

control the stimulation and to record the force of the muscle contraction. Measurement of the specific tetanic force was performed as previously described.³⁹

Gene expression array analysis. TA muscles from treated *mdx52*, age-matched WT, and untreated *mdx52* mice ($n = 3$ each) were used for these experiments. Total RNA was purified using an RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Gene expression array analysis was performed by the branch of the Agilent Technologies (Santa Clara, CA) in Japan. Three whole mouse genome oligo microarrays 44K (Agilent Technologies) were used in this study. Global normalization was performed to compare genes from chip to chip using GeneSpring 9.0 (Tomy Digital Biology, Denver, CO). All data quality controls were performed and met the Affymetrix quality assessment guidelines. Data analysis was performed using GeneSpring 9.0 (Tomy Digital Biology). Differentially expressed genes were selected if they passed Welch's *t*-test, a parametric test in which the variance is not assumed to be equal. $P < 0.01$ (with correction for multiple testing by the Benjamini and Hochberg method for the false discovery rate) and a 5% cutoff were used; a change of at least twofold between any two of the groups used in this study was considered significant.

Quantitative real-time PCR. For genes selected for the gene expression array, we used the same RNA that was isolated for the gene expression array and prepared cDNA using SuperScript III Reverse Transcriptase (Invitrogen). Real-time PCR was performed using a SYBR Premix Ex Taq II kit (Takara, Tokyo, Japan). Expression values were normalized to 18S rRNA expression and shown as a fold increase in the treated *mdx52*, untreated *mdx52*, and age-matched WT samples.

Transfection of cultured cells with AO. DMD 5017 cells were obtained from Coriell Cell Repositories (Camden, NJ). Fibroblasts were cultured in 20% growth medium, containing Dulbecco's modified Eagle's medium and F-12 in a 1:1 mixture (Invitrogen), 20% fetal bovine serum (SACF Biosciences, Lenexa, KS) and 1% penicillin/streptomycin (Sigma-Aldrich, St Louis, MO). Then, fluorescence-activated cell sorting sorted MyoD-enhanced green fluorescent protein-positive fibroblasts (MyoD-converted fibroblasts) were cultured in differentiation medium, containing Dulbecco's modified Eagle's medium/F-12 in a 1:1 mixture (Invitrogen), 2% horse serum (Invitrogen), and 1% penicillin/streptomycin (Sigma-Aldrich). hDo1, hDo2, and hAc were designed (Table 1) and synthesized by Gene Tools. MyoD-converted fibroblasts were transfected with a single or two AOs at a final concentration of 10 $\mu\text{mol/l}$. EndoPorter (Gene Tools) was added to give a final concentration of 6 $\mu\text{mol/l}$. After 48-hour incubation with the AOs, total RNA was extracted from MyoD-converted fibroblasts using Trizol (Invitrogen).

Statistical analysis. Statistical differences were assessed by one-way analysis of variance with differences among the groups assessed by a Tukey comparison. All data are reported as mean values \pm SEM. The level of significance was set at $P < 0.05$.

SUPPLEMENTARY MATERIAL

Figure S1. Dose-escalation study of exon 51-skipping by local intramuscular injection into *mdx52* mice.

Figure S2. Examination of adverse effects after systemic delivery of antisense oligonucleotides (AOs).

Figure S3. *In vitro* exon 51-skipping in DMD 5017 cells with deletion of exons 45–50.

Table S1. Length, annealing coordinates, sequences of all AOs targeting human exon 51.

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Post-translational Maturation of Dystroglycan Is Necessary for Pikachurin Binding and Ribbon Synaptic Localization^{*[5]}

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Pikachurin, the most recently identified ligand of dystroglycan, plays a crucial role in the formation of the photoreceptor ribbon synapse. It is known that glycosylation of dystroglycan is necessary for its ligand binding activity, and hypoglycosylation is associated with a group of muscular dystrophies that often involve eye abnormalities. Because little is known about the interaction between pikachurin and dystroglycan and its impact on molecular pathogenesis, here we characterize the interaction using deletion constructs and mouse models of muscular dystrophies with glycosylation defects (*Large*^{myd} and *POMGnT1*-deficient mice). Pikachurin-dystroglycan binding is calcium-dependent and relatively less sensitive to inhibition by heparin and high NaCl concentration, as compared with other dystroglycan ligand proteins. Using deletion constructs of the laminin globular domains in the pikachurin C terminus, we show that a certain steric structure formed by the second and the third laminin globular domains is necessary for the pikachurin-dystroglycan interaction. Binding assays using dystroglycan deletion constructs and tissue samples from *Large*-deficient (*Large*^{myd}) mice show that *Large*-dependent modification of dystroglycan is necessary for pikachurin binding. In addition, the ability of pikachurin to bind to dystroglycan prepared from *POMGnT1*-deficient mice is severely reduced, suggesting that modification of the GlcNAc- β 1,2-branch on *O*-mannose is also necessary for the interaction. Immunofluorescence analysis reveals a disruption of pikachurin localization in the photoreceptor ribbon synapse of these model animals. Together, our data demonstrate that post-translational modification on *O*-mannose, which is mediated by *Large* and *POMGnT1*, is essential for pikachurin binding and proper localization, and suggest that their disruption underlies the molecular pathogenesis of eye abnormalities in a group of muscular dystrophies.

Dystroglycan (DG)², a cell surface receptor for several extracellular matrix proteins, plays important roles in various tissues (1–7). DG consists of an extracellular, heavily glycosylated α subunit (α -DG) and a transmembrane β subunit (β -DG). α -DG and β -DG are encoded by a single gene and post-translationally cleaved to generate the two subunits. α -DG is a receptor for extracellular proteins such as laminin-111, laminin-211, agrin, perlecan, and neurexin. β -DG binds to α -DG in the extracellular space, anchoring α -DG at the cell surface. Inside the cell, β -DG binds to dystrophin, which in turn is linked to the actin cytoskeleton. Thus, α/β -DG functions as a molecular axis, connecting the extracellular matrix with the cytoskeleton across the plasma membrane.

DG ligand proteins commonly contain laminin globular (LG) domains, which mediate binding to α -DG. *O*-Mannosylation of α -DG is required for its interaction with ligands; however, the precise ligand-binding sites and epitope are not known. A unique *O*-mannosyl tetrasaccharide (Neu5Ac- α 2,3-Gal- β 1,4-GlcNAc- β 1,2-Man) was first identified on peripheral nerve α -DG (8). The initial Man transfer to Ser/Thr residues on the α -DG peptide backbone is catalyzed by the POMT1-POMT2 complex (9). Both *POMT1* and *POMT2* were originally identified as responsible genes in Walker-Warburg syndrome (10, 11). *POMGnT1*, a causative gene for muscle-eye-brain disease, encodes a glycosyltransferase that transfers GlcNAc to *O*-Man on α -DG (12). Because mutations in these enzymes cause abnormal glycosylation of α -DG and reduce its ligand binding activity, it is recognized that the GlcNAc- β 1,2-branch on *O*-Man is essential to α -DG function as a matrix receptor.

Additional proteins, including fukutin, FKR, and *LARGE*, are also involved in synthesizing the glycans on α -DG that are required for ligand binding activity. Recently, a GalNAc- β 1,3-GlcNAc- β 1,4-branch and a phosphodiester-linked modification on *O*-Man were identified (13). α -DG from cells with mutations in *fukutin* or *Large* shows defective post-phosphoryl modification on *O*-Man, suggesting that this phosphoryl branch serves a laminin-binding moiety. *fukutin* was originally identified as the responsible gene for Fukuyama-type congenital muscular dystrophy (14), and the *fukutin* homologue *FKR* was identified through sequence homology (15). Mutation of

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² The abbreviations used are: DG, dystroglycan; ERG, electroretinogram; LG, laminin globular.

LARGE, a putative glycosyltransferase, generates spontaneous muscular dystrophy in the *Large*^{myd} mouse model (16). A unique feature of LARGE is that its overexpression produces a hyperglycosylated α -DG that shows increased laminin binding activity, even in cells with genetic defects in the α -DG glycosylation pathway (17).

Mutations in each of these genes (*POMT1*, *POMT2*, *POMGnT1*, *fukutin*, *FKRP*, and *LARGE*) have been identified in congenital and limb-girdle forms of muscular dystrophy (18). A common characteristic of patients who have such mutations is abnormal glycosylation of α -DG; thus, these conditions are collectively referred to as dystroglycanopathies. The clinical spectrum of dystroglycanopathy is broad, ranging from severe congenital onset associated with structural brain malformations to a milder congenital variant with no brain involvement and to limb-girdle muscular dystrophy type 2 variants with childhood or adult onset (18, 19). Eye abnormalities are often associated with more severe dystroglycanopathy, such as Walker-Warburg syndrome and muscle-eye-brain disease (20). The ophthalmologic phenotype of muscular dystrophy is also known for Duchenne/Becker muscular dystrophy, which is caused by dystrophin mutations. Most patients with Duchenne/Becker muscular dystrophy have evidence of abnormal electroretinograms (ERG) (21).

Pikachurin, the most recently identified DG ligand protein, is localized in the synaptic cleft in the photoreceptor ribbon synapse (22). Like other DG ligands, pikachurin contains LG domains in its C-terminal region. Pikachurin-null mutant mice show improper apposition of the bipolar cell dendritic tips to the photoreceptor ribbon synapses, resulting in altered synaptic signal transmission and visual function. Similar retinal electrophysiological abnormalities, such as attenuated or delayed b-wave, have been observed in *Large*^{myd} (23) and *POMGnT1*-deficient mice (24). These studies imply a functional relationship between pikachurin and DG glycosylation in the retinal ribbon synapse.

In this study, we have biochemically characterized the interaction between pikachurin and α -DG. We have found that both GlcNAc- β 1,2-branch and LARGE-dependent modification on O-Man are necessary for the pikachurin-DG interaction. Furthermore, in dystroglycanopathy model animals, pikachurin localization in the retinal synaptic outer plexiform layer is severely disrupted. These data demonstrate that post-translational maturation of DG is essential for pikachurin binding and proper localization, providing a possible molecular explanation for the retinal electrophysiological abnormalities observed in dystroglycanopathy patients.

EXPERIMENTAL PROCEDURES

Vector Construction and Protein Expression—Construction of recombinant mouse pikachurin LG domains (PikaLG; residues 391–1017) has been described previously (22). Single or tandem LG domains were constructed using PCR, with a full-length PikaLG expression vector as the template cDNA. Primers used were as follows: LG1(391–627), PikaKpn (CTTGGTACCGAGCTCGGATC) and E627r (TTCTCGAGCCTCCAGGGGCCAGG-GTGTG); LG2(542–838), G542f (TTGGTACCGAGCTCGGA-TCTGGGAAGAAGATTGACATGAG) and P838r (TTCTCG-

AGCTGGGATCTCGATGGCTTCTA); LG3(799–1017), D766f (TTGGTACCGAGCTCGGATCTGACCGGACCAT-CCATGTGAAG) and PikaR (GCAACTAGAAGGCACAG-TCCG); LG1-2(391–883), PikaKpn and P838r; and LG2-3(542–1017), G542f and PikaR. PCR products were digested with KpnI/XhoI and inserted into the KpnI/XhoI sites of the pSecTag2 vector (Invitrogen).

Recombinant mouse α -DG fused to an Fc tag (DGFc) also has been described previously (22). Deletion mutants were constructed using PCR, with the wild-type DGFc vector as the template cDNA. Primers used were as follows: DG-N(1-313), pCAGf (AAGAATTCGCCGCCACCATGAGG) and DGFc313r (AATCTAGATTTGGGGAGAGTGGGCTTCTT); DG Δ N(1–28 plus 315–651), pCAGf and DGFc28r (GGCCT-GAGCCACAGCCACAGACAGGAGGAG); and DGFc315f (ACACCTACACCTGTTACTGCC) and Fcexon2r (TCCC-CCCAGGAGTTCAGGTGC); DG Δ C(1–483), pCAGf and DGFc483r (AATCTAGAAGGAATTGTCAGTGTGG-GCG); DG^{half}(1–407), pCAGf and DGFc407r (AATCTAGAA-CTGGTGGTAGTACGGATTCG). PCR products were digested with EcoRI/XbaI and inserted into the EcoRI/XbaI sites of the wild-type DGFc expression vector.

PikaLG and DGFc constructs were expressed in HEK293 cells (22). For preparation of PikaLG-containing cell lysates, transfected cells were solubilized in lysis buffer (1% Nonidet P-40, 10% glycerol, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and a proteinase inhibitor mixture). The samples were centrifuged at 15,000 rpm for 10 min at 4 °C, and the supernatants were used for binding assays.

DGFc proteins were secreted into the cell culture media and recovered with protein A or protein G beads. For the solid-phase binding assays, DGFc proteins were eluted with 0.1 M glycine HCl, pH 2.5, and then neutralized to a final concentration of 0.2 M Tris-HCl, pH 8.0. Protein concentrations of the cell lysates and the DGFc proteins were determined using Lowry's method (Bio-Rad) with BSA as a standard.

Antibodies—Antibodies used for Western blots and immunofluorescence were as follows: mouse monoclonal antibody 8D5 against β -DG (Novacastra); rabbit polyclonal antibody against β -DG (Santa Cruz Biotechnology); mouse monoclonal antibody IIH6 against α -DG (Upstate); goat polyclonal antibody against the α -DG C-terminal domain (AP-074G-C) (25); and rabbit polyclonal antibodies against pikachurin (22).

Animals—C57BL/6 mice were obtained from Japan SLC, Inc., and *Large*^{myd} mice were obtained from The Jackson Laboratory. Generation of *POMGnT1*-deficient mice has been described previously (26). Mice were maintained in accordance with the animal care guidelines of Osaka University and Kobe University.

Pikachurin Binding Assay—For the DGFc pull-down assay, secreted DGFc proteins were recovered from conditioned media using protein A beads (10 μ l). DGFc-protein A bead complexes were washed with TBS (50 mM Tris-HCl, pH 7.4, and 150 mM NaCl) and incubated with cell lysates containing PikaLG proteins in the presence of 2 mM CaCl₂ overnight at 4 °C. After five washes with washing buffer (0.1% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM CaCl₂), bound materials were eluted with SDS-sample buffer. Bound materials

Pikachurin-Dystroglycan Interaction

were analyzed by Western blotting using anti-His or anti-Myc tag antibodies and the anti-Fc antibody.

For the solid-phase binding assays, DGFc preparations (2.5 μg) were coated on ELISA microplates (Costar) for 16 h at 4 °C. Plates were washed in TBS and blocked for 2 h with 3% BSA in TBS. PikaLG-containing cell lysates (8 μg) in binding buffer (3% BSA, 1% Nonidet P-40, 2 mM CaCl_2 in TBS) were applied and incubated overnight at 4 °C. Wells were washed with TBS containing 1% BSA, 0.1% Nonidet P-40, and 1 mM CaCl_2 for three times and incubated for 30 min with 1:1,000 anti-Myc (Santa Cruz Biotechnology) followed by anti-rabbit HRP. Plates were developed with *o*-phenylenediamine dihydrochloride and H_2O_2 . Reactions were stopped with 2 N H_2SO_4 , and values were obtained using a microplate reader. BSA-coated wells were used to subtract nonspecific binding. For Ca^{2+} concentration dependence, the data were fit to the equation $A = B_{\text{max}} x / (K_d + x)$, where K_d is the concentration required to reach half-maximal binding; A is absorbance, and B_{max} is maximal binding. All data were obtained as the means of triplicate measurements. Each experiment was repeated more than three times, and data are represented as the average of at least three independent experiments with standard deviations. Statistical analysis was performed with a two-tailed paired *t* test (GraphPad prism). A *p* value of <0.05 was considered to be significant.

For binding assays with PikaLG deletion proteins, we confirmed protein expression via Western blot analysis. Cell lysates containing comparable amounts of each LG deletion protein were adjusted by adding mock-transfected cell lysate to achieve a normalized total protein concentration across reaction mixtures.

DG Enrichment and Immunoprecipitation—For solid-phase binding assays with brain DG, mouse brain tissue (200 mg) was homogenized in 1.8 ml of TBS with a proteinase inhibitor mixture and then solubilized with 1% Triton X-100. Samples were centrifuged at 15,000 rpm for 10 min, and the supernatants were incubated with 50 μl of wheat germ agglutinin-agarose beads (Vector Laboratories) overnight at 4 °C. The beads were washed three times in 1 ml of TBS containing 0.1% Triton X-100 and then eluted with 250 μl of TBS containing 0.1% Triton X-100 and 200 mM *N*-acetylglucosamine. The presence of comparable amounts of DG protein in each elution was confirmed via Western blot analysis with DG antibodies, as described above.

To immunoprecipitate α -DG from mouse tissues, mouse brains or eyes were homogenized in TBS with a proteinase inhibitor mixture and then solubilized with 1% Triton X-100. Samples were centrifuged at 15,000 rpm for 10 min, and then the supernatants were incubated with or without anti- α -DG core protein (25). α -DG was immunoprecipitated using protein G beads. The α -DG-protein G beads were washed with TBS containing 0.1% Triton X-100 three times and then tested for binding with PikaLG, as described above.

Heparin Affinity Beads—PikaLG-containing cell lysates were incubated with the heparin beads (Sigma) overnight at 4 °C. After three washes with TBS containing 0.1% Nonidet P-40, bound materials were eluted with SDS-sample buffer.

Immunofluorescent Staining—Mouse eye cups were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 30

min. Samples were cryoprotected, embedded, frozen, and sectioned 20 μm thick. Slides were incubated with blocking solution (5% normal goat serum and 0.5% Triton X-100 in PBS) for 1 h. Sections were incubated with primary antibodies at room temperature for 4 h, washed with PBS for 10 min, and incubated with secondary antibodies for 2 h. The sections were coverslipped with Gelvatol after rinsing with 0.02% Triton X-100 in PBS.

Quantitative Real Time PCR Analysis—Total RNA (1 μg) from the mouse retina was isolated using TRIzol reagent (Invitrogen) and converted to cDNA using Superscript II RTase (Invitrogen). Quantitative real time PCR was performed using SYBR Green ER qPCR Super MIX (Invitrogen) and the Thermal Cycler Dice Real Time System single MRQ TP870 (Takara) according to the manufacturer's instructions. Quantification was performed using Thermal Cycler Dice Real Time System software version 2.0 (Takara). Primers used in gene amplification were as follows: amplification of the pikachurin gene, Pikachurin-F, GGAAGATTACAGTGGATGACTACG, and Pikachurin-R, GTGTGCAGAGCGATTTCCCTTCATTC; amplification of β -actin gene, actin-F, CGTGCGTGACATCAAAGAGAA, and actin-R, TGGATGCCACAGGATTCCAT.

RESULTS

Properties of the Pikachurin-Dystroglycan Interaction—To analyze binding between pikachurin and α -DG, we used recombinant pikachurin LG domains with a myc-His tandem tag (PikaLG) and α -DG fused to an Fc tag (DGFc) (Fig. 1A) (22). Previous data suggest that the pikachurin-DG interaction requires divalent cations (22). To further characterize this requirement, we analyzed PikaLG-DGFc binding in the presence of Ca^{2+} , Mg^{2+} , or Mn^{2+} using a pulldown assay (Fig. 1B). Ca^{2+} produced the strongest binding, whereas Mn^{2+} gave only faint binding, and no binding was observed with Mg^{2+} alone. To evaluate PikaLG-DGFc binding quantitatively, we developed a solid-phase binding assay. DGFc was immobilized on microplates, and cell lysates containing PikaLG were applied for binding. Signals representing binding of PikaLG to DGFc were detected, whereas no detectable signal was obtained from mock-transfected cell lysates (supplemental Fig. 1). Immobilized Fc protein showed no difference in signal intensity between PikaLG-containing and mock-transfected cell lysates, confirming the lack of specific interactions through the Fc portion (supplemental Fig. 1). We concluded that the solid-phase binding assay is sufficient to quantitatively detect PikaLG-DGFc interactions. The solid-phase binding assays showed results comparable with the pulldown assays. We observed a reduction in binding of around 70% in the presence of Mn^{2+} and a strong reduction in the presence of Mg^{2+} , similar to that seen in the presence of EDTA for chelation of divalent cations (Fig. 1C). The solid-phase binding assays in various Ca^{2+} concentrations established that the concentration required for half-maximal binding is $\sim 80 \mu\text{M}$ (Fig. 1D). It has been reported that heparin or a high NaCl concentration (0.5 M) inhibits binding of laminin-111 and agrin to α -DG (27, 28). We examined the effects of heparin and NaCl on PikaLG-DGFc binding. The results showed that PikaLG-DGFc binding was inhibited slightly at 0.5 M NaCl ($\sim 80\%$ binding), and the inhibitory effect

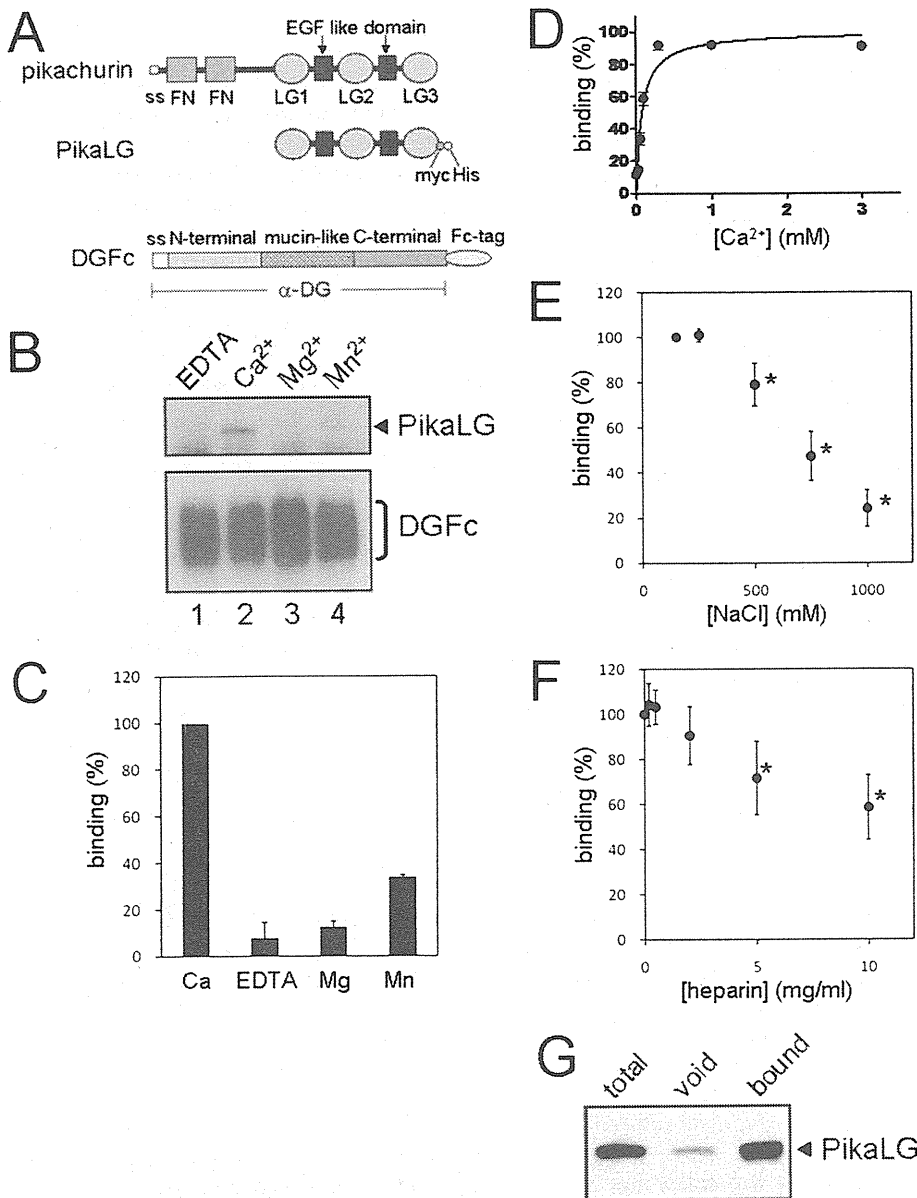


FIGURE 1. Biochemical characterization of pikachurin-dystroglycan interaction. *A*, schematic representation of recombinant pikachurin and α -DG. Pikachurin contains a signal sequence (ss), two fibronectin 3 (FN) domains, three laminin globular (LG) domains, and two calcium-binding EGF-like (EGF like) domains. Recombinant pikachurin LG domains (PikaLG) contain amino acid residues 391–1071 and a tandem myc-His tag at the C terminus. α -DG contains the signal sequence (ss), N-terminal, mucin-like, and C-terminal domains. Recombinant α -DG (DGFc) has an Fc tag at the C terminus. *B*, divalent cation is necessary for pikachurin-dystroglycan interaction. PikaLG binding to DGFc-protein A beads was tested in the presence of 2 mM EDTA (lane 1) and 2 mM each of Ca^{2+} (lane 2), Mg^{2+} (lane 3), or Mn^{2+} (lane 4). Bound PikaLG was detected by Western blotting with an anti-His tag antibody (upper panel, indicated by PikaLG). Comparable amounts of DGFc proteins on protein A beads were confirmed by staining with an anti-Fc antibody (lower panel, indicated by DGFc). *C*, quantitative solid-phase binding assays for divalent cation dependence. PikaLG binding to immobilized DGFc was tested in the presence of 2 mM EDTA and 2 mM each of Ca^{2+} , Mg^{2+} , or Mn^{2+} . Binding in the presence of Ca^{2+} was set as 100%. Data shown are the average of three independent experiments with standard deviations. *D*, Ca^{2+} -dependent binding of pikachurin to dystroglycan. PikaLG binding to DGFc was tested in various Ca^{2+} concentrations by solid-phase binding assays. The binding data were fit to the equation $Y = B_{\text{max}} x / (K_d + x)$, where K_d is the concentration required to reach half-maximal binding, and B_{max} is maximal binding. Maximal binding was set as 100%. $K_d = 78 \pm 15 \mu\text{M}$. Data shown are the average of four independent experiments with standard deviations. *E* and *F*, effects of NaCl (*E*) and heparin (*F*) on the pikachurin-dystroglycan interaction. PikaLG binding to DGFc was tested in various NaCl or heparin concentrations by solid-phase binding assays. Binding in the presence of 150 mM NaCl (*E*) or in the absence of heparin (*F*) was set as 100%. Data shown are the average of four (*E*) and six (*F*) independent experiments with standard deviations. *, $p < 0.05$. *G*, binding of pikachurin LG domains to heparin. Lysates from PikaLG-expressing cells were incubated with heparin affinity beads. Total lysate sample (total, lane 1), flow-through (void, lane 2), and bound (bound, lane 3) fractions were analyzed by Western blotting with an antibody to anti-Myc tag.

increased with higher concentrations of NaCl (Fig. 1E). No significant inhibitory effect was detected with heparin at 2 mg/ml (Fig. 1F), which is a sufficient concentration to completely inhibit α -DG binding to laminin-111 or agrin (28, 29). At 10 mg/ml heparin, PikaLG-DGFc binding was reduced to 60% (Fig. 1F). We confirmed that these conditions (0.5 M NaCl and 2 mg/ml heparin) do inhibit laminin-111 binding to DGFc (data not shown). To examine whether PikaLG has heparin binding capacity, we exposed lysates prepared from PikaLG-expressing cells to heparin affinity beads (Fig. 1G). Binding of PikaLG to heparin affinity beads was positive, indicating that PikaLG contains a heparin-binding site.

Dissection of Domains Necessary for Pikachurin-Dystroglycan Interaction—All known DG ligand proteins (laminin-111, laminin-211, agrin, perlecan, and neurexin) contain LG domains, through which they bind to α -DG. Pikachurin contains three LG domains in its C terminus. To examine which LG domain serves as the α -DG-binding site, we constructed single or tandem LG domains (Fig. 2A) and examined DGFc binding to each construct. We confirmed expression of all constructs in cells and then tested cell lysates containing comparable amounts of each LG protein for binding to DGFc (Fig. 2B). We found that the LG2-LG3 tandem construct binds to DGFc at a level similar to that of the full-length construct (Fig. 2B, right panel, lanes 5 and 6). Binding of other constructs to DGFc was minimal or undetectable. Solid-phase binding assays also confirmed that LG2-3 bound to DGFc, whereas the other deletion constructs did not (Fig. 2C). When PikaLG deletion constructs were subjected to SDS gel electrophoresis without heat denaturing, the constructs containing LG1 domains appeared at higher molecular weights than observed with heat denaturing (Fig. 2D). This result indicates that pikachurin forms oligomeric structures. Al-

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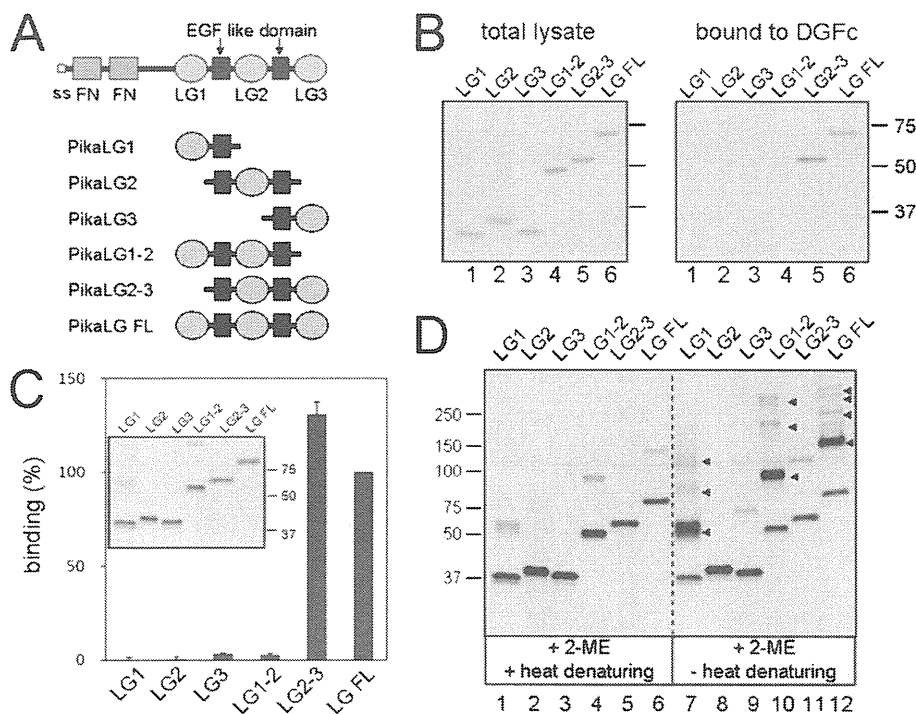


FIGURE 2. Dissection of the dystroglycan binding region in pikachurin. *A*, schematic representation of pikachurin deletion mutant proteins. All constructs contain a tandem myc-His tag at the C terminus. *ss*, signal sequence. *B*, binding of pikachurin deletion constructs to dystroglycan. Each deletion construct was expressed in HEK293 cells, and cell lysates were subjected to the DGFC binding assay. PikaLG in the reaction mixture (*left panel*) and PikaLG bound to DGFC-protein G-beads (*right panel*) were analyzed by Western blotting with an anti-Myc tag antibody. *C*, solid-phase binding assays for pikachurin deletion constructs. Cell lysates containing comparable amounts of each deletion construct were tested for DGFC binding. Binding of full-length DGFC was set as 100%. Data shown are the average of four independent experiments with standard deviations. *Inset*, Western blot analysis to confirm the amount of each LG protein used in the binding assays. *D*, oligomer formation of pikachurin. Cell lysates containing comparable amounts of each construct were dissolved in SDS sample buffer containing 2-mercaptoethanol (2-ME) and then subjected to SDS-PAGE with (+2-ME, +heat denaturing) or without (+2-ME, -heat denaturing) heat denaturing (95 °C, 5 min). Constructs containing LG1 (LG1, LG1-2, and LG FL) showed several higher molecular weight bands (*arrowheads*), which might indicate oligomeric structure formation by pikachurin.

though we observed no positive effect of the LG1 domain on PikaLG-DGFC binding, LG1-mediated oligomerization might play a physiological role in a more native situation. Overall, our results indicate that a certain steric structure formed by the LG2 and the LG3 domains is necessary for the pikachurin-DG interaction.

LARGE plays a crucial role in the DG modification process (17, 30). For LARGE-dependent modification of α -DG, two distinct domains of α -DG, the N-terminal domain and the first half of the mucin-like domain, are necessary. The N-terminal domain of α -DG is recognized by LARGE during post-translational maturation of DG and then proteolytically removed. The first half of the mucin-like domain of α -DG is modified with certain glycans necessary for acquiring ligand binding activity (30). To investigate whether these domains of α -DG are required for pikachurin binding, we used several DGFC deletion constructs (Fig. 3A). DG-N, which contains only the N-terminal domain, did not bind to PikaLG (Fig. 3B, lane 2). DG Δ N, which lacks the N-terminal domain, also failed to bind to PikaLG, even though it contains the entire mucin-like domain (Fig. 3B, lane 3). Co-expression with LARGE enhanced PikaLG binding to full-length DGFC (DG-wt) (Fig. 3B, lanes 4 and 5).

Using deletion constructs lacking the C-terminal domain (DG Δ C) or containing the N-terminal domain plus the first half of the mucin-like domain (and DG^{half}), we showed that pikachurin-binding domains are located within the first half of the mucin-like domain (Fig. 3C). We examined binding of laminin-111 to these constructs using an overlay assay and confirmed that PikaLG binds to the same constructs that are able to capture laminin-111 (Fig. 3, B and C, bottom panels).

Disruption of Pikachurin Binding and Localization in Dystroglycanopathy Animals—We investigated various aspects of the pikachurin-DG interaction in dystroglycanopathy model animals. First, we used *POMGnT1*-deficient mice to investigate whether the GlcNAc- β 1, 2-branch on O-Man is necessary for pikachurin binding. Endogenous α -DG was immunoprecipitated from brain extracts of *POMGnT1*-deficient and littermate heterozygous mice using antibodies that recognize the α -DG core protein (AP-G074). Precipitates were then incubated with lysates prepared from PikaLG-expressing cells (Fig. 4A). Western blot analysis of the immunoprecipitated materials confirmed that α -DG from the

POMGnT1-deficient samples was hypoglycosylated, as evidenced by reduced molecular size. Whereas PikaLG bound to control α -DG of normal molecular size, the PikaLG- α -DG interaction was dramatically reduced in *POMGnT1*-deficient mice.

Next, we examined whether native α -DG from *Large*^{myd} (*myd*) mice binds to pikachurin. Endogenous α -DG was immunoprecipitated from brains of *myd* or control heterozygous mice and then tested for pikachurin binding (Fig. 4B). We observed PikaLG binding to control α -DG with normal molecular size but not to hypoglycosylated α -DG from the *myd* mouse brain.

We also examined the PikaLG binding to native α -DG prepared from these mutant mice brains by solid-phase assays. Although binding signals obtained from the native DG preparations were generally weaker than those of DGFC, Ca²⁺-sensitive binding was detected in control heterozygous samples (Fig. 4C). However, no significant binding was detected in mutant samples. These data indicate that the pikachurin-DG interaction is disrupted in dystroglycanopathy animals.

We also examined pikachurin expression and localization in the ribbon synapses of *POMGnT1*-deficient and *myd* mice.

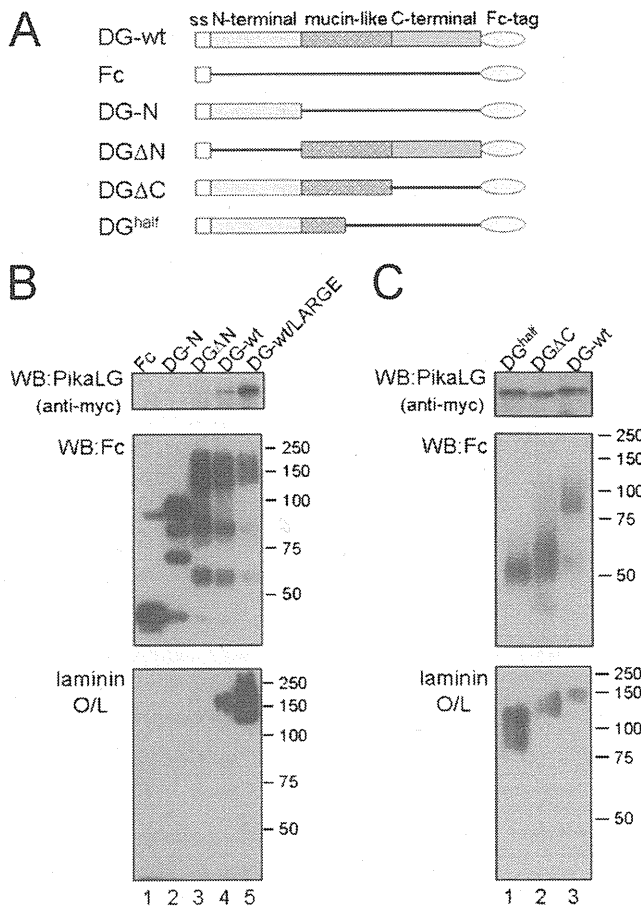


FIGURE 3. Dystroglycan functional domains for pikachurin binding. A, schematic representation of deletion mutants of DG_{FC} proteins. ss, signal sequence. B and C, dissection of dystroglycan domains necessary for pikachurin binding. Each deletion construct was expressed in HEK293 cells and recovered from the culture media using protein A beads. The DG-wt construct was expressed without or with LARGE (B, lanes 4 and 5). Lysates from PikalG-expressing cells were subjected to protein A beads that had captured each DG_{FC} mutant protein. PikalG binding was detected by Western blotting with an anti-Myc antibody (upper panel, PikalG). Comparable amounts of DG_{FC} mutant proteins on protein A beads were confirmed by Western blotting (WB) with an anti-Fc antibody (middle panel, Fc). The blot was also tested using a laminin-111 overlay assay (bottom panel, laminin O/L).

Immunofluorescence staining showed reduced pikachurin immunoreactivity in the ribbon synapse of *POMGnT1*-deficient mice, as compared with littermate heterozygous controls (Fig. 5A). Immunostaining of β -DG showed no apparent difference in DG protein expression between *POMGnT1*-heterozygous and *POMGnT1*-deficient animals. Binding assays confirmed that pikachurin binding is reduced in *POMGnT1*-deficient eye tissue (Fig. 5B). The reduced signal intensity for normal size α -DG in eye tissue relative to that in brain tissue (Fig. 4) is likely due to a lower abundance of DG proteins in the eye. Immunostaining in *myd* mice showed severely reduced pikachurin immunoreactivity in the ribbon synapse (Fig. 5C). Binding assays confirmed that pikachurin binding is also reduced in *myd* eye tissue (Fig. 5D). Real time quantitative PCR analysis showed that the amount of the pikachurin transcript was unchanged in dystroglycanopathy models (supplemental Fig. 2). Endogenous pikachurin protein has not been

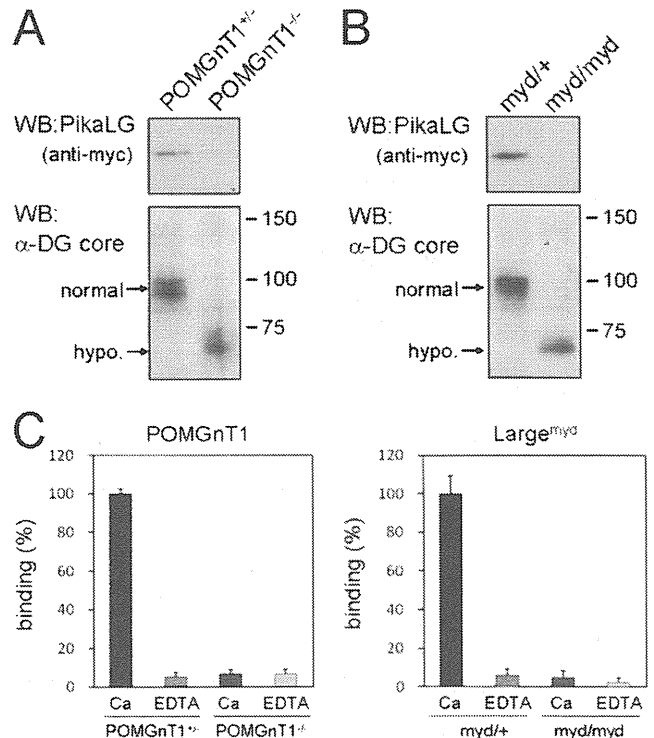


FIGURE 4. Reduced pikachurin binding to α -dystroglycan in dystroglycanopathy animals. α -DG was immunoprecipitated from the brains of *POMGnT1*-deficient (A) and *Large^{myd}* (B) mice. Littermates were used as controls. Lysates from PikalG-expressing cells were incubated with the immunoprecipitated materials to examine PikalG-DG binding. PikalG binding was detected by Western blotting (WB) with an anti-Myc antibody (upper panel, PikalG). Comparable amounts of α -DG were confirmed by Western blotting with anti- α -DG antibody (lower panel, α -DG core). Normal and hypoglycosylated (*hypo.*) sizes of α -DG are indicated on the left side of the blots. C, quantitative solid-phase binding assays for brain DG. Wheat germ agglutinin-enriched brain DG preparations from *POMGnT1*-deficient, *Large^{myd}*, and their littermates were immobilized and tested for PikalG binding. Binding to DG preparations from littermate controls in the presence of Ca^{2+} was set as 100%. Data shown are the average of three individual preparations with standard deviations.

detected by Western blotting, possibly due to low abundance and/or insolubility. These data demonstrate that pikachurin binding activity of α -DG is necessary for proper localization of pikachurin in the ribbon synapse.

DISCUSSION

In this study, we have characterized the pikachurin-DG interaction and demonstrated that both the GlcNAc- β 1, 2-branch and LARGE-dependent modification on O-Man of α -DG are necessary for the interaction to occur. Defects in these modifications result in reduced pikachurin-DG binding and disruption of pikachurin localization in the ribbon synapse, which might provide a molecular explanation for the abnormal ERG observed in dystroglycanopathy (supplemental Fig. 3).

The earlier studies have shown that binding of ligand proteins to α -DG is Ca^{2+} -dependent (27, 28, 31). A crystal structure study revealed that the laminin α 2-chain LG5 contains two Ca^{2+} -coordinating residues, Asp-2982 and Asp-3055. Other LG domains in α -DG ligand proteins commonly contain residues equivalent to these two residues (32). Sequence alignment revealed that each of the three LG domains in pikachurin con-

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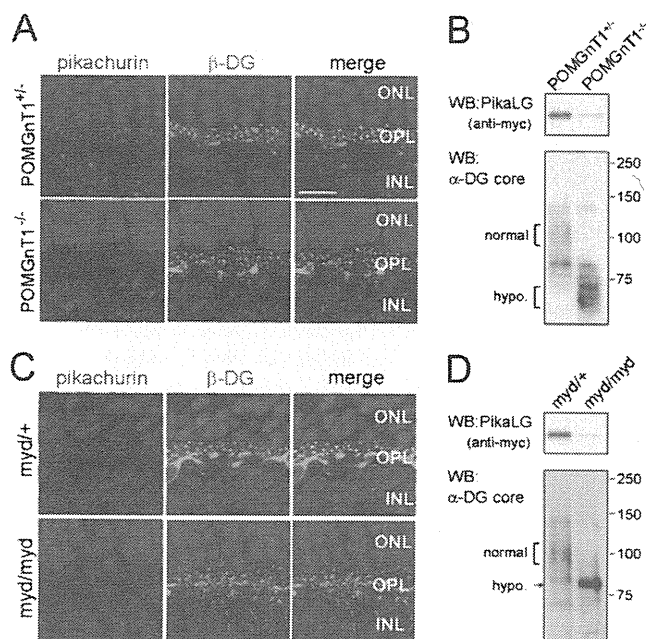


FIGURE 5. Disruption of pikachurin localization in dystroglycanopathy animals. A and C, immunofluorescence analysis of pikachurin in the outer plexiform layer (OPL). Retinal sections of *POMGnT1*-deficient ($-/-$) and *Large*^{myd} (*myd/myd*) mice, and their littermate heterozygous controls, were immunostained using antibodies to pikachurin (red, left panels) or β -DG (green, middle panels). Nuclei were stained with DAPI (blue). Merged images are shown in the right panels. Scale bar, 10 μ m. ONL, outer nuclear layer; INL, inner nuclear layer. B and D, reduced pikachurin binding to α -DG in dystroglycanopathy models. α -DG was immunoprecipitated from eyes of *POMGnT1*-deficient ($-/-$) and *Large*^{myd} (*myd/myd*) mice, and their littermate heterozygous controls. PikaLG-containing cell lysates were incubated with the immunoprecipitated materials to examine PikaLG-DG binding. PikaLG binding was detected by Western blotting (WB) with anti-Myc antibody (upper panel, PikaLG). Comparable amounts of α -DG were confirmed by Western blotting with anti- α -DG antibody (lower panel, α -DG core). Normal and hypoglycosylated (*hypo.*) sizes of α -DG are indicated on the left side of the blots.

tains an Asp residue equivalent to Asp-2982 in the laminin α 2-chain LG5, but they lack a residue equivalent to Asp-3055 (supplemental Fig. 4). The residue equivalent to Asp-3055 in pikachurin LG3 is Asn, which is capable of coordinating a Ca^{2+} , but LG1 and LG2 appear to lack the second Ca^{2+} -coordinating site. It has been shown that a single LG domain is usually insufficient for α -DG binding except laminin α 1-chain LG4. This is also the case for pikachurin (Fig. 2). Thus, the adjacent tandem LG2-LG3 domains likely allow multiple Ca^{2+} sites to form a stable pikachurin-DG connection, as is proposed for other known ligand proteins (32). Interestingly, our data show that pikachurin can form oligomeric structures. This suggests the possibility that multimerization or clustering effects may play a role in modulating pikachurin-DG interactions in the native environment.

Unlike the laminin α 1-chain and agrin (28, 29), the interaction of pikachurin with α -DG was relatively less sensitive to the inhibitory effects of heparin, although pikachurin LG domains have heparin binding capacity (Fig. 1). Heparin insensitivity at the submilligram/ml range is also observed with the laminin α 2-chain and perlecan (33). These data may indicate that the α -DG-binding site is spatially distinct from the heparin-binding site in pikachurin LG domains, thus preventing heparin

interference with the α -DG interaction. More interestingly, whereas 0.5 M NaCl strongly inhibits interaction between α -DG and other ligand proteins (33), only a modest inhibitory effect was observed for 0.5 M NaCl with pikachurin-DG binding. The strong inhibitory effects of NaCl on other DG ligand proteins indicate that, in addition to Ca^{2+} -mediated contact, an electrostatic effect may contribute partially to DG-ligand interactions. However, this may not be the case for pikachurin. Rather, it is likely that the Ca^{2+} -binding site in pikachurin primarily ensures the interaction with α -DG. There seem to be subtle differences between the binding of pikachurin to α -DG and that described for other LG domain proteins. Our ligand competition experiments show that PikaLG inhibits laminin-111 binding to DGFc, but even very high concentrations of laminin-111 do not inhibit PikaLG-binding to DGFc (supplemental Fig. 5). These data suggest that pikachurin might contain more binding sites on α -DG than does laminin-111. Alternatively, PikaLG might have much higher affinity for α -DG compared with laminin-111. Further investigation is necessary to reveal pikachurin-binding sites on α -DG in the future.

It is known that certain glycosylation events are necessary for α -DG ligand binding activity; however, the exact glycan structure necessary for the ligand binding is still not determined. Several lines of evidence have shown that among heterogeneous glycans on α -DG, O-mannosylation is an essential post-translational modification. The POMT1/2 complex catalyzes the initial Man transfer to Ser/Thr residues (9), and POMGnT1 synthesizes the GlcNAc- β 1,2-branch on O-Man (12). A very recent study demonstrated the involvement of LARGE in the synthesis of phosphodiester-linked glycan on O-Man, which would serve as a laminin-binding moiety (13). Another study showed that β 3GnT1 is involved in LARGE-dependent modification (34). β 3GnT1 interacts with LARGE, and reduced expression of β 3GnT1 leads to diminished synthesis of laminin-binding glycans. Here we have demonstrated that post-translational modification on O-Man mediated by LARGE and POMGnT1 is necessary for the pikachurin-DG interaction.

Mutations in these glycosylation pathways are causative for dystroglycanopathy, which is frequently associated with eye involvement, including abnormal retinal physiology. Several models for dystroglycanopathy, including *POMGnT1*-deficient, *Large*-mutant *Large*^{myd}, and *Large*^{vis} mice, show abnormal retinal physiology such as attenuation or delay in the electroretinogram b-wave (23, 24, 35). Previously, we reported that genetic disruption of pikachurin causes an ERG abnormality similar to those seen in other dystroglycanopathy model mice (22). In the retina, DG is expressed in the Müller glial end feet at the inner limiting membrane, in the glial end feet abutting the vasculature (36), and at ribbon synapses of photoreceptors in the outer plexiform layer (37–40). On the other hand, pikachurin localization is specific to the synaptic cleft of the photoreceptor ribbon synapse in the outer plexiform layer (22). In this study, we demonstrated that the pikachurin-DG interaction and pikachurin localization at the ribbon synapse are both disrupted in dystroglycanopathy animals. We propose that proper localization of pikachurin at the ribbon synapse, which is supported by functionally mature DG, plays important roles in the physiology of the retina.

Another physiological role of DG in the retina, apart from the ribbon synapse, was recently demonstrated (7). In that study, it has been shown that deletion of DG in the central nervous system (CNS) causes attenuation of the b-wave, which is associated with a selective loss of dystrophin and Kir4.1 clustering in glial end feet. Dystrophin is the product of the causative gene for Duchenne and Becker muscular dystrophies; it forms a protein complex with DG termed the dystrophin-glycoprotein complex. Loss of either dystrophin or DG results in reduction of the entire dystrophin-glycoprotein complex from the cell surface membrane (2, 41). Importantly, abnormalities in ERG similar to those seen in CNS-selective DG-deficient mice are frequently observed in individuals with Duchenne/Becker muscular dystrophy (42, 43). Dystrophin isoforms generated through differential promoter usage and alternative splicing are regulated in a tissue-specific and developmental manner. Dp260, which is transcribed using an internal promoter, is a retina-specific isoform located in the outer plexiform layer (44). In Dp260-disrupted mice, DG expression in the outer plexiform layer is severely reduced, and the implicit time of the b-wave is prolonged (45). These changes are also observed in dystroglycanopathy and pikachurin-deficient mouse models. Combined with our present work, these studies support the hypothesis that DG contributes to retina function via multiple mechanisms (7), including the pikachurin-DG-Dp260 molecular complex at the ribbon synapse and the DG-dystrophin-Kir4.1 clusters at glial end feet. Overall, our data not only shed light on the molecular pathogenesis of eye abnormalities in muscular dystrophy patients but also contribute to understanding the molecular mechanisms for ribbon synapse formation and maintenance.

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Review

Progress in muscular dystrophy research with special emphasis on gene therapy

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Abstract: Duchenne muscular dystrophy (DMD) is an X-linked, progressive muscle-wasting disease caused by mutations in the *DMD* gene. Since the disease was described by physicians in the 19th century, information about the subject has been accumulated. One author (Sugita) was one of the coworkers who first reported that the serum creatine kinase (CK) level is elevated in progressive muscular dystrophy patients. Even 50 years after that first report, an elevated serum CK level is still the most useful marker in the diagnosis of DMD, a sensitive index of the state of skeletal muscle, and useful to evaluate therapeutic effects. In the latter half of this article, we describe recent progress in the therapy of DMD, with an emphasis on gene therapies, particularly exon skipping.

Keywords: Duchenne muscular dystrophy, dystrophin, exon skipping, out-of-frame mutation, clinical trial, antisense oligonucleotides

Introduction

Muscular dystrophy is not a single disease but a group of genetically heterogeneous muscle diseases marked by progressive wasting and weakness of the skeletal muscles, and sometimes involvement of cardiac and smooth muscle or other tissues. In 1851, Meryon reported boys with symptoms consistent with the diagnosis of muscular dystrophy,¹⁾ and in 1868 in France, Duchenne published a detailed and systematic clinical, muscle pathological, and electrophysiological study of an "atrophie musculaire progressive", which is now generally recognized as Duchenne muscular dystrophy (DMD).²⁾ Its preva-

lence in the population is estimated to be 1.8–4.8 per 100,000. Although the gene responsible was identified in 1986^{3),4)} and the underlying pathogenesis is understood to some extent,⁵⁾ there is no effective therapy at present other than corticosteroids. In this article, we review historical aspects of the research on DMD and discuss the therapies of the near future.

Biochemistry and diagnosis of DMD

Biochemical abnormalities in patients with muscular dystrophy were first reported by Sibley and Lehninger in 1949.⁶⁾ They determined serum aldolase activity in patients with muscular dystrophy, and reported increased serum aldolase activity in the patients. Sugita measured the serum aldolase levels of patients and confirmed that patients with muscular dystrophy had elevated serum aldolase activity; this finding was reported in a Japanese journal in 1958.⁷⁾ At the end of the same year, a middle-aged male was hospitalized at the University of Tokyo Hospital directed by Prof. Dr. Shigeo Okinaka. The patient had moderately atrophic extremities without positive tendon reflexes. Curiously, he did not show any sensory disturbance. Interestingly enough, he had a markedly elevated serum aldolase level. After much deliberate consideration, Prof. Okinaka diagnosed him with a motor neuronitis, however, at grand rounds, the attending

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Abbreviations: DMD: Duchenne muscular dystrophy; CK: creatine kinase; AO: antisense oligonucleotide; BMD: Becker muscular dystrophy; LNA: locked nucleic acid; PNA: peptide nucleic acid; ENA: ethylene-bridged nucleic acid; 2'-O-MePS AO: 2'-O-methyl phosphorothioate AO; PMO: phosphorodiamidate morpholino oligomer; PPMO: a cell-penetrating peptide-linked PMO; ESE: exonic splicing enhancer; nNOS: neuronal nitric oxide synthase; GRMD: golden retriever muscular dystrophy; CXMD_J: canine X-linked muscular dystrophy in Japan; AAV: adeno-associated virus.

physician told Prof. Okinaka that the patient was suffering from muscular dystrophy, based on the serum aldolase activities that Sugita measured. Prof. Okinaka became furious and told Sugita that it was not acceptable to make a diagnosis simply on the determination of something in the blood. In retrospect, Prof. Okinaka was correct; the patient was probably suffering from polymyositis rather than muscular dystrophy.

However, the incident led to one of the monumental achievements in the history of muscular dystrophy research due to the outstanding professional intuition of Dr. Setsuro Ebashi. When Dr. Momoi, a close friend of Dr. Ebashi since middle school, told him about the serum aldolase activity in muscular dystrophy, he pointedly asked Dr. Momoi, "Why do you determine a nonspecific enzyme such as aldolase in muscular diseases? You should look at the level creatine phosphokinase, which is more specific to skeletal muscle than aldolase." It was a stroke of genius! At his suggestion, we set up a team, headed by Dr. Ebashi and including Drs. Momoi, Toyokura, and Sugita, to study creatine phosphokinase (CPK; it was also known as creatine kinase or CK). Thus, first paper on serum CK activity in progressive muscular dystrophy was published by Ebashi and coworkers in 1959.⁸⁾ Among various neuromuscular disorders, they found increased serum CK levels in patients with muscular dystrophy, and it is now regarded as the most reliable laboratory test for muscular dystrophy. The discovery of the importance of serum CK opened the door for the recent myology research, in particular pathological studies including genetics and the exploration of treatments such as gene therapy.

Discovery of dystrophin and its localization at the muscle surface membrane

A pioneer application of positional cloning to human diseases appeared in 1986, when the gene responsible for DMD/Becker muscular dystrophy (BMD) was isolated by Dr. A.P. Monaco *et al.* of Dr. L. Kunkel's group.³⁾ The *DMD* gene is 2,500 kb long and consists of 79 exons covering 1% of the x-chromosome. It is transcribed to yield a 14 kb cDNA. In 1987, Dr. E.P. Hoffman *et al.* identified a 427 kD protein encoded by the *DMD* gene, and this protein was named "dystrophin",⁴⁾ which is absent from the skeletal muscle of most patients with DMD. Almost all cases of DMD showed an out-of-frame mutation. In contrast, most patients with BMD had an in-frame mutation. Using polyclonal antibodies against the

near N-terminal portion derived from the dystrophin cDNA, Dr. Kiichi Arahata *et al.* of the muscular dystrophy research group at National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan, identified a specific immunohistochemical reaction with peptides on the surface membrane of skeletal and cardiac muscle fibers that was absent in the muscles of DMD patients.⁹⁾ These results have been confirmed by other research groups. In symptomatic carriers of DMD, a distinct mosaic pattern of immunohistochemical staining of the surface membrane of the muscle fibers can be observed. BMD exhibits a positive but faint and patchy expression pattern of dystrophin with altered protein contents and molecular weights. Thus, it became clear that DMD and BMD are caused by fragility of the muscle surface membrane due to the lack of dystrophin.

Development of therapy for DMD

Drug treatment for DMD patients is currently restricted almost completely to corticosteroids (oxandrolone and prednisone), but a variety of therapeutic approaches to muscular dystrophies have been tested over the past few decades, and some of them show great promise (recently reviewed in Ref. 10, and current situation was summarized in Table 1). For successful application of viral vector-mediated gene therapy, there are still several hurdles to be overcome.¹¹⁾ Pluri- or multipotent stem cell-based therapies are still in their immature stages, but currently some alternatives are progressing to clinical trials. Among several therapeutic approaches in preclinical or clinical stage, authors here focus on one of the most promising therapeutic approaches: exon skipping with antisense oligonucleotides (AOs).

Exon-skipping therapy using AOs

DMD is caused by the lack of dystrophin, most commonly as a result of frame-shift mutations. Deletions and duplications in the *DMD* gene result in out-of-frame mRNA, such as nonsense mutations in which a single base change alters a codon into a premature stop codon. Theoretically, in these cases, selective removal of the flanking exons can result in an in-frame mRNA transcript. Such an in-frame mRNA transcript can be translated into a quasi-dystrophin protein (reviewed in Ref. 12). AOs, which hybridize the sequences near the splice acceptor or donor sites as well as within exons, can alter gene expression *via* steric block interference with the splicing machinery, and thereby direct the exclusion

Table 1. Clinical trials for DMD/BMD

Category	Interventions	Phase (ClinicalTrials.gov)
Drug	Myostatin blocking	
	MYO-029	Completed; not effective
	Read-through	
	PTC124	Completed; not effective
	Gentamicin	Completed; not effective
	Others	
	Pentoxifylline	Completed; not effective
	Idebenone	Phase III
	Ramipril vs. Carvedilol	Phase IV
	Coenzyme Q10 and prednisone	Phase III
Coenzyme Q10 and lisinopril	Phase II/III	
Debio-025 (cyclosporine analogue)	Phase IIb	
Gene therapy	Exon skipping (systemic delivery)	
	PRO051 (2'-O-MePS AO)(exon 51 skipping)	Phase III
	PRO044 (2'-O-MePS AO)(exon 44 skipping)	Phase I/II
	AVI-4658 (PMO)(exon 51 skipping)	Phase IIb
	AAV vector	
rAAV2.5-CMV-Mini-Dystrophin	Phase I*	
Cell therapy	Satellite cells (myoblasts)	Pending
	Mesoangioblasts	In preparation
	Induced pluripotent stem (iPS) cells	Experimental

Shown are representative ongoing or just finished clinical trials for DMD. Some have finished with disappointing results. For more information, please refer to the homepage presented by the U.S. National Institute of Health, 'ClinicalTrials.gov'.

*Unwanted immune responses to dystrophin have been reported at the 2010 meeting of the American Academy of Neurology (<http://quest.mda.org/news/caution-immune-response-seen-dmd-gene-therapy>).

of one or more exons in the final transcript, resulting in restoration of the reading frame of dystrophin mRNA and the expression of a shorter, truncated but functional dystrophin.

One of the pioneering researchers who tried to restore the reading frame of the mutated DMD transcripts was Dr. Masafumi Matsuo at Kobe University.¹³⁾ His group tried to skip exon 19 of the *DMD* gene in exon 20-deleted DMD patients, based on the idea of DMD Kobe, where exon 19 has been skipped due to a 52-bp deletion within the exon. Later, proof-of-concept studies by many groups followed *in vitro* and *in vivo* (reviewed in Ref. 14).

Chemistries of AOs

For maximal effects in exon-skipping therapy, the chemistry of AOs seems to be one of most critical factors. AOs used for exon skipping are usually 20–25 bases long and chemically synthesized. Various chemistries for AOs have been proposed to overcome

the unstable nature of single-strand DNA or RNA molecules. Several modifications of AOs include bicyclic locked nucleic acid (LNA), peptide nucleic acid (PNA), ethylene-bridged nucleic acid (ENA), 2'-O-methyl phosphorothioate AO (2'-O-MePS AO), and phosphorodiamidate morpholino oligomer (PMO). Among them, 2'-O-MePS AO and PMO are the most frequently utilized because of their suitable properties (Fig. 1).

2'-O-MePS AO. The structure of 2'-O-MePS AO is similar to that of RNA, but it is methylated at the 2'-OH position of the ribose ring. 2'-O-MePS AO is widely used because it is relatively easy to synthesize and cheap to produce. 2'-O-MePS AO is stable, has a high affinity to mRNA, and is also resistant to nucleases, however the low solubility of 2'-O-MePS AO in water prevents its use at higher dosages.

PMO. PMO has a morpholine ring instead of a deoxyribose ring in DNA, and these artificial rings

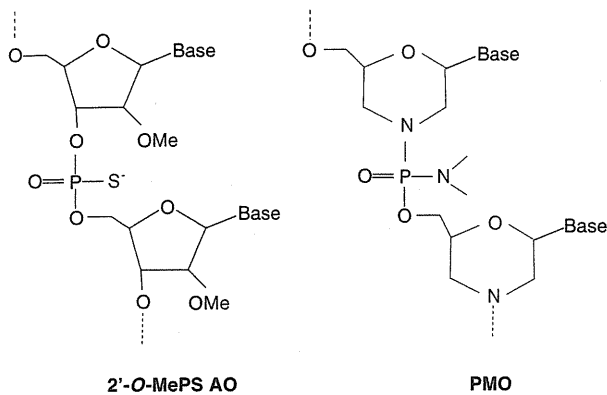


Fig. 1. Comparison of chemistry used in clinical trials. 2'-O-MePS AO: 2'-O-methyl phosphorothioate antisense oligonucleotides. PMO: phosphorodiamidate morpholino oligomers. PMO (AVI BioPharma, <http://www.avibio.com/>) has a different backbone (morpholino backbone) from RNA or DNA, enabling highly sequence-specific, stronger base pairing to the target RNA than RNA or DNA. Red highlights the differences in the chemistry from RNA or DNA.

are linked to each other through phosphorodiamidate, enabling highly sequence-specific, stronger base pairing to the target RNA than RNA or DNA. Importantly, PMO does not stimulate or activate Toll-like receptors and therefore does not activate innate immune responses. In addition, PMO is believed to be not recognized by cellular proteins and to be resistant to nuclease-mediated degradation. PMO has high solubility in water.

Other chemicals to improve efficiencies of delivery to whole-body musculature. Delivery of AOs to normal tissues *in vivo* is generally difficult because healthy tissues do not take up PMO or 2'-O-MePS AO. Although the mechanisms are not fully understood, PMO can easily enter the nuclei of skeletal muscle of DMD patients. This might be because the dystrophin-deficient muscle membrane is inherently leaky due to absence of the dystrophin-glycoprotein complex.

However, systemic administration of 2'-O-MePS AO or PMO failed to restore dystrophin expression in the heart, although it is again unclear why cardiac cells do not take up 2'-O-MePS AO or PMO. To improve the efficacy of its introduction into cardiomyocytes, a PMO covalently conjugated with a designed cell-penetrating peptide (PPMO) was injected into dystrophin-deficient *mdx* mice. Systemic delivery of the novel PPMO restored dystrophin to almost normal levels in both cardiac and skeletal muscles in *mdx* mice.¹⁵⁾ Later, the same group reported that a PMO modified with an octaguanidi-

nium dendrimer, Vivo-Morpholino, also restored dystrophin expression in cardiac and skeletal muscles.¹⁶⁾ So far, no study has clearly demonstrated toxicity after systemic delivery or immune response to PPMO or Vivo-Morpholino.

AOs designs

To obtain efficient exon skipping while lowering the dose of AOs for clinical trials, the design of the AOs (base sequence) is important. In eukaryotic organisms, the gene is transcribed in the nucleus and introns are spliced out into mRNA, and then the mRNA is exported from the nucleus to the cytoplasm. Therefore, AOs must either enter the nucleus, where they bind to their target pre-mRNA sequences and get in the way of molecules that are otherwise involved in the splicing process, or they must alter the secondary structure folding of the pre-mRNA. AOs targeting exon-intron boundary sequences can often effectively induce exon skipping. On the other hand, when web-based software, such as ESEfinder (<http://rulai.cshl.edu/tools/ESE/>), is used to design AO sequences to target an ESE, exon skipping is not always induced. Recently, Wee *et al.* have developed bioinformatic tools to optimize AOs sequences based on the pre-mRNA secondary structure.¹⁷⁾ Nevertheless, no single design tool is sufficient for designing the AOs, and often empirical analysis is required.

Proof of principle of exon skipping therapy in animal models

Cultured skeletal muscle cells from DMD patients are often used to evaluate the exon-skipping efficiency of newly-designed AOs.¹⁸⁾ However, to assess the therapeutic benefits, preclinical studies must be performed using animal models. In this section, studies using *mdx* mice and dystrophic dogs are described and discussed.

Exon skipping in *mdx* mice. The *mdx* mouse is a naturally occurring animal model that has a nonsense mutation in exon 23 of the *DMD* gene, resulting in a premature stop codon and complete absence of dystrophin.¹⁹⁾ *Mdx* mice show high levels of serum CK, active muscle degeneration/regeneration cycles, and loss of myofibers and fibrosis in the diaphragm, but still show mild, non-progressive muscle weakness of the limbs and only 20% reduction in life span. In this point, *mdx* might not be an ideal animal model for DMD, but due to the low cost of maintenance and short gestation times, many preclinical studies have been carried out using *mdx* mice.

Lu *et al.* reported the local administration of the AOs with the non-ionic polymer F127, which promotes intracellular uptake of 2'-O-MePS AO, to the skeletal muscles of 2-week-old *mdx* mice. The result showed that dystrophin together with β -dystroglycan, sarcoglycans, and nNOS was restored in 20% of the muscle fibers.²⁰⁾ Furthermore, systemic administration of anti-sequences of the same 2'-O-MePS AO with F127 revealed that dystrophin was expressed in the skeletal muscle of the whole body. 2'-O-MePS AO produced no toxicity, but its expression did not reach a therapeutic level.²¹⁾ Likewise, Wells *et al.* reported that local administration of 2'-O-MeAO using electroporation restored dystrophin expression to up to 20% of the normal level.²²⁾ Systemic induction of dystrophin expression by PMO administration reached up to 20% in whole body skeletal muscle.²³⁾

Dystrophic dogs as models for DMD.

Muscular dystrophy in dogs was originally identified in golden retrievers and designated "golden retriever muscular dystrophy" (GRMD). GRMD shows progressive skeletal muscle weakness and atrophy as well as abnormal electrocardiographic findings and myocardial fibrosis like those seen in DMD. Because the phenotype and genetic background are closer to human DMD than those of the mouse model, the dystrophic dog is a useful model to examine pathogenesis and therapeutic strategies. However, the dogs are too large to be maintained conveniently; thus, we have established a colony of medium-sized beagle-based dystrophic dogs (canine X-linked muscular dystrophy in Japan: CXMD_J) at the National Center of Neurology and Psychiatry, Tokyo, by using artificial insemination of frozen GRMD semen.²⁴⁾ Still, we have found that CXMD_J requires extra daily care, and therefore, is expensive.

Clinical features of CXMD_J. The level of serum CK in neonatal CXMD_J is very high, and 25–33% of the pups die of respiratory failure during the neonatal period, mainly due to severe degeneration and thus dysfunction of the diaphragm. Around the age of 2–3 months, atrophy and weakness of limb muscles appear; then, gait disturbance, joint contracture, macroglossia, and dysphasia appear in rapid progression until the dogs are 10 months of age; subsequently, the progression is retarded.²⁴⁾

Cardiac involvement of dystrophic dogs.

Dystrophic dogs and DMD have similar cardiac involvement, including distinct deep Q-waves on the electrocardiogram and fibrosis of the left ventricular wall. The distinct deep Q-waves are generally

ascribed to fibrosis in the posterobasal region of the left ventricular wall in DMD, but one report suggests that the deep Q-waves precede the development of fibrosis in CXMD_J.²⁵⁾ Importantly, Purkinje fibers in dystrophic dogs showed remarkable vacuolar degeneration despite the absence of detectable fibrotic lesions in the ventricular myocardium. In the degenerated Purkinje fibers, Dp71, a C-terminal truncated isoform of dystrophin, was up-regulated at the sarcolemma. In addition, the calcium-dependent protease μ -calpain was found at the cell periphery near the sarcolemma or in the vacuoles. Utrophin, a homologue of dystrophin, was also highly up-regulated in the Purkinje fibers in the early stage. Together, the selective degeneration of Purkinje fibers may be associated with distinct deep Q-waves on electrocardiograms and the fatal arrhythmia seen in dystrophinopathy.²⁵⁾

Multi-exon skipping in dystrophic dogs. The dystrophic dogs have a point mutation at the intron 6 splice acceptor site in the canine *DMD* gene, resulting in skipping of exon 7 and a premature stop codon in exon 8. Thus, dystrophin is not produced in the affected dog. Recently, Yokota *et al.* reported systemic administration of three PMOs targeting exons 6 and 8 to convert an out-of-frame mutation to an in-frame mutation in CXMD_J.²⁶⁾ The result showed that dystrophin was restored in the entire body skeletal muscle. The authors reported that the motor ability of treated dystrophic dogs was improved and that they showed no side effects. To the best of our knowledge, this is the first report that multi exon-skipping is feasible and effective in improving performance of dystrophic animals *in vivo*.

As reported in *mdx* mice, a combination of PMOs failed to restore the expression of dystrophin in cardiac muscle, even at a high dose in CXMD_J. To resolve this problem, modified PMOs (PPMO or Vivo-Morpholino) were tested (Yokota *et al.*, unpublished observation).

Viral vector-mediated exon skipping

The duration of the effects of AOs *in vivo* is limited; therefore, patients have to be injected with AOs weekly or monthly to maintain the therapeutic levels of dystrophin. An alternative strategy is to deliver AOs *via* adeno-associated virus (AAV) vector-mediated gene transfer. Based on an AAV vector-mediated approach, Goyenvalle *et al.* linked U7 small nuclear RNA to antisense sequences to achieve sustained dystrophin expression derived from skipped mRNA for more than 13 weeks in the limbs

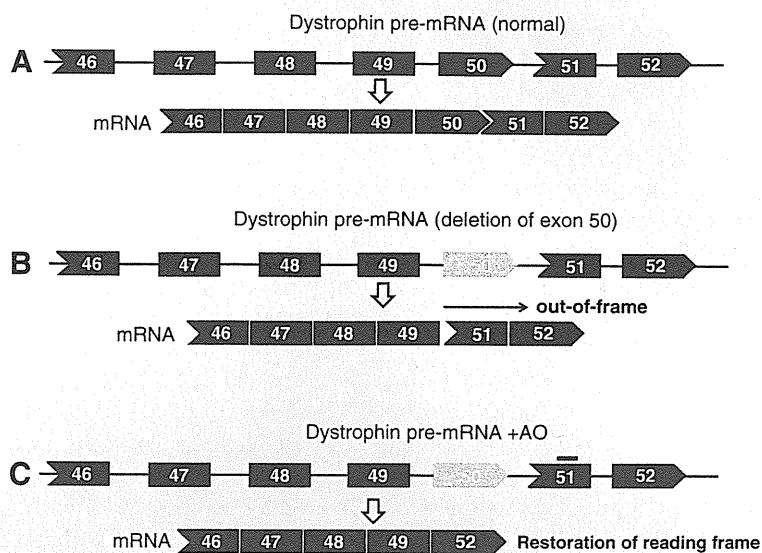


Fig. 2. Example of exon skipping therapy for DMD patients with deletion of exon 50. A. Normal dystrophin transcript and mRNA. B. Deletion of exon 50 disrupts the open reading frame, leading to a premature stop codon, unstable mRNA, and a truncated unstable protein. C. Targeted skipping of exon 51 using antisense oligonucleotides, such as AVI-4658 or PRO051 (blue line), restores the reading frame and produces a truncated but functional dystrophin that lacks exons 50 and 51.

of the *mdx* mouse.²⁷⁾ Lentiviral vectors were also used to modify muscle stem cells expressing modified small nuclear RNAs that change splicing patterns of pre-mRNAs and correct the reading frame.^{28),29)}

Test of AOs efficacy using patient cells

To apply exon skipping therapy to individuals, not only the deletion pattern in the genome but also the mRNA/cDNA level must be analyzed to determine the precise splicing pattern and to design therapeutic AOs. Subsequently, designed AOs must be tested *in vitro* on cells derived from the patient before clinical trials. Skin fibroblasts are easy to obtain, but the levels of DMD transcripts are low, and therefore the results tend to be variable. On the other hand, preparation of myoblasts through biopsy from dystrophic patients is invasive. Therefore, transformation of fibroblasts to myoblasts *in vitro* by forced MyoD expression is often used to test the efficacy of AOs.^{30),31)}

Multiple exon skipping: theory and reality

We and other research groups tried to find the most effective target of exon skipping strategy among DMD patients. Theoretically, exon 51-skipping therapy is effective for up to 15% of DMD patients having a deletion, but large numbers of DMD patients will not benefit from it. We recently reported that three unrelated patients with a deletion of exons

45–55 showed very mild skeletal muscle involvement and were ambulant until his seventies in a patient.³²⁾ Bérout *et al.* also described 15 asymptomatic or very mild patients with an exon 45–55 deletion.³³⁾ In fact, the Leiden Muscular Dystrophy database indicates that the exon 45–55 deletion produced a BMD phenotype in more than 95% of the cases. Collectively, these observations indicate that approximately 60% of DMD patients having a deletion within the hot spot may be treatable with multi-exon skipping of exons 45–55. We recently tried exon 45–55 skipping by injection of a mixture of 10 PMOs in the anterior tibial muscle of *mdx52* mice³⁴⁾ and confirmed that multi-exon skipping is feasible, at least in mice (Aoki *et al.*, unpublished observation). However, the efficacy of multi-exon skipping was lower than that of single-exon skipping, possibly because many partially spliced products, many of which are again out-of-frame, are produced.

Phase I/II clinical trials of exon skipping for DMD

Based on the success of AOs-mediated exon skipping in animal models, clinical trials have been performed or are in progress for the skipping of exon 51 of the *DMD* gene (Fig. 2 and Table 1).

2'-O-MePS AO. Skipping of the exon 51 of the *DMD* gene with 2'-O-MePS AO, PRO051, has been already tested on DMD patients by a Dutch group.³⁵⁾

Four DMD patients received a dose of 0.8 mg of PRO051 injected into the tibialis anterior muscle. A biopsy performed 28 days later revealed restoration of sarcolemmal dystrophin in 64% to 97% of myofibers of each patient. Further, PRO051 did not evoke any clinically apparent adverse events.

PMO. Recently, a UK group reported that local injection of morpholino oligomer AVI-4658 successfully restored the expression of dystrophin in treated muscles of all seven DMD patients.³⁶⁾ No adverse events related to AVI-4658 were observed. Based on these observations, systemic injection of AVI-4658 (Phase I and IIa) is now ongoing in the UK. At present, a Good Manufacturing Practice grade of PMO is produced exclusively by AVI Biopharma Inc. (<http://www.avibio.com/>).

Conclusions

Since the identification of dystrophin in 1987, various therapeutic approaches to DMD treatment have been evaluated, and now exon skipping, which is one of the most promising strategies, is in clinical trials. Because individual DMD patients have different mutations, exon skipping therapy requires a precise evaluation of mutations in the genome and the cDNA, and splicing patterns must be confirmed in each patient's muscle. In this point, exon skipping is a quite new, personalized therapeutic strategy. As clinicians and researchers involved in the study of muscular dystrophies, one of us for more than 50 years, we are pleased with recent progress in the field and hope that DMD patients benefit from this new therapy in the near future.

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Profile

Hideo Sugita was born in 1930. He was graduated from Faculty of Medicine, University of Tokyo in 1954. In 1959, Prof. S. Ebashi and coworkers, including Sugita discovered the increase in serum creatine kinase (CK) in muscular dystrophy. Even now, an elevated level of serum CK is the most useful marker in the diagnosis of muscular dystrophy. Since then, he had been concentrated his efforts to elucidate the pathogenesis of muscular dystrophy. In 1988, his research group at the National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP) clarified that dystrophin, the gene product of Duchenne muscular dystrophy (DMD) was located along the surface membrane of the skeletal and cardiac muscles and absent in DMD muscles. He was promoted to Director General of the Institute in 1989 and devoted himself to the progress of neuroscience research, health and welfare of the patients suffering from mental, neurological and developmental disorders. He was awarded Uehara Prize in 1986 and The Takeda Prize for Medical Science in 1996. He was installed as the President of NCNP in 1994 and retired in 1998. Since 2009, he is the President of Japan Foundation for Neuroscience and Mental Health.

