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

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Phosphorylated O-mannosyl trisaccharide [N-acetylgalactosamine–β3-N-acetylgalactosamine–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. We found that glycosyltransferase-like domain–containing 2 (GTD2C) is a protein O-linked mannoside β 1,4-N-acetylgalactosaminyltransferase 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 mannoside 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.

Posttranslational 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 (CMDs)—including Walker-Warburg syndrome (WWS), Fukuyama CMD, muscle-eye-brain disease, 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, perlecain, agrin, and neurexin (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-acetylgalactosamine (GlcnAc)–β4-mannose

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Supplementary Materials

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Figs. S1 to S10

Movie S1

Reference (27)

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produces a branch chain that is ultimately extended with repeating disaccharides (α-3-glucuronic acid (GlcA)-β-xylose (Xyl)—) synthesized by like-acetylgalactosaminyltransferase (LARGE), enabling α-DG to bind ECM ligands (3, 4). Mutations in several known and putative glycosyltransferases cause DG-related disorders. Recently, genetic studies identified several new causative genes, including isoprenoid synthase domain containing (ISPD) (5), transmembrane protein 5 (TMEM5) (6), β,3-N-acetylgalactosaminyltransferase (B3GNT1) (7), glycosyltransferase-like domain containing 2 (GTDC2) (8), β,3-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 mucinlike domain of human α-DG (residues 316 to 329), in which Thr317 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 (HEK) 293 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 N-acetylhexosamine to the glycopeptide (fig. S1). Repetition of this assay using uridine 5‘-diphosphate (UDP)-GlCNAC instead of GDP-Man confirmed the GTDC2 activity. Note, however, that GDP-Man was not a good substrate for GTDC2. Therefore, we focused on UDP-GlcNAc as the specific substrate for GTDC2.

**Fig. 1.** GTDC2 has a protein O-linked mannose β1,4-N-acetylgalactosaminyltransferase activity. (A) HEK293 cells expressing c-Myc-tagged GTDC2 were stained with anti-Myc (green), ERp72 (ER marker, red), and 4′,6-diamidino-2-phenylindole (DAPI, nuclei, blue). Scale bars indicate 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. A, Ala; G, Gly; H, His; I, Ile; P, Pro; T, Thr; V, Val. a.u., arbitrary units; m/z, mass to charge ratio. (C) Reactant of the GTDC2dTM assay using Man–α-MU and UDP-GlcNAc was separated on Superdex Peptide 10/300 (GE Healthcare) columns. S, unreacted acceptor substrate. P, enzymatic product. (D) Structure of the product in (C), with the sugar subunits labeled A and B. HMOC (E) and overlay (F) of the HMOC (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 represents the position on that subunit. The red peak in (E) is the folded peak. ppm, parts per million.

**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-GlcNAc as substrates was analyzed by MALDI-TOF/MS. (B) Laminin (open circles, left) and WFA (open circles, right) binding to DG-derived peptide modified with the GlcNAc–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 circles) served as positive control in the laminin-binding assay. Error bars indicate SD. (C) Fc-tagged DGFc340 was produced in [32P]orthophosphate–labeled fibroblasts derived from a control individual and GTDC2- or B3GALNT2-mutated patients. DGFc340 was isolated from the culture medium by using protein-A agarose, separated by SDS-polyacrylamide gel electrophoresis, stained with Coomassie brilliant blue (CBB), and analyzed by phosphorimaging ([32P]). Mr, relative molecular mass. (D) 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 (top) or presence (bottom) of ATP.

Fig. 4. SGK196 phosphorylates the 6-position of O-mannose. (A) The 31P/1H correlation spectroscopy spectrum of the product depicted in Fig. 3B when GalNAc-β3-GlcNAc-β4-Man-α-MU was used as the acceptor. Assigned cross-peaks were labeled as described in Fig. 1 by using the subunit designation indicated in (B). (B) Structure of the phosphorylated product, with sugar subunits labeled A to 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.

(12) However, the GalNAc-β3-GlcNAc-β sequence had not been found in mammals when the gene was cloned, leaving the biological importance 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 EC-M-linking moiety of α-DG extends from this phosphate residue (3). To test whether 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 UDP-GalNAc and the GlcNAc-β4-Man-α-MU-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 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 whether a defect in synthesis of the GalNAc-β3-GlcNAc-β-terminus prevented O-mannose from being further modified by phosphorylation. We expressed Fe-tagged recombinant DG (DGFc340), which contains the region in which the functional modification occurs (13), in [32P]-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 [32P]-phosphorylated DGFc340, indicating that phosphorylation of the α-DG O-mannose is inhibited by lack of the GalNAc-β3-GlcNAc-β-terminus from the mannos.
Conformational Motions Regulate Phosphoryl Transfer in Related Protein Tyrosine Phosphatases

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Many studies have implicated a role for conformational motions during the catalytic cycle, acting to optimize the binding pocket or facilitate product release, but a more intimate role in the chemical reaction has not been described. We address this by monitoring active-site loop motion in two protein tyrosine phosphatases (PTPs) using nuclear magnetic resonance spectroscopy. The PTPs, YopH and PTP1B, have very different catalytic rates; however, we find in both that the active-site loop closes to its catalytically competent position at rates that mirror the phosphotyrosine cleavage kinetics. This loop contains the catalytic acid, suggesting that loop closure occurs concomitantly with the protonation of the leaving group tyrosine and explains the different kinetics of two otherwise chemically and mechanistically indistinguishable enzymes.

Molecular motions are crucial for the optimal functioning of enzymes. There has been much debate regarding what role motions play in the enzymatic conversion of substrates to products (1, 2), and recent studies, primarily solution nuclear magnetic resonance (NMR) relaxation experiments, have shown that enzyme motions are critical for optimizing the active site (3–6), enabling effective substrate or cofactor binding (7), and facilitating protein dissociation (8). These motions often require collective movement of many amino acids over substantial molecular distances (9) and are

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