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SGK196 Is a Glycosylation-Specific *O*-Mannose Kinase Required for Dystroglycan Function

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Abstract

Phosphorylated *O*-mannosyl trisaccharide [*N*-acetylgalactosamine-β3-*N*-acetylglucosamine-β4-(phosphate-6-)mannose] is required for dystroglycan to bind laminin-G domain-containing extracellular proteins with high affinity in muscle and brain. However, the enzymes that produce this structure have not been fully elucidated. Here we found that glycosyltransferase-like domain containing 2 (GTDC2) is a protein O-linked mannose β 1,4-*N*-acetylglucosaminyltransferase whose product could be extended by β 1,3-*N*-acetylgalactosaminyltransferase2 (B3GALNT2) to form the *O*-mannosyl trisaccharide. Furthermore, we identified SGK196 as an atypical kinase that phosphorylated the 6-position of *O*-mannose, specifically after the mannose had been modified by both GTDC2 and B3GALNT2. These findings suggest how mutations in GTDC2, B3GALNT2, and SGK196 disrupt dystroglycan receptor function and lead to congenital muscular dystrophy.

Post-translational modification of proteins via stringently regulated biosynthetic pathways extends their range of function. Defects in the posttranslational modification of the dystroglycan (DG) protein are common to a variety of congenital muscular dystrophies (CMD)—including Walker-Warburg syndrome (WWS), Fukuyama CMD, Muscle-Eye-Brain disease (MEB), and certain types of limb-girdle muscular dystrophy— and result in the malfunction of DG as an extracellular matrix (ECM) receptor (1). DG is composed of a transmembrane β subunit and a cell-surface α subunit (2). α-DG serves as a receptor for laminin-G domain-containing ECM ligands, including laminin, perlecan, agrin, and neurexin

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(2), which involves various types of glycosylation of its mucin domain. In particular, phosphorylation at the 6-position of an *O*-mannose of the trisaccharide [*N*-acetylgalactosamine (GalNAc)-β3-*N*-acetylglucosamine (GlcNAc)-β4-mannose] produces a branch chain that is ultimately extended with repeating disaccharides [-α3-glucuronic acid (GlcA)-β3-xylose (Xyl)-] synthesized by likeacetylglucosaminyltransferase (LARGE), enabling α-DG to bind ECM ligands (3, 4). Mutations in several known and putative glycosyltransferases cause DG-related disorders. Recently genetic studies of the DGrelated diseases CMD and cobblestone lissencephaly identified several causative genes, including Isoprenoid Synthase Domain Containing (*ISPD*) (5), Transmembrane protein 5 (*TMEM5*) (6), β1,3-N-acetylglucosaminyltransferase (*B3GNT1*) (7), Glycosyltransferase-like domain containing 2 (*GTDC2*) (8), β3-*N*-acetylgalactosaminyltransferase2 (*B3GALNT2*) (9), and *SGK196* (10). However, the functions of the genes' products remain largely unknown.

O-Mannosyl glycosylation of α-DG is initiated by the endoplasmic reticulum (ER)-resident Protein *O*-Mannosyl Transferase 1/2 complex (POMT1/2), which adds mannose to Ser/Thr residues (11). To help to clarify the functions of the recently identified causative proteins, we examined their subcellular localization. GTDC2 was present in the ER (Fig. 1A), suggesting that it might modify the above-described *O*mannose. Thus, we synthesized a peptide corresponding to the mucin-like domain of human α-DG (residues 316–329), in which Thr-317 was modified by *O*-mannose but the remaining Thr/Ser residues were replaced with Ala. The glycopeptide was incubated with c-Myc-tagged GTDC2 (GTDC2-Myc) purified from human embryonic kidney 293 (HEK293) cell lysates, as well as various nucleotide sugars. Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis suggested that GTDC2-Myc transferred Nacetylhexosamine to the glycopeptide (fig. S1). Repetition of this assay using UDP-GlcNAc (Fig. 1B) or UDPGalNAc (fig. S2) as the donor confirmed that the transfer was specific to GlcNAc and that it did not occur when GTDC2-Myc carrying a mutation found in CMD patient was used (fig. S2). Next, we prepared a secreted form of GTDC2 (lacking the transmembrane domain; GTDC2dTM) in HEK293 cells (fig. S3) and conducted the transfer assay using fluorescently labeled mannoside (4-methylumbelliferyl-α-D-mannoside; Man-α-MU) as the acceptor. The product was purified by gel filtration (Fig. 1C) and then analyzed by nuclear magnetic resonance (NMR). The ^{1}H and ^{13}C resonances of the product were assigned using heteronuclear multiple quantum coherence (HMQC) and heteronuclear 2-bond correlation (H2BC) spectra (fig. S4, Fig. 1D–E, table S1). Rotating-frame Overhauser enhancement (ROE) data (fig. S5) confirmed that GlcNAc has a beta-configuration. A BH1/AC4 cross peak detected by the heteronuclear multiple bond correlation (HMBC) spectrum (Fig. 1F) indicated that the GlcNAc was linked to the 4-position of the mannose. Thus, GTDC2 possesses a protein *O*-mannose β1,4-*N*-acetylglucosaminyltransferase activity.

Human *B3GALNT2*, mutations in which cause WWS (9), has been cloned based on its β3-glycosyltransferase motifs. This enzyme is thought to act as a β1,3-*N*-acetylgalactosaminyltransferase that uses β-linked GlcNAc as its acceptor in vitro (12). However, the GalNAc-β3- GlcNAc-β-R sequence had not been found in mammals when the gene was cloned, leaving the biological significance of this enzyme unclear. α-DG contains an *O*-mannosyl glycan (GalNAc-β3-GlcNAc-β4-Man), with the mannose phosphorylated at the 6-position (3). The ECM-ligand*binding moiety of α-DG extends from this phosphate residue (3). To test if B3GALNT2 and GTDC2 act coordinately on *O*-mannose to synthesize this trisaccharide, we prepared a secreted form of B3GALNT2 (B3GALNT2dTM) (fig. S6) and incubated this protein with UDPGalNAc and the GlcNAc-β4-Man-O-peptide produced by the GTDC2dTM reaction. MALDI-TOF/MS analysis confirmed that B3GALNT2 could transfer a GalNAc residue to the acceptor (Fig. 2A), suggesting that B3GALNT2 and GTDC2 can synthesize GalNAc-β3-GlcNAc-β4-Man. CMD patients who have mutations in

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these genes produce α-DG with pathological defects in ECM-binding (8, 9). We next used a solid-phase laminin-binding assay to test whether the GalNAc-β3-GlcNAc-β- terminus contributes directly to the binding of α-DG to ECM ligands. Whereas the GalNAc-β3-GlcNAc-β4-Man-modified peptide exhibited significant affinity for Wisteria floribunda lectin (WFA, which recognizes terminal GalNAc residues), this was not the case for laminin-111 (Fig. 2B). Next we asked if a defect in synthesis of the GalNAc-β3-GlcNAc-β-terminus prevented *O*-mannose from being further modified by phosphorylation. We expressed Fc-tagged recombinant DG (DGFc340), which contains the region in which the functional modification occurs (13), in [^{32}P]-orthophosphate-labeled control fibroblasts and CMD patient fibroblasts with mutations in GTDC2 or B3GALNT2 (Fig. 2C). Indeed, the cells from the CMD patients did not produce [^{32}P]-phosphorylated DGFc340, indicating that phosphorylation of the α-DG *O*-mannose is inhibited by lack of the GalNAc-β3-GlcNAc-β-terminus from the mannose.

To understand how the *O*-glycan on α-DG is phosphorylated, we synthesized fluorescently labeled GalNAc-β3-GlcNAc-β4-Man, using GTDC2dTM, B3GALNT2dTM and the acceptor Man-α-MU. We first tested ATP as a phosphate donor, performing the assay on total membrane fractions obtained from rabbit brain, using GalNAc-β3-GlcNAc- β4-Man-α-MU as the acceptor. Separation of the reactant on a C18 column revealed that GalNAc-β3-GlcNAc-β4-Man-α-MU (Fig. 2D) was phosphorylated in the presence of ATP. We further separated the ER and Golgi complex in mouse liver membrane fractions and found that the phophorylation activity resided in the ER fractions (fig. S7). Causative proteins of DG-related disorders whose functions remain unknown include FKRP, FKTN, TMEM5, ISPD, and SGK196. Among these, only SGK196 shares homology with known protein kinases, although it is believed to be inactive because of the high divergence of its putative kinase domains from the consensus sequence (14). We tested its phosphorylation activity in lysates from control fibroblasts and CMD patient fibroblasts with mutations in SGK196, GTDC2, or B3GALNT2. The activity toward GalNAc-β3-GlcNAc-β4-Man-α-MU was lacking only in the SGK196-mutated cells and this loss was rescued by ectopic expression of c-Myc-DDK-tagged SGK196 (SGK196-Myc-DDK) (Fig. 3A). When SGK196-Myc-DDK produced in HEK293 cells was incubated with ATP and/or Man-α-MU derivatives, SGK196 exhibited significant phosphorylation activity toward GalNAc-β3-GlcNAc-β4-Man-α-MU. Moreover, this activity was not observed when GlcNAc-β4-Man-α-MU or Man-α-MU was used as the acceptor (Fig. 3B). Phosphorylated Gal- NAc-β3-GlcNAc-β4-Man-α-MU was not detected when SGK196-Myc- DDK carrying a mutation (L137R) found in a CMD patient (10) was used in the assay (fig. S8). To elucidate the enzymatic properties of SGK196, we produced a soluble form of SGK196 (SGK196dTM, fig. S9). Its phosphorylation activity depended on divalent ions with an apparent Km for ATP of 4.1 ± 1.4 μM (fig. S10). To pinpoint which hydroxyl group of the trisaccharide is phosphorylated by SGK196, we isolated the product obtained in the experiment depicted in Fig. 3B and analyzed its structure by NMR. The ^{1}H and ^{13}C resonances of the product were assigned based on HMQC, HMBC and H2BC spectra (fig. S11 and table S2), and the anomeric configurations were determined by ROESY (fig. S12). The BH1/AC4 and CH1/BC3 cross peaks detected in the HMBC spectrum confirmed that the GalNAc and GlcNAc residues were attached to GlcNAc and Man via β1-3 and β1-4 linkages, respectively (fig. S11). The phosphate group added by SGK196 was attached to the 6-position of the mannose residue (Fig. 4A and B).

Here, we have shown that GTDC2 has a protein *O*-mannose β1,4-*N*-acetylglucosaminyltransferase activity, which leads us to designate it as POMGNT2, and that GTDC2 and B3GALNT2 can synthesize a Gal- NAc-β3-GlcNAc-β-terminus at the 4-position of protein *O*-mannose. SGK196 phosphorylated the 6-position of *O*-mannose using ATP, based on which we propose to designate it as a protein *O*-mannose kinase (POMK).

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Because SGK196 lacks certain residues required for catalysis by kinases (14), the mechanism used to catalyze the phosphotransfer reaction is unclear. SGK196 exhibited the phosphorylation activity only when the GalNAc-β3-GlcNAc-β-terminus was linked to the 4-position of *O*-mannose, indicating that this disaccharide serves as the substrate recognition motif of SGK196. This strict specificity of SGK196 may explain why mutations in GTDC2 and B3GALNT2 cause DG-related disorders although their product does not directly recognize the ECM ligand. Because multiple types of *O*-mannosyl glycans exist, we propose to designate the *O*-mannosyl glycan structures as cores M1–M3 (Fig. 4C). Although sialylated core M1 of DG was originally proposed to be responsible for binding to ligands in the ECM (15), recent glycomics analysis suggests that proteins besides DG are subject to modification of sialylated cores M1 and M2 (16). The fact that LARGE alone could not modify phosphorylated GalNAc-β3-GlcNAc-β4-Man-α-MU using UDPXyl and UDP-GlcA as substrate (fig. S13) suggests that CMD causative proteins (FKRP, FKTN, TMEM5, and B3GNT1) besides LARGE are likely to contribute to maturation of the ECM-binding moiety on the phosphorylated core M3 glycan.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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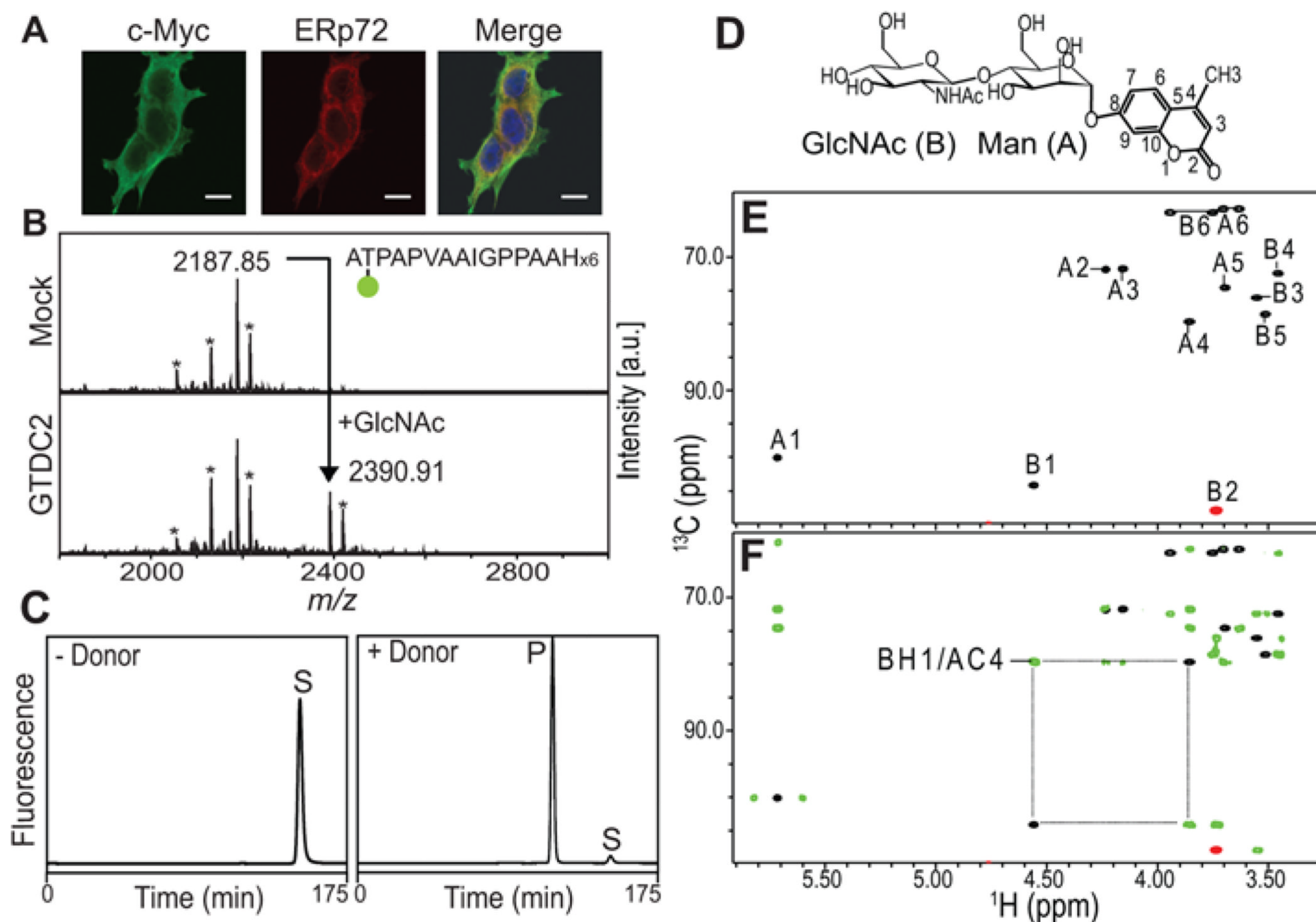

Fig. 1.

GTDC2 has a protein O-linked mannose β1,4-*N*-acetylglucosaminyltransferase activity. (**A**) HEK293 cells expressing c-Myc tagged GTDC2 were stained with anti-Myc (green), ERp72 (ER marker, red), and DAPI (nuclei, blue). Bar indicates 10 μm. (**B**) The product of the GTDC2 in vitro assay when a DG-derived peptide modified with O-linked mannose and UDP-GlcNAc were used as substrates was analyzed by MALDI-TOF/MS. (**C**) Reactant of the GTDC2dTM assay using Man-α-MU and UDP-GlcNAc was separated on Superdex Peptide 10/300 columns. S, unreacted acceptor substrate. P, enzymatic product. (**D**) Structure of the product in (C), with the sugar subunits labeled A and B. (**E–F**) HMQC (**E**) and overlay (**F**) of the HMQC (black and red) and HMBC (green) spectra of the product. Assigned cross peaks are labeled with a first letter representing the subunit (as designated in D), and the rest of the label representing the position on that subunit. The red peak in (E) is the folded peak. ppm, parts per million.

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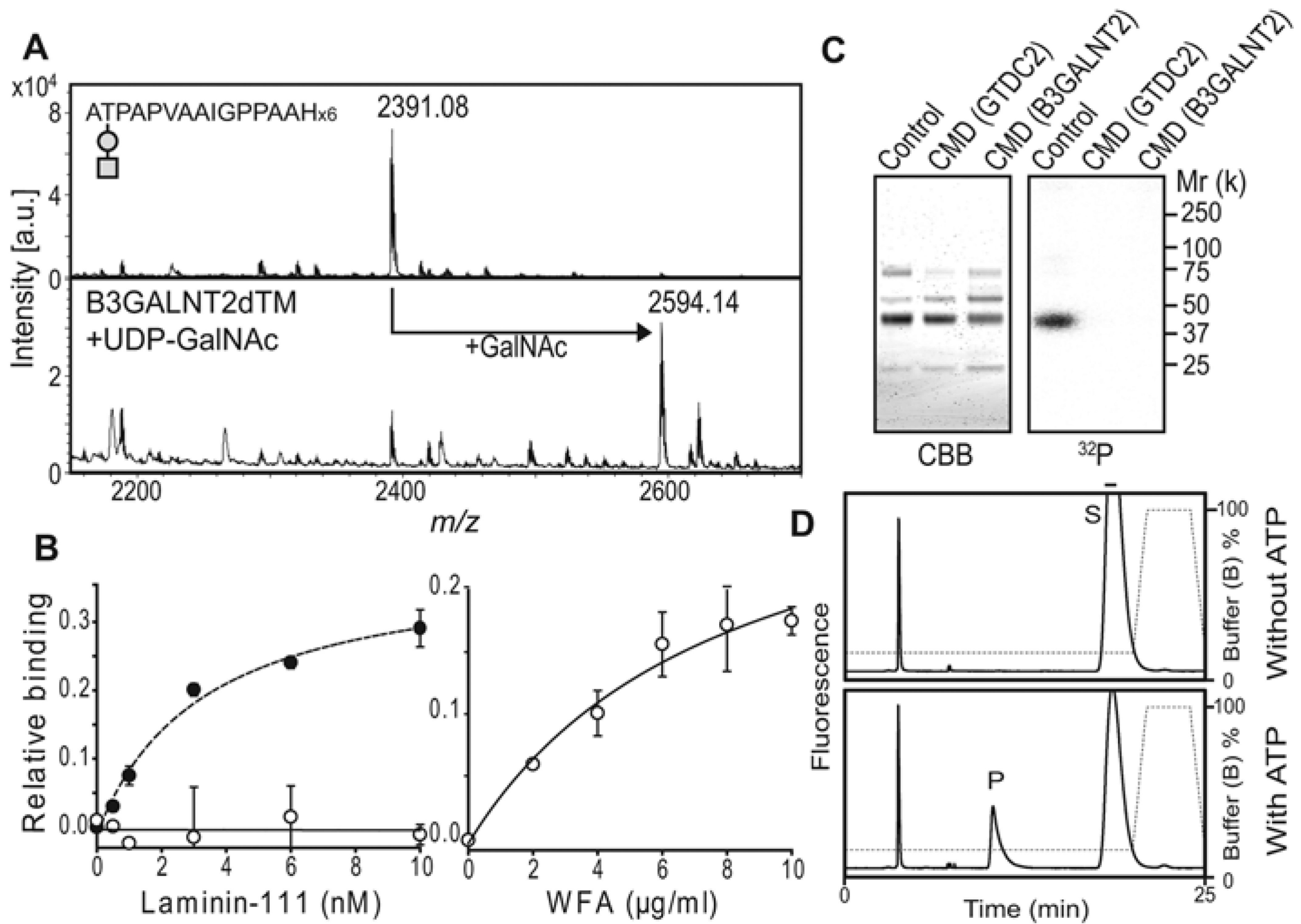

Fig. 2.

Mutations in GTDC2 and B3GALNT2 cause defects in the synthesis of phosphorylated α-DG. (**A**) The product of the B3GALNT2dTM in vitro assay using the product depicted in Fig. 1B and UDP-GalNAc as substrates was analyzed by MALDI-TOF/MS. (**B**) Laminin- (open circle, left) and WFA- (open circle, right) binding to DG-derived peptide modified with the GalNAc-β3-GlcNAc-β4-Mannose was measured by solid-phase assay (n = 3). The trisaccharide-modified peptide produced by the GTDC2dTM and B3GALNT2dTM reactions was conjugated to maleimide-activated plates. The peptide modified with mannose was used for background subtraction. Wild type muscle glycoproteins (solid circle) served as positive control in the laminin-binding assay. Error bars indicate standard deviation (SD). (**C**) Fc-tagged DGFc340 was produced in [^{32}P]-orthophosphate-labeled fibroblasts derived from control individual and GTDC2- or B3GALNT2-mutated patient. DGFc340 was isolated from the culture medium using protein-A agarose, separated by SDS-PAGE, stained with Coomassie Brilliant Blue (CBB), and analyzed by phosphorimaging ([^{32}P]). (B) Reactants of rabbit brain total membrane fraction incubated with ATP and GalNAc-β3-GlcNAc-β4-Man-α-MU at 37°C for 6 hours were separated on a C18 reverse-phase column. S, unreacted acceptor substrate. P, enzymatic product.

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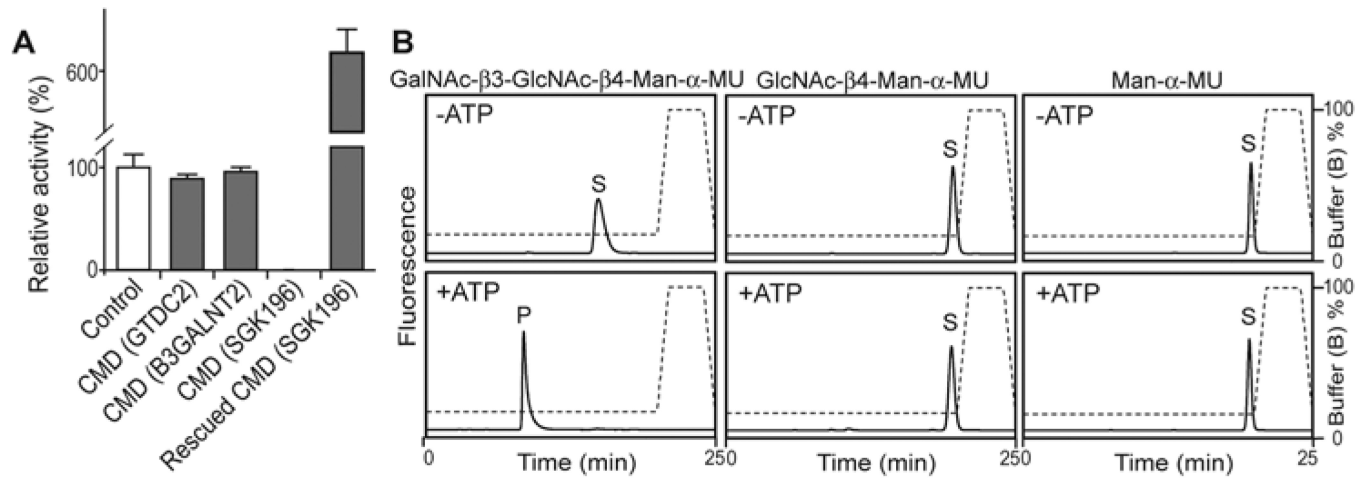

Fig. 3.

SGK196 phosphorylates GalNAc-β3-GlcNAc-β4-Man. (**A**) Cell lysates from control fibroblasts and fibroblasts derived from patients with a mutation in SGK196, GTDC2, or B3GALNT2, as well as SGK196 patient-derived fibroblasts ectopically expressing SGK196- Myc-DDK, were subjected to a kinase assay using GalNAc-β3-GlcNAc-β4-Man-α-MU. Data obtained from three individual experiments are shown, with error bars indicating SD. (**B**) Reactants from a phosphorylation assay in which SGK196-Myc-DDK was used were separated on a C18 reverse-phase column. GalNAc-β3-GlcNAc-β4-Man-α-MU (left), GlcNAc-β4-Man-α-MU (middle), or Man-α-MU (right) was used as acceptor, in the absence (upper) or presence (lower) of ATP.

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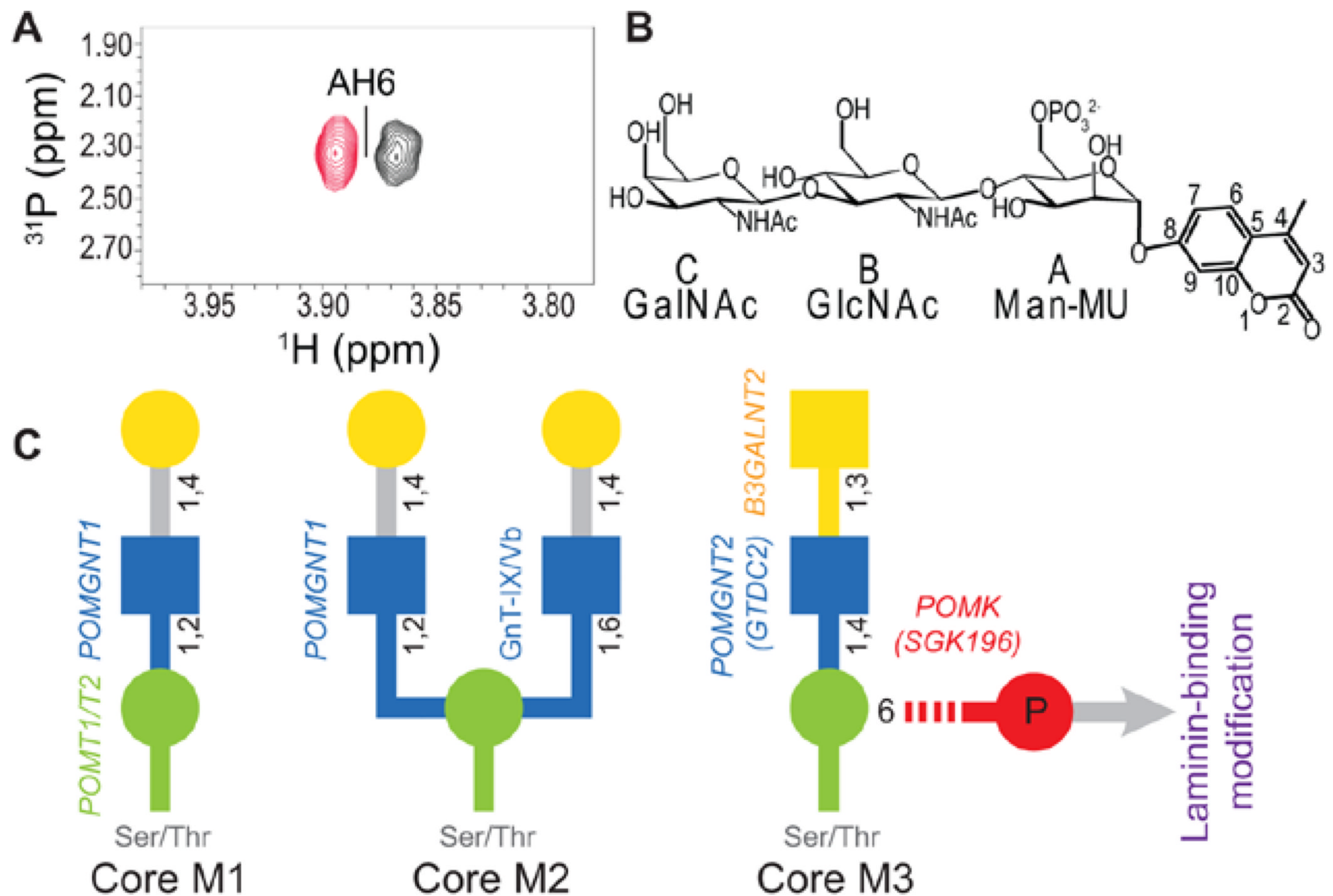

Fig. 4.

SGK196 phosphorylates the 6-position of *O*-mannose. (**A**) ^{31}P/^{1}H COSY spectrum of the product depicted in Fig. 3B when GalNAc-β3-GlcNAc-β4-Man-α-MU was used as the acceptor. Assigned cross peaks are labeled as described in Fig. 1, using the subunit designation indicated in B. (**B**) Structure of the phosphorylated product, with sugar subunits labeled A–C. (**C**) Model of α-DG glycan structures. Proposed classification of each *O*-mannosyl core structure is indicated at bottom. Enzymes responsible for forming the respective linkages are indicated at left; those identified as causing (POMT1/2, POMGNT1 and 2, B3GALNT2, and POMK) DG-related disorders are indicated in italics. Green circle, Man; blue square, GlcNAc; yellow circle, Gal; yellow square, GalNAc, red circle, phosphate.