THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 41, pp. 31208–31216, October 8, 2010

Post-translational Maturation of Dystroglycan Is Necessary for Pikachurin Binding and Ribbon Synaptic Localization*[S]

Received for publication, February 20, 2010, and in revised form, July 23, 2010 Published, JBC Papers in Press, August 3, 2010, DOI 10.1074/jbc.M110.116343

Motoi Kanagawa[‡], Yoshihiro Omori[§], Shigeru Sato[§], Kazuhiro Kobayashi[‡], Yuko Miyagoe-Suzuki[¶], Shin'ichi Takeda[¶], Tamao Endo[‖], Takahisa Furukawa[§], and Tatsushi Toda[‡1]

From the [‡]Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe 650-0017, the [§]Department of Developmental Biology, Osaka Bioscience Institute, Japan Science and Technology Agency, Core Research for Evolutional Science and Technology, Suita 565-0874, the [¶]Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira 187-8502, and [‖]Molecular Glycobiology, Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015, Japan

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-β1,2-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.

* This work was supported by Ministry of Health, Labor, and Welfare of Japan Intramural Research Grant for Neurological and Psychiatric Disorders of the National Center of Neurology and Psychiatry 20B-13, Research on Psychiatric and Neurological Diseases and Mental Health Grant H20-016 (to T. T.), Japan Society for the Promotion of Science Grant-in-aid for Young Scientists (B) 21790318, and Grant-in-aid for Scientific Research on Priority Areas 20056020 (to M. K.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5.

[1] To whom correspondence should be addressed: 7-5-1 Kusunoki-chou, Chuo-ku, Kobe 650-0017, Japan. Tel.: 81-78-382-6287; Fax: 81-78-382-6288; E-mail: toda@med.kobe-u.ac.jp.

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-α2,3-Gal-β1,4-GlcNAc-β1,2-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-β1,2-branch on *O*-Man is essential to α-DG function as a matrix receptor.

Additional proteins, including fukutin, FKRP, and LARGE, are also involved in synthesizing the glycans on α-DG that are required for ligand binding activity. Recently, a GalNAc-β1,3-GlcNAc-β1,4-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 *FKRP* was identified through sequence homology (15). Mutation of

[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-β1,2-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 (TTCTCGAGCCTCCAGGGGCCAGGGTGTG); LG2(542–838), G542f (TTGGTACCGAGCTCGGATCTGGGAAGAAGATTGACATGAG) and P838r (TTCTCGAGCTGGGATCTCGATGGCTTCTA); LG3(799–1017), D766f (TTGGTACCGAGCTCGGATCTGACCGGACCATCCATGTGAAG) and PikaR (GCAACTAGAAGGCACAGTCG); 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); DGΔN(1–28 plus 315–651), pCAGf and DGFc28r (GGCCTGAGCCACAGCCACAGACAGGAGGAG); and DGFc315f (ACACCTACACCTGTTACTGCC) and Fcexon2r (TCCCCCCAGGAGTTCAGGTGC); DGΔC(1–483), pCAGf and DGFc483r (AATCTAGAAGGAATTGTCAGTGTTGGGCG); DG^{half}(1–407), pCAGf and DGFc407r (AATCTAGAACTGGTGGTAGTACGGATTCG). 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 pulldown assay, secreted DGFc proteins were recovered from conditioned media using protein A beads (10 μl). 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 $CaCl_2$ 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 $CaCl_2$), bound materials were eluted with SDS-sample buffer. Bound materials

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 $A = B_{max}x/(K_d + x)$, 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, GTGTGCAGAGCGATTTCCTTCATTC; 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. 1*A*) (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. 1*B*). 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. 1*C*). The solid-phase binding assays in various Ca^{2+} concentrations established that the concentration required for half-maximal binding is ~80 μM (Fig. 1*D*). 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 (~80% binding), and the inhibitory effect

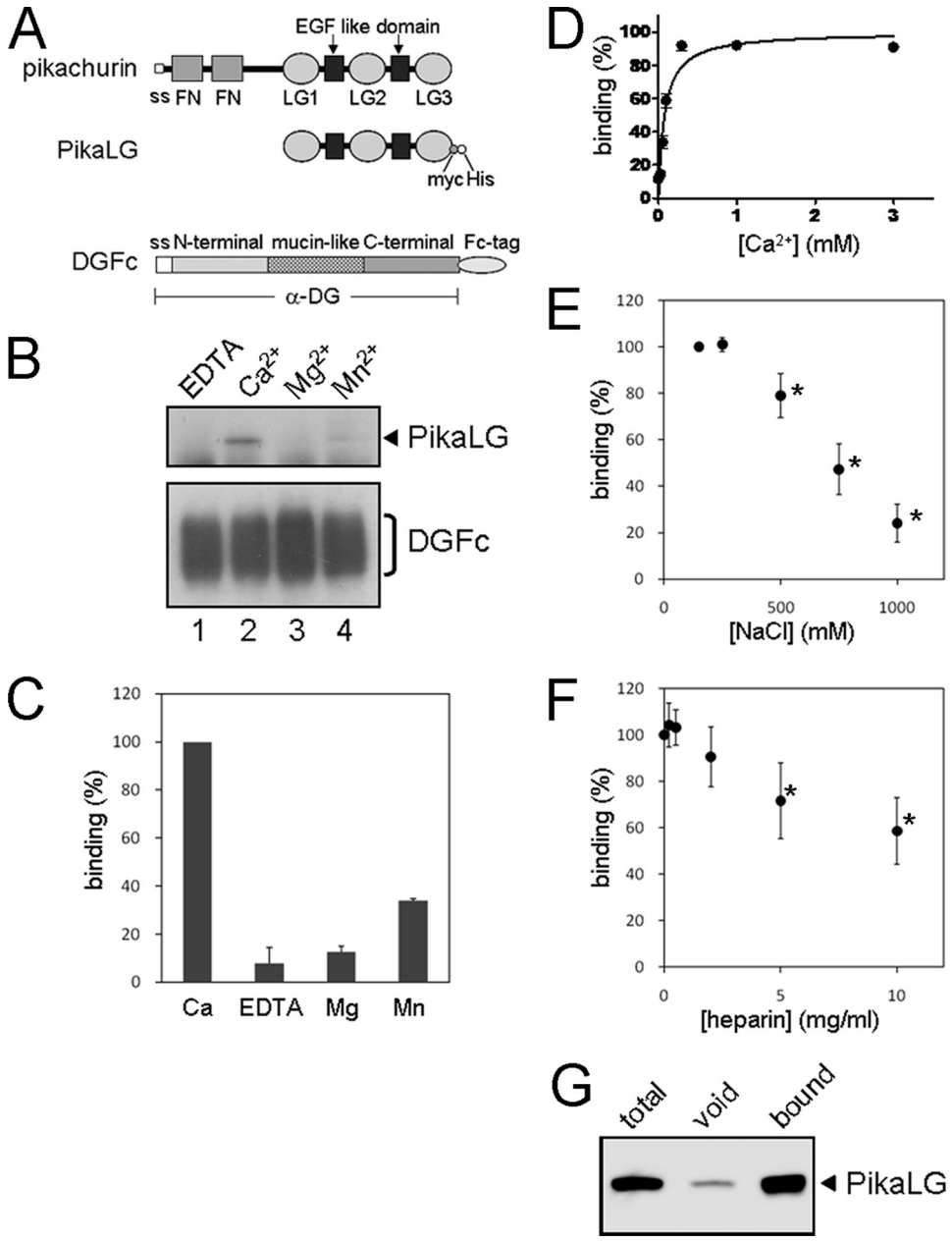

FIGURE 1. **Biochemical characterization of pikachurin-dystroglycan interaction.** *A*, schematic representation of recombinant pikachurin and α-DG. Pikachurin contains a signal sequence (*ss*), two fibronectin 3 (*FN*) domains, three laminin globular (*LG*) domains, and two calcium-binding EGF-like (*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 $Y = B_{max}\,x/(K_d + x)$, where K_d is the concentration required to reach half-maximal binding, and B_{max} is maximal binding. Maximal binding was set as 100%. $K_d = 78 \pm 15$ μM. 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. 1*E*). No significant inhibitory effect was detected with heparin at 2 mg/ml (Fig. 1*F*), 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. 1*F*). 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. 1*G*). 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. 2*A*) 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. 2*B*). We found that the LG2-LG3 tandem construct binds to DGFc at a level similar to that of the full-length construct (Fig. 2*B, 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. 2*C*). 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. 2*D*). This result indicates that pikachurin forms oligomeric structures. Al-

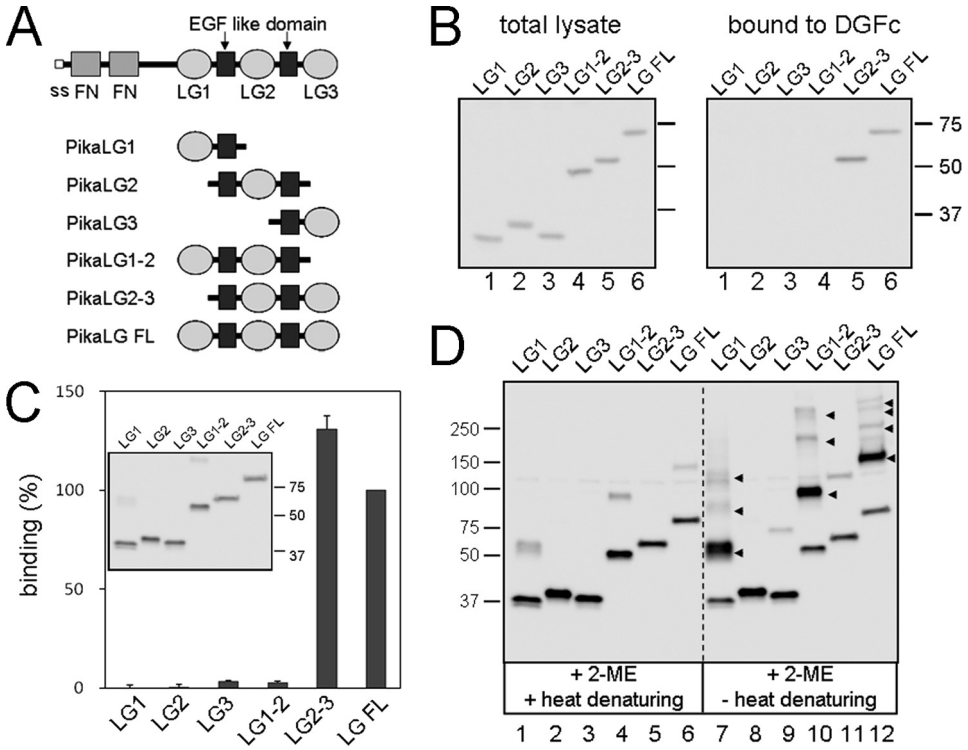

FIGURE 2. **Dissection of the dystroglycan binding region in pikachurin.** *A*, schematic representation of pikachurin deletion mutant proteins. All constructs contain a tandem myc-His tag at the C terminus. *ss*, signal sequence. *B*, binding of pikachurin deletion constructs to dystroglycan. Each deletion 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. 3*A*). DG-N, which contains only the N-terminal domain, did not bind to PikaLG (Fig. 3*B*, *lane 2*). DGΔN, which lacks the N-terminal domain, also failed to bind to PikaLG, even though it contains the entire mucin-like domain (Fig. 3*B*, *lane 3*). Co-expression with LARGE enhanced PikaLG binding to full-length DGFc (DG-wt) (Fig. 3*B*, *lanes 4* and *5*). Using deletion constructs lacking the C-terminal domain (DGΔC) 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. 3*C*). 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-β1, 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. 4*A*). 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. 4*B*). 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, Ca^{2+}-sensitive binding was detected in control heterozygous samples (Fig. 4*C*). 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.

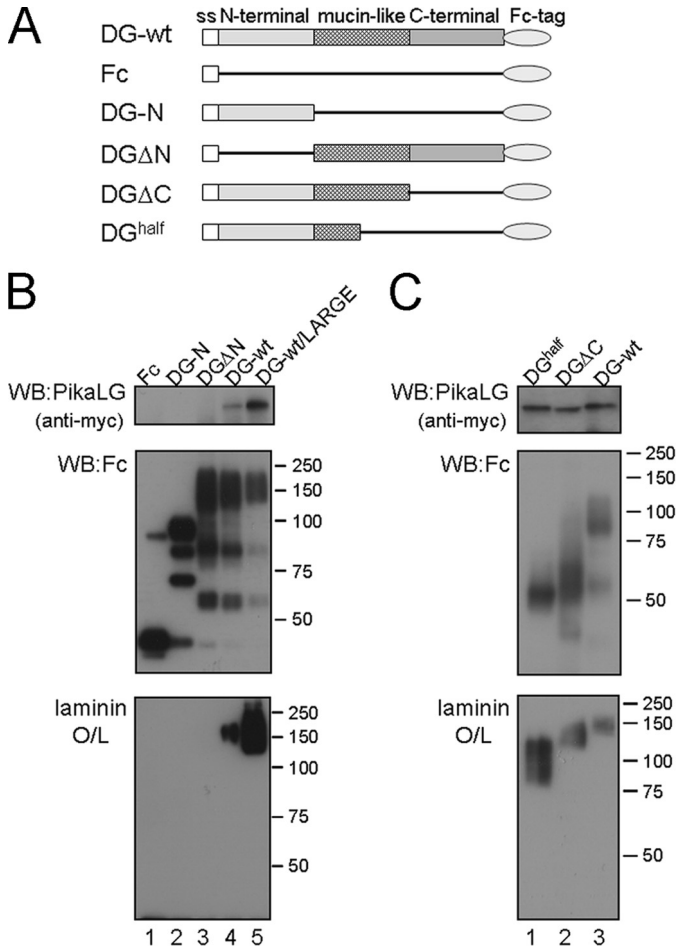

FIGURE 3. **Dystroglycan functional domains for pikachurin binding.** *A*, schematic representation of deletion mutants of DGFc proteins. *ss*, signal sequence. *B* and *C*, dissection of dystroglycan domains necessary for pikachurin binding. Each deletion construct was expressed in HEK293 cells and 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 DGFc mutant protein. PikaLG binding was detected by Western blotting with an anti-Myc antibody (*upper panel, PikaLG*). Comparable amounts of DGFc 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*).

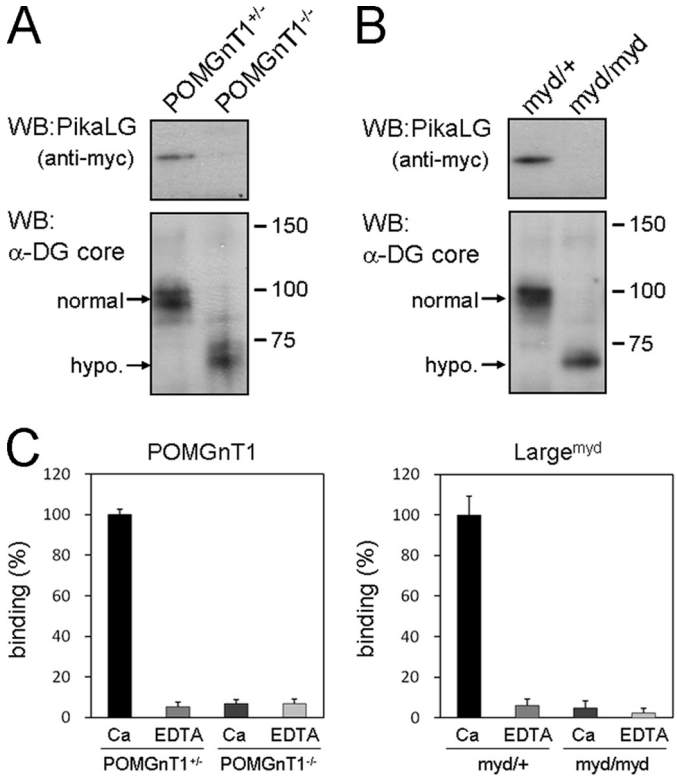

FIGURE 4. **Reduced pikachurin binding to α-dystroglycan in dystroglycanopathy animals.** α-DG was immunoprecipitated from the brains of *POMGnT1*-deficient (*A*) and *Large*^{myd} (*B*) mice. Littermates were used as controls. Lysates from PikaLG-expressing cells were incubated with the immunoprecipitated 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.

Immunofluorescence staining showed reduced pikachurin immunoreactivity in the ribbon synapse of *POMGnT1*-deficient mice, as compared with littermate heterozygous controls (Fig. 5*A*). 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. 5*B*). 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. 5*C*). Binding assays confirmed that pikachurin binding is also reduced in myd eye tissue (Fig. 5*D*). 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 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-β1, 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 α2-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-

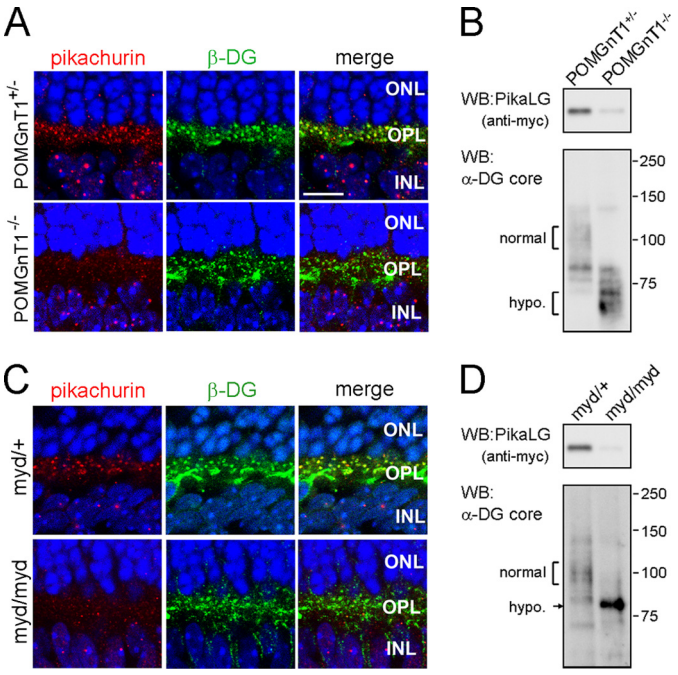

FIGURE 5. **Disruption of pikachurin localization in dystroglycanopathy animals.** *A* and *C*, immunofluorescence analysis of pikachurin in the outer plexiform layer (*OPL*). Retinal sections of *POMGnT1*-deficient (−/−) and *Large*^{myd} (myd/myd) mice, and their littermate heterozygous controls, were 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 μm. *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 α2-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 α1-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 α1-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 α2-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-β1,2-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 β3GnT1 is involved in LARGE-dependent modification (34). β3GnT1 interacts with LARGE, and reduced expression of β3GnT1 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*^{vls} 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.

Acknowledgments—We thank past and present members of the Toda laboratory for fruitful discussions and scientific contributions. We also thank Chiyomi Ito for technical support and Dr. Jennifer Logan for help with editing the manuscript.

REFERENCES

1. Barresi, R., and Campbell, K. P. (2006) *J. Cell. Sci.* **119,** 199–207
2. Cohn, R. D., Henry, M. D., Michele, D. E., Barresi, R., Saito, F., Moore, S. A., Flanagan, J. D., Skwarchuk, M. W., Robbins, M. E., Mendell, J. R., Williamson, R. A., and Campbell, K. P. (2002) *Cell* **110,** 639–648
3. Han, R., Kanagawa, M., Yoshida-Moriguchi, T., Rader, E. P., Ng, R. A., Michele, D. E., Muirhead, D. E., Kunz, S., Moore, S. A., Iannaccone, S. T., Miyake, K., McNeil, P. L., Mayer, U., Oldstone, M. B., Faulkner, J. A., and Campbell, K. P. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 12573–12579
4. Michele, D. E., Kabaeva, Z., Davis, S. L., Weiss, R. M., and Campbell, K. P. (2009) *Circ. Res.* **105,** 984–993
5. 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., Hoshi, T., and Campbell, K. P. (2002) *Nature* **418,** 422–425
6. Saito, F., Moore, S. A., Barresi, R., Henry, M. D., Messing, A., Ross-Barta, S. E., Cohn, R. D., Williamson, R. A., Sluka, K. A., Sherman, D. L., Brophy, P. J., Schmelzer, J. D., Low, P. A., Wrabetz, L., Feltri, M. L., and Campbell, K. P. (2003) *Neuron* **38,** 747–758
7. Satz, J. S., Philp, A. R., Nguyen, H., Kusano, H., Lee, J., Turk, R., Riker, M. J., Hernández, J., Weiss, R. M., Anderson, M. G., Mullins, R. F., Moore, S. A., Stone, E. M., and Campbell, K. P. (2009) *J. Neurosci.* **29,** 13136–13146
8. Chiba, A., Matsumura, K., Yamada, H., Inazu, T., Shimizu, T., Kusunoki, S., Kanazawa, I., Kobata, A., and Endo, T. (1997) *J. Biol. Chem.* **272,** 2156–2162
9. Manya, H., Chiba, A., Yoshida, A., Wang, X., Chiba, Y., Jigami, Y., Margolis, R. U., and Endo, T. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101,** 500–505
10. 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., Cormand, B., Lehesjoki, A. E., Cruces, J., Voit, T., Walsh, C. A., van Bokhoven, H., and Brunner, H. G. (2002) *Am. J. Hum. Genet.* **71,** 1033–1043
11. van Reeuwijk, J., Janssen, M., van den Elzen, C., Beltran-Valero de Bernabé, D., Sabatelli, P., Merlini, L., Boon, M., Scheffer, H., Brockington, M., Muntoni, F., Huynen, M. A., Verrips, A., Walsh, C. A., Barth, P. G., Brunner, H. G., and van Bokhoven, H. (2005) *J. Med. Genet.* **42,** 907–912
12. Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M., Herrmann, R., Straub, V., Talim, B., Voit, T., Topaloglu, H., Toda, T., and Endo, T. (2001) *Dev. Cell* **1,** 717–724
13. Yoshida-Moriguchi, T., Yu, L., Stalnaker, S. H., Davis, S., Kunz, S., Madson, M., Oldstone, M. B., Schachter, H., Wells, L., and Campbell, K. P. (2010) *Science* **327,** 88–92
14. Kobayashi, K., Nakahori, Y., Miyake, M., Matsumura, K., Kondo-Iida, E., Nomura, Y., Segawa, M., Yoshioka, M., Saito, K., Osawa, M., Hamano, K., Sakakihara, Y., Nonaka, I., Nakagome, Y., Kanazawa, I., Nakamura, Y., Tokunaga, K., and Toda, T. (1998) *Nature* **394,** 388–392
15. Brockington, M., Blake, D. J., Prandini, P., Brown, S. C., Torelli, S., Benson, M. A., Ponting, C. P., Estournet, B., Romero, N. B., Mercuri, E., Voit, T., Sewry, C. A., Guicheney, P., and Muntoni, F. (2001) *Am. J. Hum. Genet.* **69,** 1198–1209
16. Grewal, P. K., Holzfeind, P. J., Bittner, R. E., and Hewitt, J. E. (2001) *Nat. Genet.* **28,** 151–154
17. Barresi, R., Michele, D. E., Kanagawa, M., Harper, H. A., Dovico, S. A., Satz, J. S., Moore, S. A., Zhang, W., Schachter, H., Dumanski, J. P., Cohn, R. D., Nishino, I., and Campbell, K. P. (2004) *Nat. Med.* **10,** 696–703
18. Muntoni, F., Torelli, S., and Brockington, M. (2008) *Neurotherapeutics* **5,** 627–632
19. Godfrey, C., Clement, E., Mein, R., Brockington, M., Smith, J., Talim, B., Straub, V., Robb, S., Quinlivan, R., Feng, L., Jimenez-Mallebrera, C., Mercuri, E., Manzur, A. Y., Kinali, M., Torelli, S., Brown, S. C., Sewry, C. A., Bushby, K., Topaloglu, H., North, K., Abbs, S., and Muntoni, F. (2007) *Brain* **130,** 2725–2735
20. Lisi, M. T., and Cohn, R. D. (2007) *Biochim. Biophys. Acta* **1772,** 159–172
21. Sigesmund, D. A., Weleber, R. G., Pillers, D. A., Westall, C. A., Panton, C. M., Powell, B. R., Héon, E., Murphey, W. H., Musarella, M. A., and Ray, P. N. (1994) *Ophthalmology* **101,** 856–865
22. Sato, S., Omori, Y., Katoh, K., Kondo, M., Kanagawa, M., Miyata, K., Funabiki, K., Koyasu, T., Kajimura, N., Miyoshi, T., Sawai, H., Kobayashi, K., Tani, A., Toda, T., Usukura, J., Tano, Y., Fujikado, T., and Furukawa, T. (2008) *Nat. Neurosci.* **11,** 923–931
23. Lee, Y., Kameya, S., Cox, G. A., Hsu, J., Hicks, W., Maddatu, T. P., Smith, R. S., Naggert, J. K., Peachey, N. S., and Nishina, P. M. (2005) *Mol. Cell. Neurosci.* **30,** 160–172
24. Liu, J., Ball, S. L., Yang, Y., Mei, P., Zhang, L., Shi, H., Kaminski, H. J., Lemmon, V. P., and Hu, H. (2006) *Mech. Dev.* **123,** 228–240
25. Kanagawa, M., Nishimoto, A., Chiyonobu, T., Takeda, S., Miyagoe-Suzuki, Y., Wang, F., Fujikake, N., Taniguchi, M., Lu, Z., Tachikawa, M., Nagai, Y., Tashiro, F., Miyazaki, J., Tajima, Y., Takeda, S., Endo, T., Kobayashi, K., Campbell, K. P., and Toda, T. (2009) *Hum. Mol. Genet.* **18,** 621–631
26. Miyagoe-Suzuki, Y., Masubuchi, N., Miyamoto, K., Wada, M. R., Yuasa, S., Saito, F., Matsumura, K., Kanesaki, H., Kudo, A., Manya, H., Endo, T., and Takeda, S. (2009) *Mech. Dev.* **126,** 107–116
27. Ervasti, J. M., and Campbell, K. P. (1993) *J. Cell Biol.* **122,** 809–823
28. Gee, S. H., Montanaro, F., Lindenbaum, M. H., and Carbonetto, S. (1994) *Cell* **77,** 675–686
29. Ervasti, J. M., Burwell, A. L., and Geissler, A. L. (1997) *J. Biol. Chem.* **272,** 22315–22321
30. Kanagawa, M., Saito, F., Kunz, S., Yoshida-Moriguchi, T., Barresi, R., Kobayashi, Y. M., Muschler, J., Dumanski, J. P., Michele, D. E., Oldstone, M. B., and Campbell, K. P. (2004) *Cell* **117,** 953–964
31. Sugita, S., Saito, F., Tang, J., Satz, J., Campbell, K., and Südhof, T. C. (2001)

J. Cell Biol. **154,** 435–445
32. Hohenester, E., Tisi, D., Talts, J. F., and Timpl, R. (1999) *Mol. Cell* **4,** 783–792
33. Talts, J. F., Andac, Z., Göhring, W., Brancaccio, A., and Timpl, R. (1999) *EMBO J.* **18,** 863–870
34. Bao, X., Kobayashi, M., Hatakeyama, S., Angata, K., Gullberg, D., Nakayama, J., Fukuda, M. N., and Fukuda, M. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 12109–12114
35. Holzfeind, P. J., Grewal, P. K., Reitsamer, H. A., Kechvar, J., Lassmann, H., Hoeger, H., Hewitt, J. E., and Bittner, R. E. (2002) *Hum. Mol. Genet.* **11,** 2673–2687
36. Montanaro, F., Carbonetto, S., Campbell, K. P., and Lindenbaum, M. (1995) *J. Neurosci. Res.* **42,** 528–538
37. Blank, M., Koulen, P., and Kröger, S. (1997) *J. Comp. Neurol.* **389,** 668–678
38. Koulen, P., Blank, M., and Kröger, S. (1998) *J. Neurosci. Res.* **51,** 735–747
39. Blank, M., Koulen, P., Blake, D. J., and Kröger, S. (1999) *Eur. J. Neurosci.* **11,** 2121–2133
40. Jastrow, H., Koulen, P., Altrock, W. D., and Kröger, S. (2006) *Invest. Ophthalmol. Vis. Sci.* **47,** 17–24
41. Ohlendieck, K., and Campbell, K. P. (1991) *J. Cell. Biol.* **115,** 1685–1694
42. Cibis, G. W., Fitzgerald, K. M., Harris, D. J., Rothberg, P. G., and Rupani, M. (1993) *Invest. Ophthalmol. Vis. Sci.* **34,** 3646–3652
43. Pillers, D. A., Fitzgerald, K. M., Duncan, N. M., Rash, S. M., White, R. A., Dwinnell, S. J., Powell, B. R., Schnur, R. E., Ray, P. N., Cibis, G. W., and Weleber, R. G. (1999) *Hum. Genet.* **105,** 2–9
44. D'Souza, V. N., Nguyen, T. M., Morris, G. E., Karges, W., Pillers, D. A., and Ray, P. N. (1995) *Hum. Mol. Genet.* **4,** 837–842
45. Kameya, S., Araki, E., Katsuki, M., Mizota, A., Adachi, E., Nakahara, K., Nonaka, I., Sakuragi, S., Takeda, S., and Nabeshima, Y. (1997) *Hum. Mol. Genet.* **6,** 2195–2203