Absence of Post-phosphoryl Modification in Dystroglycanopathy Mouse Models and Wild-type Tissues Expressing Non-laminin Binding Form of α-Dystroglycan

Received for publication, June 14, 2011, and in revised form, January 13, 2012. Published, JBC Papers in Press, January 23, 2012, DOI 10.1074/jbc.M111.271767

Atsushi Kuga,‡ Motoi Kanagawa,‡ Atsushi Sudo,‡ Yiumo Michael Chan,§ Michiko Tajiri,¶ Hiroshi Manya,¶ Yamato Kikkawa, Motoyoshi Nomizu, Kazuhiro Kobayashi, Tamao Endo,‡ Qi L. Lu,¶ Yoshinao Wada,‡ and Tatsushi Toda‡

From the ‡Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan, the §McColl-Lockwood Laboratory for Muscular Dystrophy Research, Neuromuscular/ALS Center, Carolinas Medical Center, Charlotte, North Carolina 28231, the ¶Department of Molecular Medicine, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka 594-1101, Japan, the Molecular Glycobiology, Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015, Japan, and the **Laboratory of Clinical Biochemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Tokyo 192-0392, Japan

Background: The biosynthetic pathway for the ligand-binding moiety of α-dystroglycan, defects in which cause dystroglycanopathy, remains unclear.

Results: The phosphodiester-linked moiety on O-mannose is absent in dystroglycanopathy models and in wild-type lung and testis.

Conclusion: Post-phosphoryl modification is a key determinant of the functional expression of α-dystroglycan as a laminin receptor.

Significance: This work expands our understanding of the molecular mechanism of a unique post-translational modification.

α-Dystroglycan (α-DG) is a membrane-association glycoprotein that interacts with several extracellular matrix proteins, including laminin and agrin. Aberrant glycosylation of α-DG disrupts its interaction with ligands and causes a certain type of muscular dystrophy commonly referred to as dystroglycanopathy. It has been reported that a unique O-mannosyl tetrascartridge (Neu5Ac-α2,3-Gal-β1,4-GlcNAc-β1,2-Man) and a phosphodiester-linked modification on O-mannose play important roles in the laminin binding activity of α-DG. In this study, we use several dystroglycanopathy mouse models to demonstrate that, in addition to fukutin and LARGE, FKRP (fukutin-related protein) is also involved in the post-phosphoryl modification of O-mannose on α-DG. Furthermore, we have found that the glycosylation status of α-DG in lung and testis is minimally affected by defects in fukutin, LARGE, or FKRP. α-DG prepared from wild-type lung- or testis-derived cells lacks the post-phosphoryl moiety and shows little laminin-binding activity. These results show that FKRP is involved in post-phosphoryl modification rather than in O-mannosyl tetrascartridge synthesis. Our data also demonstrate that post-phosphoryl modification not only plays critical roles in the pathogenesis of dystroglycanopathy but also is a key determinant of α-DG functional expression as a laminin receptor in normal tissues and cells.

Dystroglycan (DG), a cell surface receptor for several extracellular matrix proteins, plays important roles in various tissues (1). DG consists of a heavily glycosylated extracellular 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 (2). α-DG binds to extracellular proteins such as laminin, agrin, perlecan, neurexin, and pikachurin (2–7). β-DG anchors α-DG at the cell surface and binds intracellularly to dystrophin, which in turn binds to the actin cytoskeleton. Thus, α/β-DG functions as a molecular axis, connecting the extracellular matrix with the cytoskeleton across the plasma membrane (1).

O-Glycosylation of α-DG is necessary for its interaction with ligands, and genetic disruption of the glycosylation pathway for DG is associated with a group of muscular dystrophies known as “dystroglycanopathy” (8–10). Six genes (POMT1, POMT2, POMGnT1, fukutin, FKRP, and LARGE) have been identified as causative genes for dystroglycanopathy. A common biochemical characteristic of these disorders is abnormal glycosylation and reduced laminin-binding activity of α-DG; however, the precise glycan structure required for α-DG ligand binding is not completely determined. Two unique O-mannosyl modifications have been identified in α-DG: an O-mannosyl tetrascartridge (Neu5Ac-α2,3-Gal-β1,4-GlcNAc-β1,2-Man) (11), and

‡This work was supported by Ministry of Health, Labor, and Welfare of Japan Intramural Research Grant (23B-5) for Neurological and Mental Disorders and The Research on Psychiatric and Neurological Diseases and Mental Health H20-016 (to T. T.), Grant-in-aid for Scientific Research (A) 23249049 (to T. T.) and a Grant-in-aid for Young Scientists (B) 21790318 (to M. K.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and the Takeda Science Foundation (to M. K.).

This article contains supplemental Fig. 1.

1To 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.

* This work was supported by Ministry of Health, Labor, and Welfare of Japan Intramural Research Grant (23B-5) for Neurological and Mental Disorders and The Research on Psychiatric and Neurological Diseases and Mental Health H20-016 (to T. T.), Grant-in-aid for Scientific Research (A) 23249049 (to T. T.) and a Grant-in-aid for Young Scientists (B) 21790318 (to M. K.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and the Takeda Science Foundation (to M. K.).

**This article contains supplemental Fig. 1.

The abbreviations used are: DG, dystroglycan; IMAC, immobilized metal affinity chromatography; MW, molecular weight; HFaq, aqueous hydrofluoric acid.
a phosphodiester-linked branch structure present at the C6 hydroxyl residue of O-mannose (12). The O-mannosyl tetrasaccharide was first identified on peripheral nerve α-DG (11). The initial mannoside transferred to Ser/Thr residues on the α-DG polypeptide backbone is catalyzed by the POMT1/POMT2 complex (13). Mutations in POMT1 and POMT2 were originally identified as causative for Walker-Warburg syndrome (14, 15). POMGnT1, established as a causative gene for muscle-eye-brain disease, encodes a glycosyltransferase that transfers GlcNAc to O-mannose on α-DG (16). In these disorders, α-DG lacks laminin-binding activity (17); therefore, the tetrasaccharide plays an important role in the post-translational maturation of α-DG as a laminin receptor. On the other hand, recent studies have suggested that the Neu5Ac-α2,3-Gal-β1,4-GlcNAc branch on O-mannose per se is not likely the laminin-binding glycan of α-DG (12, 18).

fukutin was originally identified as the causative gene for Fukuyama-type congenital muscular dystrophy (19), and FKRP was identified as the causative gene for both MDC1C (congenital muscular dystrophy type 1C) (20) and LGMD2I (limb-girdle muscular dystrophy in the spontaneous mouse model) (21). The precise function of fukutin and FKRP is still uncertain. Mutation of LARGE causes muscular dystrophy in the spontaneous Largeemouse model (22) and in human congenital muscular dystrophy type 1D (23). Recently, a phosphodiester-linked modification on an O-mannose was identified (12). It was shown that α-DG in fukutin-mutated Fukuyama-type congenital muscular dystrophy and Largeemuscle cells exhibits defective post-phosphoryl modification on the O-mannose, suggesting that this phosphorylated branch serves as the laminin-binding moiety. To explore the role of phosphorylated O-mannose in functional α-DG ligand-binding and in other forms of dystroglycanopathy, we have investigated α-DG glycosylation in several dystroglycanopathy mouse models.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—TM3 and CHL cell lines were purchased from European collection of cell cultures and the RIKEN BioResource Center, respectively. TM3 cells were cultured in Ham’s F12/DMEM (1:1) containing 5% horse serum and 2.5% fetal bovine serum. CHL cells were cultured in DMEM containing 10% fetal bovine serum. Expression vectors for LARGE were grown at 37 °C and harvested at 48 h after transfection. The transfected cells were solubilized in TBS with 1% Triton X-100. The transfected cells were centrifuged at 15,000 rpm for 10 min at 4 °C. Supernatants were collected, and protein concentrations were measured by Lowry methods, using BSA as a standard.

**Animals**—Largeemice were obtained from The Jackson Laboratory. Generation of FKRP-neo-P448L knock-in mice, Hpl−/− mice, and POMGnTI-deficient mice has been described previously (25–27). Mice were maintained in accordance with the animal care guidelines of Kobe University. All animal studies using FKRP-neo-P448L knock-in mice were approved by the Institutional Animal Care and Use Committee of the Carolinas Medical Center.

**Protein Enrichment**—Frozen tissue samples were solubilized in TBS (pH 7.4) with 1% Triton X-100. The solubilized materials were incubated with wheat germ agglutinin beads, and the DG-enriched fraction was then eluted with 0.3 M N-acetyl-d-glucosamine in TBS containing 0.1% Triton X-100. For the immobilized metal affinity chromatography (IMAC)-binding assay, aqueous hydrofluoric acid (HFaq) treatment, and deglycosylation assay, the DG-enriched fractions were diluted in 0.25% CHAPS/water (w/v) and then desalted and concentrated using Amicon-ultra filters (Millipore).

**IMAC-binding Assay**—Samples were diluted in a solution containing 250 mM acetic acid, 30% acetonitrile, and 0.15% CHAPS and incubated with PHOS-Select iron affinity gel (Sigma) at room temperature for 0.5 h. Bound materials were directly eluted with SDS-loading buffer. Equal ratios of the void and the bound samples were used for Western blot analysis.

**HFaq Treatment**—Samples were incubated with 48% aqueous hydrofluoric acid (Wako) on ice for 12 h. Control samples were incubated with water instead of hydrofluoric acid. After removal of the reagents under a stream of nitrogen gas, residues were dissolved with SDS-loading buffer for Western blot analysis.

**Deglycosylation Assay**—Glycopeptidase F (peptide-N-glycosidase; Wako), α-2 (3, 6, 8, 9) neuraminidase (Calbiochem), β1–4 galactosidase (New England Biolabs), β-N-acetyl-hexosaminidase (Seikagaku Corp.), and O-glycosidase (Roche Applied Science) were used according to the manufacturer’s protocol.

**Antibodies**—Antibodies used for Western blotting were mouse monoclonal antibody IHH6 against glycosylated α-DG (Millipore) and goat polyclonal antibody against the C-terminal domain of the α-DG polypeptide (AP-074G-C) (26).

**Laminin and Agrin Overlay Assays**—Recombinant mouse laminin LG4–5 domains of laminin α1 and laminin α2 chains fused to Fc tags were recovered from the cell culture media using protein A beads (28). Recombinant agrin was purchased from R&D Systems. Laminin and agrin overlay assays were performed as described previously (26).

**RT-PCR Analysis**—Total RNA was isolated from wild-type mouse testis and TM3 cells using the RNeasy Plus mini kit (Qiagen) and converted to cDNA using Superscript III reverse transcriptase (Invitrogen). The forward and reverse primers used in gene amplification were as follows: Large (5’-TCAAT-CTTTCTGCGAAACGTG-3’ and 5’-TCCAACTTTAGCAGCATGCCGCT-3’), POMT1 (5’-CGGGTGTCCTTTGCTTCTG-3’ and 5’-AGTGACTGACCGCCGCA-3’), POMT2 (5’-CGGAACTTGCAGCTCA-3’ and 5’-ATCCGCCAGAAGTCATTG-3’), POMGnTI (5’-CCAAGGGGTATCTCCACAGA-3’ and 5’-GGTCTCCTTCCAGAACACCA-3’), fukutin (5’-CGCAGTTCGATCTAC-3’ and 5’-GGTGGCCGCAGGGT-3’), FKRP (5’-CTTCTGTCCTTGCCTTTCCTG-3’ and 5’-CAAAGAGGGGACATGAGGTTG-3’), GFPP (5’-TTCAATCGAATCAGCCAGGTA-3’ and 5’-TCTCTAATCCTTCCATCA-3’), GAPDH (5’-CGT-AGAATAAATGTTAAGG-3’ and 5’-TCTTTGCTAGAG-3’).
α-Dystroglycan in FKRP Mutants

GACCTTG3'), and DAG1 (5′-ACCAAGCACCACATCAG-3′ and 5′-GTTCACCCAGGCATCTAC-3′).

RESULTS

Defects of Post-phosphoryl Modification in FKRP-deficient Mice—To examine whether dystroglycanopathy models share a common defect in the post-phosphoryl modification of α-DG, we performed an IMAC bead-binding assay. IMAC beads bind to monooester-linked, but not diester-linked, phosphorylated compounds, and it has been shown that post-phosphoryl modification binds to IMAC beads (12). First, we used Large

We next examined whether FKRP is also involved in the post-phosphoryl modification of α-DG. Consistent with previous observations, α-DG from the skeletal muscle of homozygous FKRP-neo-P448L knock-in mice (FKRP-P448L mice) was aberrantly glycosylated, as indicated by the loss of IIH6 reactivity (25). The hypoglycosylated α-DG, showing a lower MW of 90,000 compared with wild-type α-DG at 150,000, bound to the IMAC beads (Fig. 2A, lower panel). In brain tissue, IIH6-positive α-DG shows a MW of 100,000, whereas hypoglycosylated α-DG shows a MW of 70,000. As was the case in skeletal muscle, hypoglycosylated α-DG from the homogygous mouse bound to IMAC beads (Fig. 2A). It has been reported that treatment with cold HFaq cleaves the phosphodiester linker in α-DG (12). After HFaq treatment, the MW of α-DG was reduced to ∼90,000, and α-DG lost IIH6-reactivity (Fig. 2B, left panel). In contrast to the mature α-DG from heterozygous controls, the hypoglycosylated α-DG from homozygous FKRP-P448L muscle showed almost no change in MW after the HFaq treatment (Fig. 2B, right panel). Treatment with several mixtures of glycosidase predicted to remove N-glycan, mucin type O-glycan, and the trisaccharide at the non-reducing end of the Neu5Acα2,3-Galβ1,4-GlcnAcβ1,2-Man glycan (12, 18) generated stepwise decreases in the MW of α-DG through multi-step digestions (Fig. 2C). These results indicate for the first time that FKRP is involved in the post-phosphoryl modification of α-DG rather than in the synthesis of the Neu5Acα2,3-Galβ1,4-GlcnAcβ1,2-Man glycan. This concept is supported by the previous observation that neither POMT1 nor POMGnT1 activity was reduced in lymphoblast cells from patients with FKRP mutations (29). Overall, our results establish and confirm that a defect in post-phosphoryl modification on O-mannose is a common biochemical characteristic in dystroglycanopathy caused by mutations in LARGE, POMGnT1, fukutin, and FKRP.

Defects of Post-phosphoryl Modification in FKRP-deficient Mice—We have demonstrated that disruption of Large, fukutin, or FKRP decreases the MW of α-DG in skeletal muscle and brain due to the lack of post-phosphoryl modification. It is known that the MW of α-DG and its reactivity to the monoclonal antibody IIH6 vary among different tissues (1, 30). We hypothesized that the low MW of α-DG in some tissues may result from the lack of post-phosphoryl modification and/or the Neu5Acα2,3-Galβ1,4-GlcnAcβ1,2-Man glycan. Several tissues from dystroglycanopathy model mice were therefore investigated. We found that the decreases in the MW of α-DG were relatively minor in lung and very scarce in testis from FKRP-P448L mice and Hp/l−/− mice when compared with litter controls (Fig. 3A). Minor changes in α-DG MW in the lung and testis of Large-deficient mice have been also observed elsewhere (30), supporting our findings. On the other hand, α-DG in lung and testis from POMGnT1-deficient mice clearly shows a lower MW compared with litter heterozygous controls and other mutant mouse strains (Fig. 3B). These results suggested that the GlcnAcβ1,2 branch on O-mannose is present in wild-type α-DG in lung and testis, but post-phosphoryl modification is absent.

We examined these tissues in wild-type mice using an IMAC bead-binding assay and HFaq treatment. In Fig. 4A, Western blot analysis of wild-type tissues showed that α-DG in testis has a MW of 90,000 and was not recognized by the IIH6 antibody,
FIGURE 2. Defects of post-phosphoryl modification in the FKRP-deficient disease model. A, IMAC bead-binding assay for FKRP-deficient mice. α-DG enriched samples from skeletal muscle and brain of FKRP-P448L homozygous (homo) and litter control heterozygous (hetero) mice were tested for binding to IMAC beads. The void (v) and bound (b) fractions were collected. B, chemical dephosphorylation of α-DG from FKRP-deficient mice. α-DG enriched samples from skeletal muscle of FKRP-P448L homozygous (homo) and litter control heterozygous (hetero) mice were treated with HFaq. *, these bands are not likely derived from α-DG because they are not recognized by antibodies against the α-DG core protein. C, enzymatic deglycosylation of α-DG from FKRP-deficient mice. α-DG-enriched samples from skeletal muscle of FKRP-P448L homozygous mice were digested with glycosidase mixtures (peptide-N-glycosidase (PNGase F), neuraminidase, β1–4 galactosidase/β-N-acetylhexosaminidase, and O-glycosidase). Following the IMAC bead-binding assay, HFaq treatment, and enzymatic digestions, the samples were analyzed by Western blot, using antibodies against the α-DG core protein (Core) or the functionally glycosylated form (IIH6). v, void fraction; b, bound fraction.

and that lung α-DG consists of two major detectable populations (IIH6-positive > 100,000, minor form, arrow; IIH6-negative < 100,000, major form, arrowhead). Both testis and lung IIH6-negative α-DG were found to bind to IMAC beads, in contrast with IIH6-positive α-DG in skeletal muscle, brain, liver, and lung (Fig. 4A). Furthermore, HFaq treatment reduced the MW of α-DG to ~75,000 in wild-type skeletal muscle, brain, and liver. On the other hand, the MW shift observed in testis α-DG and IIH6-negative lung α-DG was relatively minor following HFaq treatment (Fig. 4B). These data indicate the absence of post-phosphoryl modification on α-DG in some wild-type tissues. Ligand overlay assays showed that IIH6-positive α-DGs in skeletal muscle, brain, and lung bound to the ligand proteins laminin α1, α2, and agrin, whereas IIH6-negative α-DG in testis and lung did not bind to these ligands (Fig. 5). Altogether, these data confirm that IIH6-reactivity and laminin-binding activity in α-DG are associated with post-phosphoryl modification.

Because lung and testis tissues contain heterogeneous cell types, we also examined the established cell lines CHL (lung epithelial cells derived from Chinese hamster) and TM3 (Leydig cells derived from mouse testis). Both CHL and TM3 cells showed detectable amounts of endogenous α-DG using the core antibody, but they did not react with IIH6 (Fig. 6, A and D). RT-PCR analysis showed that known genes (Large, POMT1, POMT2, POMGnT1, fukutin, FKRP, and β3GnT1) involved in α-DG glycosylation were expressed in TM3 cells (Fig. 6B). β3GnT1 has been reported to be required for laminin-binding glycans formation through the complex of α-DG with LARGE (31). We did not examine expression in CHL cells because the sequences of these genes have not yet been determined in the hamster. Endogenous α-DG in CHL and TM3 cells bound to IMAC beads (Fig. 6A). HFaq treatment resulted in almost no change in the MW of α-DG in both CHL and TM3 cells (Fig. 6C), as was similarly seen in lung and testis tissues (Fig. 4B). Following sequential digestion with glycosidases, α-DG in both CHL and TM3 cells showed stepwise reductions in MW (Fig. 6D). These data suggest that the post-phosphoryl modification is absent from IIH6-negative α-DG in CHL and TM3 cells.

DISCUSSION

In the present study, we demonstrate for the first time that FKRP is involved in post-phosphoryl modification on O-mannose of α-DG. We also show that even in wild type, α-DG in certain tissues such as lung and testis lacks the post-phosphoryl modification.

Abnormal glycosylation of α-DG in dystroglycanopathies is usually determined by a loss of reactivity against monoclonal antibodies VIA4–1 or IIH6. Mutations in the POMT1/POMT2 complex result in O-mannosylation defects (13–15); therefore, O-mannosyl phosphorylation does not occur. α-DG in cells with mutations in Large, fukutin, or POMGnT1 does not undergo further modification from phospho-mannose residues...
**α-Dystroglycan in FKRP Mutants**

Our data add new evidence that mutations in *FKRP* also result in the absence of the post-phosphoryl moiety. It remains unclear how defects in *Large*, *fukutin*, *POMGnT1*, or *FKRP* all result in the same loss of the post-phosphoryl modification. A possible explanation is that these proteins may form a complex or be sequentially activated to create the post-phosphoryl moiety. *POMGnT1* catalyzes GlcNAc transfer to *O*-mannose, and thus, it may not have direct involvement in the synthesis of the post-phosphoryl structure; however, the defects in post-phosphoryl modification in *POMGnT1*-deficient cells or tissues, shown here and in another study (12), indicate that the GlcNAc-β1,2 branch on *O*-mannose might provide favorable circumstances for the post-phosphoryl modification. Together, these studies have suggested that recognition by IIH6 requires at least the post-phosphoryl structure on *O*-mannose.

The range of α-DG molecular size and its reactivity to the monoclonal antibody IIH6 varies widely among different tissues. This has been thought to result from tissue-specific glycosylation on α-DG (1, 30). Our results indicate that post-phosphoryl modification is tissue-specific and thus suggest that the difference is largely determined by the extent and/or the proportion of post-phosphoryl modification. In light of the lack of post-phosphoryl modification in normal tissues such as lung and testis, even in the presence of transcripts of all known genes responsible for α-DG glycosylation, possible explanations are that they may not be properly translated; their protein products may be inactive (e.g. improper cellular location and lack of modification); or protein levels are not sufficient for α-DG glycosylation. Another possibility is that there could exist other yet-to-be identified mechanisms for α-DG modification, for example, a negative regulator, or novel genes. Supporting this idea, a large-scale genetic study has indicated that almost half of dystroglycanopathy cases can be explained by unidentified disease-causing genes or factors (32). Some of these cases might be caused by mutations in unidentified disease-causing genes, whose products are involved in post-phosphoryl modification, and such genes might not be expressed in wild-type tissues lacking post-phosphoryl modification of α-DG. This situation is exemplified in studies using cancer cells. It has been reported that several malignant cancer cell types lose the laminin-binding glycan of α-DG due to epigenetic down-regulation of LARGE or defects in the LARGE-binding protein β3GnT1, raising the possibility of defects in post-phosphoryl modification of α-DG in those cells (31, 33).

Reduction or loss of IIH6 reactivity can be rescued by forced expression of LARGE (34, 35). It has been shown that exogenously expressed LARGE can overcome defects in the laminin-binding activity of α-DG in *fukutin* or *POMGnT1*-deficient cells or tissues (26, 34). On the other hand, if cells lack a gene that acts via direct interaction with LARGE, such as β3GnT1, forced expression of LARGE would fail to produce IIH6 reactivity (31). We observed that forced expression of LARGE could produce IIH6 reactivity in CHL cells, and newly produced IIH6-reactive α-DG no longer bound to IMAC-beads (supplemental Fig. 1). The effect of LARGE overexpression on α-DG glycosylation was also observed in TM3 cells. These data indicate that CHL and TM3 cells might lack gene activity that is involved in the post-phosphoryl modification, but such defects can be compensated by overexpression of LARGE.

Our results also raised a question about the function of the non-laminin-binding form of α-DG. It is generally thought that α-DG function relies on its glycosylation-dependent laminin-binding activity; on the other hand, several studies have suggested that dystroglycan possesses functions beyond that of a laminin receptor. The N-terminal domain of α-DG, which can be shed from the core protein into the extracellular space and body fluid (36), has been shown to promote neurite extension in PC12 cells, suggesting that it has a biological function (37).
α-Dystroglycan in FKRP Mutants

**FIGURE 4.** Absence of post-phosphoryl modification in wild-type lung and testis. A, IMAC bead-binding assays for α-DG from wild-type tissues. α-DG-enriched samples from skeletal muscle (skm), brain, liver, lung, and testis of C57BL/6 mice were tested for binding to IMAC beads. The void (v) and bound (b) fractions were collected. The arrow indicates the IIH6-positive population of lung α-DG. The arrowhead indicates the IIH6-negative fraction of lung α-DG bound to beads. An asterisk indicates a background signal that is not specific for IIH6 antibody. B, chemical dephosphorylation of α-DG from wild-type tissues. α-DG-enriched samples from skeletal muscle (skm), brain, liver, lung and testis of C57BL/6 mice were treated with HFaq and then analyzed by Western blot using anti-DG core antibody.

**FIGURE 5.** Ligand-binding assays for lung and testis α-DG. Ligand binding (laminin α1, α2, and agrin) was assessed in α-DG-enriched samples from skeletal muscle (skm), brain, lung, and testis using ligand overlay assays.

α-DG might have ligand proteins that do not require O-mannosyl modification; for example, a chondroitin sulfate proteoglycan biglycan has been shown to interact with protein core of the α-DG C-terminal domain in a glycosylation-independent manner (38). DG is also thought to serve as a signaling molecule (39). For example, the cytoplasmic tail of β-DG interacts with several signaling molecules, including caveolin-3, Grb2, and mitogen-activated protein (MAP) kinase kinase 2 (40). Although the significance of these interactions is not well understood, it is possible that DG serves as a scaffold to position interacting proteins at their proper cellular location (9, 41). Taken together, these observations suggest that the presence of...
DG without post-phosphoryl modification could be functionally important in various tissue types. 

Future work to determine the molecular structure of the post-phosphoryl moiety, and to identify genes involved in its biosynthesis, will contribute to understanding the biological basis of this unique post-translational modification and disease pathogenesis. Our present data contributes to the foundation for such research. Recently, it has been shown that LARGE can act as a bifunctional glycosyltransferase, with both xylosyltransferase and glucuronyltransferase activities (42). Involvement of these activities in the post-phosphoryl modification also should be clarified in the future.

Overall, our results indicate that phosphorylated O-mannose not only plays critical roles in the pathogenesis of dystroglycanopathy but also is a key determinant in the maturation of α-DG as a laminin receptor in normal tissues and cells.

Acknowledgments—We thank Chiyomi Ito and Elizabeth Keramaris for technical support. We also thank Dr. Yuko Miyagoe-Suzuki and Dr. Shin’ichi Takeda for providing POMGnT1 knock-out mice and Dr. Jennifer Logan for help in editing the manuscript.

REFERENCES

1. Barresi, R., and Campbell, K. P. (2006) Dystroglycan: From biosynthesis to pathogenesis of human disease. J. Cell Sci. 119, 199–207
2. Ibraghimov-Beskrovnaya, O., Ervasti, J. M., Leveille, C. I., Slaughter, C. A., Sernetz, S. W., and Campbell, K. P. (1992) Primary structure of dystro-phin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature 355, 696–702
3. Gee, S. H., Montanaro, F., Lindenbaum, M. H., and Carbonetto, S. (1994) Dystroglycan-α, a dystrophin-associated glycoprotein, is a functional agrin receptor. Cell 77, 657–686
4. Bowe, M. A., Deys, K. A., Leszyn, J. D., and Fallon, J. R. (1994) Identification and purification of an agrin receptor from Torpedo postsynaptic membranes: A heteromeric complex related to the dystroglycans. Neuron 12, 1173–1180
5. Talts, J. F., Andac, Z., Gohring, W., Brancaccio, A., and Timpl, R. (1999) Binding of the G domains of laminin α1 and α2 chains and perlecanc to heparin, sulfatides, α-dystroglycan, and several extracellular matrix proteins. EMBO J. 18, 863–870
6. Sugita, S., Saito, F., Tang, I., Sato, J., Campbell, K., and Südhof, T. C. (2001) A stoichiometric complex of neurexins and dystroglycan in brain. J. Cell Biol. 154, 435–445
7. Sato, S., Omoji, Y., Kato, K., Kondo, M., Kanagawa, M., Miyata, K., Funabiki, K., Koyasu, T., Kajimura, N., Minao, K., Koyasu, T., Kajimura, N., Kanazawa, I., Kobata, A., and Endo, T. (1997) Structures of sialylated O-linked oligosaccharides of bovine peripheral nerve α-dystroglycan. The role of a novel O-mannosyl-type oligosaccharide in the binding of α-dystroglycan with laminin. J. Biol. Chem. 272, 2156–2162
α-Dystroglycan in FKRP Mutants

Bushby, K., Topaloglu, H., North, K., Abbs, S., and Muntoni, F. (2007) Refining genotype phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan. Brain 130, 2725–2732

32. de Bernabé, D. B., Inamori, K., Yoshida-Moriguchi, T., Weydert, C. J., Harper, H. A., Willer, T., Henry, M. D., and Campbell, K. P. (2009) Loss of α-dystroglycan laminin binding in epithelium-derived cancers is caused by silencing of LARGE. J. Biol. Chem. 284, 11279–11284

33. 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) LARGE can functionally bypass α-dystroglycan glycosylation defects in distinct congenital muscular dystrophies. Nat. Med. 10, 696–703

34. Patnaik, S. K., and Stanley, P. (2005) Mouse large can modify complex N- and mucin O-glycans on α-dystroglycan to induce laminin binding. J. Biol. Chem. 280, 20851–20859

35. Saito, F., Saito-Arai, Y., Nakamura, A., Shimizu, T., and Matsumura, K. (2008) Processing and secretion of the N-terminal domain of α-dystroglycan in cell culture media. FEBS Lett. 582, 439–444

36. Hall, H., Bozic, D., Michel, K., and Hubbell, J. A. (2003) N-terminal α-dystroglycan binds to different extracellular matrix molecules expressed in regenerating peripheral nerves in a protein-mediated manner and promotes neurite extension of PC12 cells. Mol. Cell Neurosci. 24, 1062–1073

37. Bowe, M. A., Mendis, D. B., and Fallon, J. R. (2000) The small leucine-rich repeat proteoglycan biglycan binds to α-dystroglycan and is up-regulated in dystrophic muscle. J. Cell Biol. 148, 801–810

38. Higginson, J. R., and Winder, S. J. (2005) Dystroglycan: A multifunctional adaptor protein. Biochem. Soc. Trans. 33, 1254–1255

39. Spence, H. J., Dhillon, A. S., James, M., and Winder, S. J. (2004) Dystroglycan, a scaffold for the ERK-MAP kinase cascade. EMBO Rep. 5, 484–489

40. Moore, C. J., and Winder, S. J. (2010) Dystroglycan versatility in cell adhesion: A tale of multiple motifs. Cell Commun. Signal. 8, 3

41. Inamori, K., Yoshida-Moriguchi, T., Hara, Y., Anderson, M. E., Yu, L., Campbell, K. P. (2012) Dystroglycan function requires xylosyl- and glucuronyltransferase activities of LARGE. Science 335, 93–96