Disease mutations in CMP-sialic acid transporter SLC35A1 result in abnormal α-dystroglycan O-mannosylation, independent from sialic acid

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Abstract
Binding of cellular α-dystroglycan (α-DG) to its extracellular matrix ligands is fully dependent on a unique O-mannose-linked glycan. Disrupted O-mannosylation is the hallmark of the muscular dystrophy-dystroglycanopathy (MDDG) syndromes. SLC35A1, encoding the transporter of cytidine 5′-monophosphate-sialic acid, was recently identified as MDDG candidate gene. This is surprising, since sialic acid itself is dispensable for α-DG-ligand binding. In a novel SLC35A1-deficient cell model, we demonstrated a lack of α-DG O-mannosylation, ligand binding and incorporation of sialic acids. Removal of sialic acids from HAP1 wild-type cells after incorporation or preventing sialylation during synthesis did not affect α-DG O-mannosylation or ligand binding but did affect sialylation. Lentiviral-mediated complementation with the only known disease mutation p.Q101H failed to restore deficient O-mannosylation in SLC35A1 knockout cells and partly restored sialylation. These data indicate a role for SLC35A1 in α-DG O-mannosylation that is distinct from sialic acid metabolism. In addition, human SLC35A1 deficiency can be considered as a combined disorder of α-DG O-mannosylation and sialylation, a novel variant of the MDDG syndromes.

Introduction
α-Dystroglycan (α-DG) is a heavily glycosylated extracellular protein and binds several extracellular matrix ligands, including laminin (1), agrin (2,3), perlecan (4), neurexin (5), pikachurin (6) and slit (7). α-DG shows a peripheral non-covalent binding with transmembrane protein β-dystroglycan (β-DG), that is bound to dystrophin and F-actin (1,8). This complex, also known as the dystrophin-glycoprotein complex (DGC), connects the extracellular matrix to the cytoskeleton. α-DG-ligand binding requires a specific O-mannose-linked glycan. Two types of O-mannose
glycans are described: a tetrasaccharide Neu5Acα2-3Galβ1-4GlcNAcβ1-2-Man-Ser/Thr (9) and a trisaccharide GalNAcβ1-3GlcNAcβ1-4-Man-Ser/Thr (10). The mannos residue of the trisaccharide contains a 6-O-linked phosphate group (10) that is probably bound to an as far not completely known, but crucial part of the laminin-binding glycan epitope, containing xylose and glucuronic acid residues (11,12).

Defective O-mannosylation results in reduced ligand binding and causes muscular dystrophy-dystroglycanopathy (MDDG) syndromes, a group of severe congenital disorders characterized by muscular dystrophy and brain and eye abnormalities (13). The O-mannose ligand-binding structures are also used by Lassa virus for entry into host cells (14). A recent genome-wide screen for genes involved in Lassa virus entry (15) revealed a large set of genes involved in α-DG O-mannosylation that were subsequently associated with disease mutations in various MDDG syndromes. An exception was the cytidine 5’-monophosphate (CMP)-sialic acid transporter SLC35A1, which has not yet been associated with the MDDG syndromes. Instead, a patient with a homozygous p.Q101H mutation in SLC35A1 was recently diagnosed as a congenital disorder of glycosylation (CDG) type II (16) with reduced sialylation of protein-linked N-glycans and mucin type O-glycans. Clinical symptoms included proteinuria, macroscopic hematuria, and glucuronic acid residues (11,12).

To analyze the effect of the previously described SLC35A1 missense mutation on α-DG O-mannosylation, we complemented a HAP1 SLC35A1 knockout cell line with wild-type and mutant (p.Q101H) V5-tagged SLC35A1 constructs using lentiviral transduction. Comparable SLC35A1 expression levels were confirmed by anti-V5 immunoblotting (Fig. 2A), and both constructs localized to the Golgi (data not shown) as previously shown in CHO cells (16).

The sialylation levels of the different cell lines were investigated using lectin staining and metabolic labeling. Complementation with wild-type SLC35A1 fully restored the amount of MALII positive cells, while mutant SLC35A1 showed only partial restoration (Fig. 2B and C). In parallel, complementation with wild-type SLC35A1 strongly reduced the amount of PNA positive cells, whereas mutant SLC35A1 complementation resulted only in a slight reduction (Fig. 2B and C).

Metabolic labeling was performed using a novel sialic acid analog, Ac5SiaNPoc, that specifically labels protein- and lipid-linked sialic acids. Cells were incubated with peracetylated Ac5SiaNPoc (Buell, et al., manuscript in preparation) and reacted with a biotinylated-labeled azide, followed by streptavidin-phycocerythrin for analysis by flow cytometry. The incorporation of Ac5SiaNPoc was completely absent in the knockout cell lines (Fig. 2D). In knockout cell lines complemented with a wild-type SLC35A1 construct, Ac5SiaNPoc incorporation was completely restored, whereas complementation with mutant SLC35A1 restored Ac5SiaNPoc incorporation for only ∼20% (Fig. 2C). These results confirm the effect of the p.Q101H mutation in SLC35A1 on glycan sialylation as previously shown by mass spectrometry of patient serum N-glycans (16). Next, expression of the laminin-binding O-mannose glycan was analyzed. In SLC35A1-deficient cells complemented with the wild-type construct, both laminin binding and IIH6 immunoreactivity were restored, but the signal was less intense than for wild-type cells. Possibly less α-DG is present in the sample of the wild-type complemented cells, as also the α-DG core antibody showed a less intense signal. In the mutant complemented cell line, the α-DG core signal was comparable with that of the knockout cell line and both laminin and IIH6 binding were nearly absent (Fig. 2E). These data show that the p.Q101H mutation in SLC35A1 affects not only the transport of CMP-sialic acid, thereby reducing sialic acid levels on glycoproteins, but also the synthesis of the O-mannosyl glycan on α-DG.
Discussion

SLC35A1 is known as a nucleotide sugar transporter that has been considered to be highly selective for CMP-sialic acid (21,22). Although sialic acid is not directly involved in α-DG-laminin binding, as has been shown by sialidase treatment of fully glycosylated α-DG from rabbit skeletal muscle (18), surprisingly a recent Lassa virus screen revealed SLC35A1 as essential gene for functional O-mannosylation of α-DG (15). Here, we show that SLC35A1 is required for functional O-mannosylation of α-DG, while sialic acid is neither directly nor indirectly needed for the binding of α-DG to laminin.

Previously, Lec2 SLC35A1-deficient CHO cells did not show a defect in O-mannosylation in a model where LARGE was overexpressed (17). However, it is known that LARGE overexpression bypasses several O-mannosylation defects (23), and possibly an impairment of SLC35A1 activity on proper synthesis of O-mannose glycans on α-DG may therefore go unnoticed. Here, we employed HAP1 cells for studying sialylation and α-DG O-mannosylation without the need of LARGE overexpression (17) to reach sufficient expression levels of functional O-mannosylated α-DG. Knockout of SLC35A1 in HAP1 cells clearly showed a defect in sialylation and O-mannosylation, while neither sialidase treatment nor inhibition of the sialylation resulted in defective α-DG-laminin binding. Complementation of knockout cells with mutant (p.Q101H) SLC35A1 restored sialylation levels partially, in agreement with our previous research showing 50% residual activity in transport of CMP-sialic acid (16). In addition, laminin binding could not be repaired, indicating that α-DG-laminin binding is disturbed in patients with SLC35A1-CDG.

In analogy with the interaction between LARGE and the α-DG domain that is required for glycosylation of distant residues (24), sialic acids on specific α-DG domains could hypothetically provide a scaffold for binding of other glycosyltransferases. However, this seems unlikely, since incubation with the sialylation inhibitor did not affect α-DG-laminin binding. The considerable difference between the molecular weight of α-DG in sialidase-treated or sialylation-inhibited cells and in SLC35A1 knockout cells, indicates that the knockout cells not only lack sialic acids on the O-mannose glycan. Possibly, SLC35A1 could transport, additional to CMP-sialic acid, another nucleotide sugar, that might be incorporated in the O-mannose glycan and might be crucial for binding of α-DG to laminin.

Previously, other nucleotide transporters have been shown to transport multiple nucleotide sugars. SLC35B4 is a UDP-xyllose and UDP-GlcNAc transporter (25) and HFRC1 transports UDP-glucose and UDP-GlcNAc (26). A recent report suggests that for CMP-sialic acid transport by SLC35A1, CMP-recognition is more important than recognition of the sialic acid substrate (27). However, to our knowledge CMP-sialic acid is the only known substrate for the mammalian SLC35A1 transporter (22). Remarkably, some SLC35A1 homologs from plants are shown to have CMP-sialic acid transport activity in Lec2 cells, but as sialic acids are absent in plants, these transporters are suggested to have physiological CMP-Kdo (2-keto-3-deoxymanno-octulosonic acid) transporter activity (28,29). Kdo is not present in mammals, which could indicate that SLC35A1 has potential activity towards another, yet to be identified cytidine nucleotide sugar that might be required for functional α-DG-O-mannosylation.

At the time we identified the SLC35A1 missense mutation in a patient from our CDG cohort (16), we did not foresee a role for SLC35A1 in the O-mannosylation pathway. With the current knowledge, a reappraisal of the dominant neurological phenotype is warranted to incorporate the combined biochemical deficiencies in sialylation and O-mannosylation. The reported macrothrombocytopenia and proteinuria are uncommon in MDDG syndromes and therefore likely correlated with deficient sialylation. The neurological phenotype of intellectual disability and epilepsy is known in the MDDG syndromes as well as in CDG. Therefore, these symptoms cannot easily be linked to a single pathway and maybe the effect on multiple pathways in brain development and function result in the dominant neurological...
phenotype in this patient. The SLC35A1 missense mutation in this adult patient is relatively mild and might therefore not cause specific O-mannosylation characteristics, such as muscular dystrophy. Recently, genetic defects in combined pathways for N-glycosylation and O-mannosylation have been described resulting in human disorders with mixed clinical phenotypes. Thus far, these have been limited to defects in the synthesis of dolichol-P-mannose required for the initial step in O-mannosylation, including defects in DOLK (30), DPM1 (31), DPM2 (32) and DPM3 (33). This study shows that mutations in SLC35A1 can be added to this growing group of combined CDG and MDDG syndromes.

In conclusion, we showed that SLC35A1 has a crucial role in the O-mannosylation pathway without involvement of sialic acid. SLC35A1 defects can be added to the growing group of diseases that show overlap between MDDG syndromes and N-linked CDG. These novel findings are important for elucidation of the O-mannosylation pathway and the identification of the ligand-binding epitope on α-DG.

Materials and Methods

Cell culture

HAP1 cells (34) were cultured in Iscove’s modified Dulbecco’s medium (IMDM, Gibco) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/l-glutamine (Gibco) at 37°C under 5% CO2 atmosphere. SLC35A1 knockout cells (15) were a kind gift from T.R. Brummelkamp (Netherlands Cancer Institute, Amsterdam, the Netherlands).

Inhibition of sialic acid transfer

P-3F₆-ac-Neu5Ac was synthesized as described before (20). To inhibit sialylation, cells were cultured 3 days in the presence or absence (control) of 100 µM of P-3F₆-ac-Neu5Ac.

Metabolic labeling of sialoglycans

To assess incorporation of propargyloxycabonyl sialic acids into cell surface sialoglycans, HAP1 cells were cultured 3 days in the presence or absence (control) of 100 µM Ac₅SiaNPoc (Büll et al., manuscript in preparation), washed in 1× PBS and incubated with 100 µl CuAAC reaction buffer (1× PBS containing 250 µM CuSO₄, 250 µM l-histidine, 500 µM sodium ascorbate and 100 µM azide-PEG3-biotin) for 30 min at room temperature. Cells were washed in FACS buffer (1× PBS containing 1% FBS and 0.02% sodium azide) and biotinylated glycoconjugates were conjugated for 10 min at 4°C with 2 µg/ml streptavidin–phycoerythrin (BD Pharmingen). Mean fluorescence intensities were measured using a CyAn ADP flow cytometer (BD Biosciences, San Jose, CA) and quantified utilizing FlowJo software (Tree Star).
Inc., Ashland, OR). Azide-PEG3-biotin, sodium 1-ascorbate, 1-histidine and copper(II) sulfate pentahydrate were purchased from Sigma-Aldrich (St. Louis, MO).

Complementation of SLC35A1 knockout HAP1 cells

Full-length wild-type and mutant (p.Q101H) SLC35A1 mRNAs were obtained from pYESUcFlag K SLC35A1 vectors (16) by PCR. The coding sequence was cloned in a pLenti6.2/V5-DEST expression vector containing a C-terminal V5-tag, using the Gateway system (Invitrogen). Constructs were verified by sequence analysis. HEK293FT cells were transfected with pLP1, pLP2, pVSV/G and either the SLC35A1 wild-type or mutant vector to produce virus particles according to manufacturer’s protocol (Life Technologies). Viruses were harvested after 72 h. HAP1 KO cells #1 were transduced by incubation with either the SLC35A1 wild-type or mutant viruses in the presence of 6 µg/ml polybrene. After 24 h incubation, virus was removed and medium was refreshed. Next day, 30 µg/ml blasticidin was added to select virus-transduced cells.

Sample preparation for western blotting

Cells were harvested by scraping in buffer containing 0.35 m sucrose, 30 mM Tris and 3 mM EDTA and enriched using Triticum vulgare (wheat) lectin (WGA)-beads as described previously (30,35). Subsequent sialidase treatment was performed overnight using 10 µM/µg α(2–3,6,8,9) neuraminidase from Arthrobacter ureafaciens (Sigma-Aldrich) at room temperature. A control reaction was performed without sialidase.

Enrichment of membrane-bound organellar proteins was performed as described previously (36). In short, HAP1 cells from a confluent 175 cm2 flask were harvested by trypsinization. The cell pellet was washed once using PBS and subsequently solubilized in 300 µl digitonin-buffer containing 25 µg/ml digitonin for 10 min. Centrifugation was performed for 10 min at 2000g at 4°C, supernatant, containing the cytosolic proteins, was removed. The remaining pellet was solubilized in 300 µl 1% Triton X-100 buffer. After centrifugation for 5 min at 7000g at 4°C the supernatant was collected and used for western blotting.

Western blotting

WGA-enriched samples were run on a 7.5% homogeneous gel on a PhastSystem gel electrophoresis system (GE Healthcare) following standard protocols and blotted onto nitrocellulose (0.2 µm Protran Whatman) by diffusion blotting (60-90 min at 60°C).

Microsomal fractions were run on 10% polyacrylamide gel, either with 4 M Urea (anti-V5) or without (Calnexin). After SDS-PAGE, proteins were transferred to a nitrocellulose membrane by western blotting (Biorad Mini PROTEAN® 3).

Lectins and antibodies used: peroxidase-labeled PNA (Sigma-Aldrich), 0.2 µg/ml), biotin-labeled MALI (Vector Labs, 10 µg/ml), anti-V5 (Life Technologies, 1:5000 dilution), Calnexin (Novus Biologicals, NB100_1965, 1:1000 dilution), IIH6 (Millipore, IIH6C4, 1:2500 dilution), DAG1 (Genetex, GTX105038, 1:333 dilution), j-DC (Novacastro Laboratories, 1:250 dilution). A laminin overlay assay was performed as described previously (30,35).

Lectin flow cytometry analysis

Cells were washed using PBS and subsequently harvested by scraping in cold PBS. Cells were washed twice using blocking buffer (1% Fetal bovine serum, 1 mM CaCl₂ and 1 mM MgCl₂ in PBS). Cells were either incubated with 20 µg/ml tetramethylrhodamine (TRITC)-labeled PNA (Sigma-Aldrich) for 90 min or with 20 µg/ml biotin-labeled MALII (Vector labs) for 45 min. After MALII incubation, cells were washed three times using 1% FBS in PBS and subsequently incubated with 10 µg/ml fluorescein isothiocyanate (FITC)-labeled streptavidin (Sigma-Aldrich) for 10 min. Before measuring, all cells were washed three times using 1% FBS-PBS. The fluorescent signal was measured at a CyAn flow cytometer (Beckman-Coulter). A total of 40 000 cells were analyzed using Summit 4.3 software. Cells incubated with blocking solution or Streptavidin–FITC only were used to determine the background signal. Average of two independent experiments is shown.

Conflict of Interest statement. None declared.

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