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Identification of a Post-translational Modification with Ribitol-Phosphate and Its Defect in Muscular Dystrophy

Graphical Abstract

- Ribitol 5-phosphate is a functional glycan unit in mammals
- α-dystroglycan function requires tandem ribitol 5-phosphate structure
- Muscular dystrophy proteins are involved in ribitol 5-phosphate glycosylation
- Supplementation with ribitol 5-phosphate metabolites may be a therapeutic strategy

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In Brief
Kanagawa et al. show that ribitol 5-phosphate is a functional glycan unit in mammals and that defects in its post-translational modification pathway are associated with muscular dystrophy.

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Identification of a Post-translational Modification with Ribitol-Phosphate and Its Defect in Muscular Dystrophy

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SUMMARY

Glycosylation is an essential post-translational modification that underlies many biological processes and diseases. α-dystroglycan (α-DG) is a receptor for matrix and synaptic proteins that causes muscular dystrophy and lissencephaly upon its abnormal glycosylation (α-dystroglycanopathies). Here we identify the glycan unit ribitol 5-phosphate (Rbo5P), a phosphoric ester of pentose alcohol, in α-DG. Rbo5P forms a tandem repeat and functions as a scaffold for the formation of the ligand-binding moiety. We show that enzyme activities of three major α-dystroglycanopathy-causing proteins are involved in the synthesis of tandem Rbo5P. Isoprenoid synthase domain-containing (ISPD) is cytidine diphosphate ribitol (CDP-Rbo) synthase. Fukutin and fukutin-related protein are sequentially acting Rbo5P transfersases that use CDP-Rbo. Consequently, Rbo5P glycosylation is defective in α-dystroglycanopathy models. Supplementation of CDP-Rbo to ISPD-deficient cells restored α-DG glycosylation. These findings establish the molecular basis of mammalian Rbo5P glycosylation and provide insight into pathogenesis and therapeutic strategies in α-DG-associated diseases.

INTRODUCTION

α-dystroglycan (α-DG) was originally identified as a laminin receptor in the dystrophin-glycoprotein complex that is defective in Duchenne muscular dystrophy (Ibraghimov-Beskrovnaya et al., 1992). Subsequently, several matrix and synaptic proteins such as agrin, perlecan, and neurexin were identified as α-DG ligands (Yoshida-Moriguchi and Campbell, 2015). α-DG also functions as a receptor for Lassa virus (Cao et al., 1998). O-mannose (Man)-type glycosylation of α-DG is required for its ligand-binding activities, and abnormal glycosylation is associated with muscular dystrophy, lissencephaly, and cancer metastasis (Michele et al., 2002; Bao et al., 2009). Several genes have been identified to be responsible for α-dystroglycanopathy, a group of muscular dystrophies caused by aberrant α-DG glycosylation (Yoshida-Moriguchi and Campbell, 2015). α-dystroglycanopathy includes Fukuyama congenital muscular dystrophy (FCMD), muscle-eye-brain disease, Walker-Warburg syndrome, congenital muscular dystrophy (MDC) 1C/1D, and limb-girdle muscular dystrophy (LGMD) 21. Fukutin is the causative gene for FCMD, and we have demonstrated that splicing-modulation therapy by antisense oligonucleotides is a promising clinical treatment for FCMD (Kobayashi et al., 1998; Taniguchi-Ikeda et al., 2011).

Some α-dystroglycanopathy gene products (POMGnT1, POMT1, and POMT2) are glycosyltransferases that synthesize the initially identified O-Man-type CoreM1 glycan [Galβ1,4GlcNAcβ1,2Man-] (Yoshida et al., 2001; Manya et al., 2004; Chiba et al., 1997). Recently, another O-Man glycan [GalNAcβ1,3GlcNAcβ1,4Man-], CoreM3, was identified in recombinant α-DG (Yoshida-Moriguchi et al., 2010), and several other α-dystroglycanopathy genes were also subsequently identified (Jae et al., 2013; Willer et al., 2012; Roscioli et al., 2012). The recently identified α-dystroglycanopathy genes GTDC2 (POMGNT2) and B3GALNT2 encode glycosyltransferases that synthesize the CoreM3 glycan (Yoshida-Moriguchi et al., 2013).
SGK196 (POMK) encodes a kinase that phosphorylates the C6 position of O-Man in CoreM3 (Yoshida-Moriguchi et al., 2013). The glycan structure directly involved in ligand binding is [-3Xyl2,1,3GlcA]1−4, (hereafter referred to as the Xyl/GlcA repeat), which is synthesized by the glycosyltransferase LARGE (Inamori et al., 2012). It has been hypothesized that the Xyl/GlcA repeat branches from the phosphorylated O-Man in CoreM3 via a putative phosphodiester-linked moiety (Yoshida-Moriguchi et al., 2010). This is called a “post-phosphoryl moiety”; however, its structure is still unknown. In addition, the presence of α-dystroglycanopathy genes with unknown functions (fukutin, FKRFP, ISPD, and TMEM5) suggests that an additional moiety and a synthetic pathway for α-DG maturation might exist. To answer these questions and to better understand the pathogenesis of α-dystroglycanopathies, we attempted to delineate the full functional glycan structure of α-DG.

RESULTS

Identification of Ribitol 5-Phosphate in the Sugar Chain of α-DG

For mass spectrometry (MS)-based structural analysis, we designed a small recombinant α-DG/Fc fusion protein, DGFcWT, which contains the wild-type (WT) N-terminal domain and the first 20 amino acids of the mucin-like domain (from Ala314 to Pro333) (Figure 1A). Mature α-DG-Fc peptides begin with Gln311 because the N-terminal domain is processed during maturation (Kanagawa et al., 2004) and LARGE-dependent glycosylation takes place on the first two Thr residues in the mucin-like domain (Thr315 and Thr317) (Hara et al., 2011b). The results of western blotting using the IIH6 antibody, which recognizes the Xyl/GlcA repeat and, a laminin overlay assay suggested that DGFcWT is modified with the Xyl/GlcA repeat and possesses laminin-binding activity in NIH 3T3 cells without LARGE overexpression (Figure 1B). To obtain glycopeptides that contain a functional moiety, we subjected the samples to a putative phosphodiester-linked moiety (Yoshida-Moriguchi et al., 2010). This is called a “post-phosphoryl moiety”; however, its structure is still unknown. In addition, the presence of α-dystroglycanopathy genes with unknown functions (fukutin, FKRFP, ISPD, and TMEM5) suggests that an additional moiety and a synthetic pathway for α-DG maturation likely exist. To answer these questions and to better understand the pathogenesis of α-dystroglycanopathies, we attempted to delineate the full functional glycan structure of α-DG.

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To more easily enable tandem MS for the GPs, we constructed a mutant, DGFc120G, which results in a small peptide following lysylendopeptidase (LEP) digestion (Figure 1A). This DGFc was properly modified with the laminin-binding glycan (Figure 1B). We prepared DG120G-GP of 2,986 Da (GP2986), which corresponds to a glycopeptide (QIHATPTPKV) with one phosphorylated CoreM3, one CoreM1, and Structure736, and then subjected it to MS/MS. The results confirmed that GP2986 contains the 80 Da and 134 Da repeat, which is linked to the terminal Xyl/GlcA unit and the core HexNAc-HexNAc at each end (Figure S2E). More intriguingly, the presence of the product ion at m/z 1,452.2 from the precursor indicates that Structure736 is not attached to the phosphate of the innermost O-Man residue.

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There were three candidate residues with the chemical formula C6H12O3 (hydrated C5H10O4): xylitol, D/L-arabitol, and ribitol (Rbo). However, mammalian cells are not known to utilize these compounds for post-translational modifications. Interestingly, microorganisms, such as Gram-positive bacteria, use a repeating unit of ribitol 5-phosphate (Rbo5P), which forms a phosphodiester-linked polyol structure, teichoic acid, as a major cell wall component (Brown et al., 2013). We hence performed composition analysis of DGFc120G using gas chromatography (GC)-MS and detected three peaks (Gp1, Gp2, and Gp3) with
similar retention times as the standard Rbo5P (Rp1, Rp2, and Rp3) (Figure 3A). The mass spectra of these peaks are highly matched to those of species with the structural formulas of anhydrous acetylated Rbo and acetylated Rbo (Figure 3B). We also confirmed that this GC analysis is able to distinguish Rbo from xylitol and arabitol (data not shown). These data indicate that
DGFC<sup>T190M</sup> contains Rbo residues and that the moiety consisting of 80 Da and 134 Da structures is Rbo phosphate (RboP).

**ISPD Is a CDP-Ribitol Synthase**

In Gram-positive bacteria, the RboS polymer is synthesized by the TarK and TarL enzymes (Tar; Teichoic acid ribitol), which use CDP-Rbo as a donor substrate, and CDP-Rbo is synthesized by the TarI enzyme from cytidine triphosphate (CTP) and Rbo<sub>5</sub>P (Brown et al., 2013). We found that bacterial TarI shared homology with mammal ISPD, which is one of the causative gene products for a-dystroglycanopathy (Figure S3A) (Willer et al., 2012; Roscioli et al., 2012). To determine whether ISPD possesses CDP-Rbo synthase activity, we prepared His-tagged human ISPD, incubated it with CTP and Rbo<sub>5</sub>P, and then analyzed the products by high-performance liquid chromatography (HPLC). After the reaction of ISPD with both Rbo<sub>5</sub>P and CTP, a novel peak was detected (Figure 4A, indicated by “P”), and the same peak was observed in the TarI enzyme products (Figure S3B). MS results of both products showed the same mass of 537 Da, which was compatible with CDP-Rbo (Figures 4B and S3C). Thus, the enzymatic properties of human ISPD were identical to those of bacterial TarI. Pathogenic mutations of the *ISPD* gene impaired CDP-Rbo synthesizing activity (Figures 4C and S3A). These data indicate that ISPD is a mammalian CDP-Rbo synthase.

**Fukutin and FKRP Are Ribitol 5-Phosphate Transferases**

The fukutin family is predicted to encode a phosphoryl-ligand transferase (Aravind and Koonin, 1999; Kuchta et al., 2009). Fukutin-related protein (FKRP) was originally identified as a causative gene for MDC1C/LGMD2I based on its sequence homology with fukutin (Brockington et al., 2001a). Both fukutin and FKRP proteins contain the putative catalytic DXD motif, which is a conserved motif found in many families of glycosyltransferases (Wiggins and Munro, 1998; Tachikawa et al., 2012; Esapa et al., 2002). Therefore, we examined whether fukutin and FKRP are involved in RboP modification. A synthetic peptide mimicking mouse a-DG (ATPAPVAAIGPK) was enzymatically modified with phosphorylated CoreM3 (phosphoCoreM3-peptide) (see Supplemental Experimental Procedures) and then used as an acceptor substrate. Fukutin and FKRP were prepared as secreted proteins with a His/myc-tag at the N terminus (s-fukutin and sFKRP, respectively). First, we incubated s-fukutin with CDP-Rbo and phosphoCoreM3-peptide and then conducted HPLC and MS analyses. A novel peak was detected in the presence of s-fukutin (Figure 5A, indicated as “P1”), and this product showed an increase in 214 Da (compatible with a RboP) from phosphoCoreM3-peptide (1,997.0 minus 1,782.9; Figure 5B). These data indicate that s-fukutin transfers Rbo<sub>5</sub>P from CDP-Rbo to phosphoCoreM3-peptide. Missense mutations found in FCMD patients and in the putative catalytic sites reduced s-fukutin enzyme activity (Figures 5C and S4A).

To examine whether FKRP also has Rbo5P transferase activity, we incubated sFKRP with CDP-Rbo and either phosphoCoreM3-peptide or Rbo<sub>5</sub>P-phosphoCoreM3-peptide. Although sFKRP did not react with phosphoCoreM3-peptide, its reaction with Rbo5P-phosphoCoreM3-peptide produced a

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**Figure 2. High-Resolution MS Analysis of a-DG**

(A) Schematic representation of the sugar components of DG-GP; Structure736 (blue), the CoreM3 glycan (green), and the Xyl/GlcA repeat (orange).

(B) High-resolution MS. The product ion mass spectrum from the precursors at m/z 367 for double charge or m/z 735 for single charge suggests that Structure736 contains a Xyl/GlcA unit (orange arrows) and moieties consisting of 80 Da and 134 Da (blue arrows). The fragments of the 134 Da and 80 Da moieties (indicated by a, b, and c) were cross-referenced to the exact mass database, and the results are shown in the table. See also Figure S2.
Figure 3. Identification of Ribitol-Phosphate in α-DG

(A) Gas chromatogram of the alditol acetate derivatives of Rbo5P (top) and DGFcT190M (bottom). The Rp1, Rp2, and Rp3 peaks correspond with those of Gp1, Gp2, and Gp3, respectively.

(B) GC-MS of Rbo5P and DGFcT190M. The Rp1, Gp1, Rp3, and Gp3 peaks shown in (A) were analyzed by MS. The mass spectra of these peaks were cross-referenced to the NIST database. The matched spectra with structural formulas are shown in the bottom panels.
novel peak (Figure 5D, indicated as “P2”) and the product showed an increase in mass of 214 Da (2,211.1 minus 1,997.0; Figures 5B and 5E). These data indicate that sFKRP transfers the second Rbo5P to the s-fukutin products. Missense mutations found in α-dystroglycanopathy patients and in the putative catalytic sites reduced sFKRP enzyme activity (Figures 5F and S4A). Finally, we fractionated mouse liver membrane preparations and performed fukutin and FKRP enzyme assays (Figure S4B). The distributions of both enzyme activities were similar to the distribution of a Golgi marker protein, which suggests that fukutin and FKRP activities reside in the Golgi apparatus.

Determination of the Tandem Ribitol Structure

To determine the precise structure of the fukutin-dependent moiety, we conducted large-scale preparation of s-fukutin products using the acceptor substrate GalNAcβ1,3GlcNAcβ-pNp (pNp, p-nitrophenyl). We confirmed that s-fukutin can transfer Rbo5P to GalNAcβ1,3GlcNAcβ-pNp (Figures S4C and S4D). The original acceptor substrate (GalNAcβ1,3GlcNAcβ-pNp) and the purified s-fukutin product were analyzed by a series of NMR measurements, including 1H-13C heteronuclear single quantum correlation (HSQC) and 2D 1H-31P heteronuclear multiple-bond correlation (HMBC). In the 1H-13C HSQC spectra, significant chemical shift changes were observed for the signals originating from the first Rbo5P residue (Rbo-C1 in Glycan2 and Glycan3), and additional peaks were detected from the second Rbo5P residue (Rbo5P-peptide; Figures 6B and S5A). Furthermore, a through-bond 1H-31P correlation was observed in the s-fukutin product between the phosphorus of the second Rbo5P residue (Rbo5P) and the H1 of the first Rbo5P residue (Figure 6B). These NMR data revealed that the second Rbo (Rbo5P) is attached to the C1 position of the first Rbo5P (CDP-Rbo: Rbo5P-1Rbo5P transferase).

We further analyzed the Rbo5P-modified products that were enzymatically synthesized, by 1D 31P NMR analysis (Figure 6C). PhosphoCoreM3-peptide showed a signal originating from Man-6-phosphate (Man6P). A peak at 3 ppm was observed in both Rbo5P-3GalNAcβ1,3GlcNAcβ-pNp and Rbo5P-phosphoCoreM3-peptide, which was assigned to the phosphorous of the first Rbo5P residue. The products of the sFKRP reaction (Rbo5P-1Rbo5P-3GalNAcβ1,3GlcNAcβ-pNp and Rbo5P-1Rbo5P-phosphoCoreM3-peptide) showed an additional peak (~2 ppm), which was assigned to the phosphorous from the second Rbo5P (Rbo5P). The 1D 31P spectrum of purified DG(190M)-GP6138 showed three signals, likely originating from Man6P, the first Rbo5P, and the second

Importantly, a through-bond 1H-31P correlation was observed in the s-fukutin product between the phosphorus and GalNAc H3 (Figure 6A). Thus, Rbo5P is linked to the C3 position of GalNAc via a phosphodiester linkage (Rbo5P-3GalNAc). Therefore, fukutin is an enzyme that transfers Rbo5P from CDP-Rbo to the C3 position of GalNAc in the CoreM3 glycan (CDP-Rbo: GalNAc-3 Rbo5P transferase).

To determine the precise structure of the FKRP-dependent moiety, we performed large-scale preparation of sFKRP products from Rbo5P-3GalNAcβ1,3GlcNAcβ-pNp (Figures S4E and S4F), and analyzed them by 1H-13C HSQC and 2D 1H-31P HMBC experiments. In the 1H-13C HSQC spectra, significant chemical shift changes were observed for the signals originating from the first Rbo5P residue (Rbo-C1 in Glycan2 and Glycan3), and additional peaks were detected from the second Rbo5P residue (Rbo5P-peptide; Figures 6B and S5A). Furthermore, a through-bond 1H-31P correlation was observed in the sFKRP product between the phosphorus of the second Rbo5P residue (Rbo5P) and the H1 of the first Rbo5P residue (Figure 6B). These NMR data revealed that the second Rbo (Rbo5P) is attached to the C1 position of the first Rbo5P residue via a phosphodiester linkage (Figures 6B and S5A). Therefore, FKRP is an enzyme that transfers the Rbo5P from CDP-Rbo to the C1 position of the first Rbo5P (CDP-Rbo: Rbo5P-1Rbo5P transferase).

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Defects of Ribitol 5-Phosphate Glycosylation in α-Dystroglycanopathy Models and Potential Therapeutic Strategies

To examine whether Rbo5P glycosylation is affected in α-dystroglycanopathy, we generated HAP1 cells with a disruption in ISPD, fukutin, or FKRP genes as disease models, by CRISPR/Cas9 gene editing (Figure S6). We confirmed the disruption of functional α-DG glycosylation in these cells by western blot analysis using the IIH6 antibody. Rescue experiments confirmed that the abnormal glycosylation was caused by a loss of the targeted genes. We first expressed DGFc\(^{WT}\) in normal HAP1 cells and analyzed DG\(^{WT}\)-GPs from the 0.25 M and 0.5 M NH\(_4\)Ac eluates by MS (Figures 7A and 7B). The data indicated the presence of ions containing Structure\(736\) in the DG\(^{WT}\)-GP preparations and that the Xyl/GlcA units were modified on these GPs (Figure 7B). We then performed MS analyses on DG\(^{WT}\)-GPs prepared from the HAP1 cells with each α-dystroglycanopathy gene defect. Although the ions containing the phosphorylated CoreM3 glycan were detected in the ISPD- or fukutin-disrupted cells, no signals were assigned to GPs containing the Rbo5P moiety (Figures 7C and 7D). These data indicate that DG\(^{WT}\)-GPs prepared from ISPD- or fukutin-disrupted HAP1 cells lacked Structure\(736\) and also support the idea that fukutin acts as a priming enzyme for Rbo5P modification. In the FKRP-disrupted cells, GPs containing a portion of Structure\(736\) (single Rbo5P, 214 Da) were observed (Figure 7E), which supports the idea that FKRP synthesizes the second Rbo5P in the tandem structure. Taken together, these results confirmed that ISPD, fukutin, and FKRP are required for the synthesis of the tandem Rbo5P structure and indicate that Rbo5P glycosylation defects cause the loss of functional glycosylation of α-DG, resulting in α-dystroglycanopathy.

Our data also suggest that formation of the tandem Rbo5P structure requires CDP-Rbo and that ISPD-deficient α-dystroglycanopathy is caused by a lack of cellular CDP-Rbo. To examine whether supplementation of CDP-Rbo restores the functional glycosylation of α-DG, we treated ISPD-deficient HAP1 or HEK293 cells with exogenous CDP-Rbo (Figure 7F). The addition of CDP-Rbo to the cell culture medium restored the functional glycosylation of α-DG. In HEK293 cells, pretreatment of CDP-Rbo with a transfection reagent increased the efficiency of the restoration of α-DG glycosylation. These results raise the possibility of CDP-Rbo supplementation as a treatment for α-dystroglycanopathy.

DISCUSSION

Our data demonstrate that tandem Rbo5P is a “post-phosphoryl moiety.” The structure required for the functional maturation of α-DG was determined to be “Rbo5P-1Rbo5P-3GalNACα1,3GlcNAcα1,4Man(6P)-O” (Figure 6D). Post-phosphoryl modifications, including LARGE-dependent Xyl/GlcA repeats, have been thought to extend from phosphorylated Man (Yoshida-Moriguchi et al., 2010); however, our present data indicate that the monoester-linked phosphate on the C6-position of O-Man exists independently of the HF-sensitive Rbo5P tandem structure and that the Xyl/GlcA repeats extend from the tandem Rbo5P. We speculate that mannosyl phosphorylation determines the substrate specificity of Rbo5P and/or Xyl/GlcA modifications. Recently, B4GAT1 was shown to be a GlcA transferase, which synthesizes an acceptor primer necessary for LARGE-dependent Xyl/GlcA repeat formation (Praissman et al., 2014; Willer et al., 2014). Structure\(736\) contains a LARGE-independent Xyl/GlcA unit, which can be elongated by LARGE activity (Figures 2 and S1). These data also confirm that the Rbo5P tandem structure is a central part of the post-phosphoryl moiety and is required for the functions of α-DG.

We subsequently identified the activities of three α-dystroglycanopathy-causing proteins, namely, ISPD, fukutin, and FKRP, to be involved in Rbo5P glycosylation (Figure 6D). ISPD is a CDP-Rbo synthase that uses Rbo5P and CTP. This finding suggests that cellular levels of Rbo5P and CTP may control the Rbo5P modification of α-DG. In bacteria, Rbo5P is synthesized by the TarJ enzyme from ribulose 5-phosphate and nicotinamide adenine dinucleotide phosphate (Brown et al., 2013). In mammals, however, neither the ortholog of TarJ nor the biosynthetic pathway of Rbo5P is currently known. It is hence important in the future to identify proteins that are involved in mammalian Rbo5P biosynthesis, because they may regulate the functional glycosylation of α-DG and thus may cause α-dystroglycanopathy upon their mutations.

Fukutin and FKRP are both Rbo5P transfers that use CDP-Rbo as a common donor substrate. On the other hand, the selectivity of fukutin and FKRP for acceptor substrates showed a distinct difference: FKRP and fukutin are not able to transfer Rbo5P to GalNac and to the first Rbo5P residue, respectively. Thus, formation of the tandem Rbo5P structure likely needs to be performed in a sequential manner. In addition, we have not observed polymer formation of more than two Rbo5P residues by the activities of fukutin or FKRP. These data suggest the existence of strict mechanisms that determine substrate specificity or regulate the formation of the tandem Rbo5P structure. It is unknown whether negative regulatory factors of the tandem Rbo5P structure exist, for example, phosphodiesterase-like activities that cleave the internal phosphodiester linkage of the Rbo5P tandem structure. If such activities exist, they may be able to regulate the functional glycosylation of α-DG, and the elevated expression or hyperactivation of such enzymes may result in the abnormal glycosylation of α-DG.

Several studies have suggested the Golgi localization of fukutin and FKRP based on the localization of exogenously expressed proteins (Kobayashi et al., 1998; Esapa et al., 2002). Our fractionation and enzyme assays also suggest that...
Figure 5. Fukutin and FKRP Are Rbo5P Transferases

(A and B) Enzyme reaction of fukutin. PhosphoCoreM3-peptide was used as an acceptor substrate for s-fukutin. S, unreacted acceptor substrate. P1, s-fukutin enzyme product. PhosphoCoreM3-peptide and the fraction containing the s-fukutin products (S and P1 in A) were analyzed by MS in the negative ion mode (B).

(C) The activities of s-fukutin with disease-causing mutations (p.M133T and p.R307Q) and with a mutation in the putative catalytic sites (p.D317A).

(legend continued on next page)
endogenous fukutin and FKRP activities are localized in the Golgi. Given that the LARGE-dependent Xyl/GlcA repeat is also synthesized in the Golgi (Yoshida-Moriguchi et al., 2013), we propose that the Rbo5P modification occurs in the Golgi apparatus. We showed that some disease-causing missense mutations in fukutin or FKRP proteins directly reduced their enzyme activity, but the mislocalization of these proteins containing disease-causing missense mutations has been also reported (Esapa et al., 2002; Tachikawa et al., 2012). A protein motif search and deglycosylation experiments suggested that both fukutin and FKRP are N-glycosylated proteins (data not shown). Mutations in their potential glycosylation sites may cause α-dystroglycanopathy by altering the cellular localization of or the protein stability of fukutin and FKRP. Indeed, a missense mutation in the potential glycosylation site of FKRP (p.S174C) has been reported (Quijano-Roy et al., 2006).

Recent studies reported that ISPD mutations are a relatively common cause of α-dystroglycanopathy (Willer et al., 2012; Roscioli et al., 2012; Cirak et al., 2013). In Japan, most cases of α-dystroglycanopathy are confirmed to be FCMD, which is caused by mutations in fukutin (Kobayashi et al., 1998; Matsu-moto et al., 2005). LGMD2I, which is caused by mutations in FKRP, is a common form of LGMD in western countries (Brock-ington et al., 2001b). Taken together, a substantial proportion of α-dystroglycanopathy is caused by abnormalities in the Rbo5P glycosylation pathway. Although 18 genes are currently known to cause abnormal glycosylation of α-DG upon their mutation, resulting in α-dystroglycanopathy (Yoshida-Moriguchi and Campbell, 2015), the causative genes for a proportion of α-dystroglycanopathy cases still remain to be identified. Some of the so-far-unidentified causative genes of α-dystroglycanopathy may function in the Rbo5P glycosylation pathway, including CDP-Rbo biosynthesis. Clarifying the Rbo5P glycosylation pathway may accelerate our understanding of the pathogenesis of α-dystroglycanopathy.

We showed that the addition of CDP-Rbo restored the functional glycosylation of α-DG in ISPD-deficient cells, which indicates that metabolic components such as Rbo5P and CDP-Rbo in the Rbo5P glycosylation pathway can be targets of molecular supplementation therapy for α-dystroglycanopathy. Although the glycosylation pattern of α-DG varies among different cells and tissues, the IIIH6-sensitive functional glycan is present in many cells and tissues (Kuga et al., 2012). α-DG in HEK293 and HAP cells contains functional glycans, and gene editing and rescue experiments validated their suitability for their use as disease model cells; however, further studies using animal models will be necessary for the development of CDP-ribitol supplementation therapy. Several lines of evidence have established that abnormal glycosylation of α-DG contributes to the invasive and proliferative phenotypes of cancer cells (Singh et al., 2004; Bao et al., 2009; Miller et al., 2015). Recently, an association between the downregulation of ISPD and mortality in clear cell renal cell carcinoma was reported (Miller et al., 2015). Thus, our findings suggest the involvement of the Rbo5P glycosylation pathway in tumor formation, as well as the possibility of molecular CDP-Rbo supplementation as a therapy for cancers.

Because Rbo5P has only been found in bacteria (Brown et al., 2013) and some plants such as Adonis vernalis (Negm and Mar-low, 1985), it is surprising that mammalian cells use Rbo5P as a functional component of post-translational modifications. Rbo5P is a component of the teichoic acids in bacterial cell walls, which play various roles in bacterial physiology such as cell shape determination, cell division, and host defenses (Brown et al., 2013). How vertebrates acquired the Rbo5P modification during evolution, including the possibility of its horizontal transmission, would be interesting to investigate. As for its physiological functions, Rbo5P glycosylation is expected to be involved in many biological processes, such as embryonic and postnatal tissue development, as demonstrated by studies using fukutin and FKRP transgenic animals (Kurashiki et al., 2005; Ackroyd et al., 2009; Kanagawa et al., 2013). An intriguing question arises as to whether Rbo5P-containing glycoconjugates other than α-DG also exist. Given that DG-deficient mice recapitulate aspects of fukutin- or FKRP-deficient α-dystroglycanopathies (Moore et al., 2002; Cohn et al., 2002), α-DG is thought to be a primary target of Rbo5P modification. We cannot exclude the possibility of the existence of a Rbo5P transferase other than fukutin and FKRP, as well as that of a Rbo5P-containing glycoprotein or glycolipid other than α-DG. Investigating these possibilities will lead to a deeper understanding of Rbo5P modifications.

The complexity of the glycosylation machinery and the possible existence of unidentified sugar units may hinder our understanding of glycosylation-dependent biological processes and the pathogenesis of human diseases. We propose that Rbo5P glycosylation is an essential modification in mammals and that defects in the Rbo5P glycosylation pathway underlie the pathogenesis of muscular dystrophy and possibly of cancer metastasis and virus infection. Together, our results expand our knowledge on post-translational modification and establish an additional disease mechanism.

**EXPERIMENTAL PROCEDURES**

**Glycopeptide Purification from the DGFc Proteins**

DGFc proteins were secreted into the cell culture media and recovered with Protein A-beads. The DGFc proteins were eluted with 0.1 M glycine-HCl (pH 2.5) and then neutralized with a final concentration of 0.2 M Tris HCl (pH 8.0). PreScission protease treatment to cleave the Fc tag was performed according to the manufacturer’s instructions (GE Healthcare Life Science). The cleaved Fc tag and the protease were removed by Protein A-beads and glutathione beads, respectively. The samples were desalted using a Zeba spin column (Life Technologies) and then freeze-dried. The freeze-dried samples were dissolved with 20 mM phosphate buffer (pH 7.0) and treated...
with α2-3,6,8,9-neuraminidase (Merck Millipore) at 37°C for 6 hr and then with O-glycosidase (New England Biolabs) at 37°C for 12 hr to remove the terminal sialic acids and O-GalNAc-type glycans. The samples were subjected to an anion exchange column (HiTrap Q XL beads, GE Healthcare Life Sciences) and were fractionated by stepwise elution with NH4Ac (0.1, 0.25, 0.5, and 0.75 M). The fraction of the 0.5 M NH4Ac elution contained the DG-GPs with Xyli/GlcA repeats, whereas the fraction of the 0.25 M NH4Ac elution contained DG-GPs without Xyli/GlcA repeats. The fractions were freeze-dried and then analyzed by MS or further purified by HPLC. For HPLC purification, the freeze-dried samples were dissolved with 0.1% trifluoroacetic acid (TFA), loaded onto an Inertsil ODS column (2.0 x 150 mm, GL Sciences), and eluted with a linear gradient elution of acetonitrile (10%–50%) in 0.1% TFA. To obtain a short glycopeptide (QIHATPTPKV), the glycopeptide (QIHATPTPKVKAIGPPTTAIQEP) was separated by anion exchange chromatography was digested with LEP (Wako Pure Chemicals Industries) in 50 mM ammonium bicarbonate and purified by HPLC using a PBR column (COSMISIL, Nacalai Tesque) with a linear gradient elution of acetonitrile (10%–50%) in 0.1% TFA. 

**Mass Spectrometry**

MALDI-TOF or nanoelectrospray ionization (nanoESI) MS was used to elucidate the glycopeptide structures. MALDI linear TOF measurements were carried out on a Voyager DE Pro MALDI mass spectrometer equipped with a nitrogen pulsed laser (337 nm) (Applied Biosystems). Typically, 0.1–1 pmol of the glycopeptide samples was dissolved in a 1 μl solution of 10 mg/ml of 2,5-dihydroxybenzoic acid (DHB) in a 0.1% TFA and 30% acetonitrile solution on a MALDI sample target and dried. The measurements were carried out in positive ion mode. For MS/MS analysis, nanoESI MS was carried out using an LTQ XL Ion trap mass spectrometer (Thermo Fisher Scientific). The samples were dissolved in a 50% methanol solution and directly infused into the mass spectrometer using a PicoTip emitter (New Objective). The measurements were carried out in the negative ion mode. Typical nanoESI source conditions were 1.6 kV source voltage, −42.8 V capillary voltage, and 200°C capillary temperature. A high-resolution MS of Structure736 was carried out on an LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific) equipped with a nanoESI ion source in the negative ion mode. The spray voltage was set at 0.8 kV, with a distance of 3–5 mm between the top of the needle and the inlet of the mass spectrometer. The resolution of the equipment was set at 100,000, and the capillary temperature was set at 200°C. The product was dissolved in a 20% methanol solution and then directly infused into the mass spectrometer using a nanoESI tip (Cellicoms Tip, HUMANIX). The electron multiplier gain, Fourier transform, storage transmission, and mass accuracy were calibrated using polytyrosine (m/z 180.06662, m/z 506.19327, and m/z 995.38326), according to the manufacturer’s instructions. The prediction of the elemental compositions from the measured accurate masses was performed by Kazusa Molecular Formula Searcher and the exact mass database of Kazusa DNA Research Institute.

**Enzyme Assays for Tar1 and ISPD**

The cDNA encoding Tarl of Bacillus subtilis subasp. spizizenii TU-B-10 was chemically synthesized by GenScript, cloned into the pET-22b (+) vector (Merck Millipore), which was modified to lack the pelB signal sequence, and expressed in Rosetta E. coli cells (Merck Millipore). The cells were ultra-sonicated in Tris-buffered saline (TBS), and the recombinant Tarl was purified using COSMOGEL His-Select. The expression vectors for human ISPD and its mutants were transfected into HEK293T cells using Effectene. The cells were ultra-sonicated in TBS, and the ISPD proteins were purified using COSMOGEL His-Select. The bound proteins were eluted with 0.5 M imidazole in TBS, concentrated, and buffer-exchanged into 20 mM Tris-HCl (pH 7.4) and 50 mM NaCl using an Amicon Ultra centrifugal filter unit. The ISPD reaction was performed as previously reported, with modifications (Baur et al., 2009). Briefly, the reaction mixture contained 100 mM MOPS-NaOH (pH 7.4), 10 mM MgCl2, 1 mM CTP, and 2 mM RiboSP in a total volume of 40 μl and was incubated for 2 hr at 37°C. CDP-Rbo synthase activity was monitored using an HPLC-based assay. The product was run on a 4.6 x 150 mm COSMISIL PBR column (Nacalai Tesque) by isocratic elution with 3% methanol/97% 50 mM acetic acid-triethylamine (pH 7.3) at 30°C and detected by absorbance at 254 nm using the Prominence HPLC system. The separated peak product was collected and lyophilized for MS.

For the reaction using pNP-oligosaccharides as the acceptor substrates, secreted s-fukutin and sFKRP were purified using COSMOGEL His-Select or cOmplete His-Tag Purification Resin (Roche). The bound protein was eluted with 0.5 M imidazole in TBS, concentrated, and buffer-exchanged into 20 mM Tris-HCl (pH 7.4) and 50 mM NaCl using an Amicon Ultra centrifugal filter unit. The s-fukutin enzymatic reaction was performed in 2 mM GaINAc/1,3GlcNAc-p-n-pn (Tokyo Chemical Industry), 4 mM CDP-Rbo, 10 mM MnCl2, 10 mM MgCl2, and 100 mM MOPS-NaOH (pH 7.4) for 16 hr. PhosphoCoreM3-peptide and Ribo5P-phosphoCoreM3-peptide were used as acceptors for s-fukutin and sFKRP, respectively. Each product was separated by reversed-phase HPLC with a Mightysil RP-180GP Aquan column (5 x 250 mm) (Kanto Chemical). Solvent A was 0.1% TFA in distilled water, and solvent B was 0.1% TFA in acetonitrile. The peptide was eluted at a flow rate of 1 ml/min using a linear gradient of 0%–40% solvent B. The peptide separation was monitored by absorbance at 214 nm, and the products P1 and P2 were analyzed by MS.

Figure 6. Structure of the Tandem Rbo5P Motif

(A and B) 2D 1H-13C HSQC and 1H-31P HMBC spectra for the s-fukutin (A) and sFKRP (B) products. NMR signals of the s-fukutin acceptor substrate (Glycan1: GalNAc[1,3GlcNAc]p-n-pn, black signals) and s-fukutin reaction product (Glycan2: Ribo5P-3GalNAc[1,3GlcNAc]p-n-pn, red signals) are shown in layers (A). NMR spectra of the sFKRP product (Glycan3: Ribo5P-1Rbo5P-3GalNAc[1,3GlcNAc]p-n-pn) are shown (B).

(C) 1D 31P-NMR analysis. The s-fukutin and sFKRP products from the acceptor substrates (GalNAc[1,3GlcNAc]p-n-pn and phosphoCoreM3-peptide) and purified DG100K.GP6136 were analyzed. The variations in the 31P chemical shift of the ManIβP group between samples is most likely due to the differences in the pH of each sample, which could influence the chemical shift (Mo et al., 2011).

(D) Schematic representation of the enzymatic activities of ISPD, fukutin, and FKRP, and the structure of the tandem Rbo5P moiety. See also Figure S5.
probe, a BBO probe, and a Prodigy probe. The probe temperature was set at 25 °C. The samples were dissolved in D$_2$O (99.98 atom% D), and the $^1$H chemical shifts were reported relative to the external standard of 4,4-dimethyl-4-silapentane-1-sulfonic acid. The $^{13}$C and $^{31}$P chemical shifts were calibrated using an indirect reference based on the X/$^1$H resonance ratio of 0.25149530 ($^{13}$C/$^1$H) and 0.404808636 ($^{31}$P/$^1$H). NMR signals were assigned by a series of one- and two-dimensional measurements including 1D $^1$H, 1D $^{31}$P, 2D $^1$H-$^1$H double quantum filtered-correlation spectroscopy (DQF-COSY), homonuclear Hartmann-Hahn spectroscopy (HOHAHA), nuclear Overhauser effect spectroscopy (NOESY), $^{13}$C-$^1$H HSQC, $^{13}$C-$^31$P HSQC-total correlation spectroscopy (TOCSY), and $^{13}$C-$^{31}$P HMBC spectra. The NMR data were processed with XWIN-NMR (ver. 3.5) and TopSpin (ver 2.1), and the spectra were displayed using XWIN- PLOT (ver. 3.5).

**Animal Study**

All animal procedures were approved by the Animal Care and Use Committee of Kobe University Graduate School of Medicine in accordance with the guidelines of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) and the Japan Society for the Promotion of Science (JSPS).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.02.017.

**AUTHOR CONTRIBUTIONS**

M.K., K.K., M.T., H.M., and A.K. designed the project, carried out the experimental work, analyzed and interpreted the data, and wrote the manuscript. Particularly, M.K. and A.K. generated the essential reagents for structural analysis; K.K. and H.M. identified the enzyme activities and generated the glycopeptides for NMR; and M.T. performed the MS experiments. Y.Y. performed the NMR experiments and wrote the manuscript. J.F. and Y.S. conducted the MS experiments and analyzed the data. K.A.-M. performed the enzyme assay. M.M. and H.K. generated the essential reagents. Y.W., T.E., and T.T. designed the project, edited the manuscript, and jointly supervised the research. Y.W. is responsible for all the reagents and data. All authors discussed the data and the manuscript.

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**Figure 7. Abnormal RboP Glycosylation in Cells with Defects in the $\alpha$-Dystroglycanopathy Genes and a Possible Therapeutic Strategy Involving CDP-Rbo Supplementation**

(A–E) MS of DG-CDPs expressed in normal HAP1 (A and B) and HAP1 cells with defects in ISPD (C), fukutin (D), and FKRP (E). (A, C–E) GPs in the 0.25 M NH$_4$Ac eluate. The ions containing Structure 736 are indicated in red, blue, and green (A and B). The data also suggested that the Xyl/GlcA units were modified on these GPs (arrows in B). The ions containing phosphoCoreM3 are indicated with their estimated compositions in parentheses (C–E). Ions indicated in red correspond to GP6138 (see Figures 1 and 2A), GP6138 with a Xyl/GlcA unit, or GP6138 without Structure 736, pep, peptide; H, Hex; N, HexNAc; P, phosphate. Note that variations exist in the Hex/HexNAc modification patterns on DG-GPs (indicated in parentheses).

(F) Rescue of the functional glycosylation of $\alpha$-DG in ISPD-deficient cells by CDP-Rbo supplementation. ISPD-deficient HAP1 or HEK293 cells were treated with exogenous CDP-Rbo. The $\alpha$-DG preparations were analyzed by western blotting with antibodies to DGs and by the laminin overlay (OL) assay. Lane 1, normal control; lane 2, ISPD-deficient; lane 3, ISPD-deficient cells with CDP-Rbo; lane 4, ISPD-deficient cells with CDP-Rbo and Lipofectamine 2000; lane 5, ISPD-deficient cells with Lipofectamine 2000.

See also Figure S6.


