bovine peripheral nerve alpha-dystroglycan The role of a novel O-mannosyl-type oligosaccharide in the binding of alpha-dystroglycan with laminin. J Biol Chem 1997;272(4):2156–62.

[21] Sasaki T, Yamada H, Matsumura K, Shimizu T, Kobata A, Endo T. Detection of O-mannosyl glycans in rabbit skeletal muscle alpha-dystroglycan. Biochim Biophys Acta 1998;1425(3):599–606.