Supplemental material

Alkynyl monosaccharide analogues as a tool for evaluating Golgi glycosylation efficiency. Application to Congenital Disorders of Glycosylation (CDG)

Jorick Vanbeselaere, Dorothee Vicogne, Gert Matthijs, Christophe Biot, Francois Foulquier*, and Yann Guerardel*

METHODS

Chemistry

All chemicals were purchased as reagent grade and used without further purification. SiaNAI was synthesized according to procedures reported by Chang et al. with slight modifications. In brief, ManNAI or N-4-Pentynoylmannosamine was obtained by reacting a mixture of D-mannosamine hydrochloride, N-succinimidyl-4-pentynoate, and triethylamine in DMF (N,N-dimethylformamide) at room temperature overnight. Biosynthesis of SiaNAI from ManNAI was catalyzed by Neu5Ac aldolase (EC 4.1.3.3) in the presence of sodium pyruvate and NaN₃ in potassium phosphate buffer. SiaNAI was purified by anion-exchange chromatography (AG-1 X2 resin; Bio-Rad) using a gradient of formic acid in water from 1.0 M to 2.5 M. Fractions were analyzed for the presence of SiaNAI using the periodate-resorcinol method. Only the β-anomer was obtained. BTTAA was synthesized according to procedures reported by Besanceney-Weble et al. ¹H- or ¹³C-NMR spectra were recorded on a BRUKER AVANCE 300MHz using D₂O as the solvent (¹H, 300 MHz; ¹³C, 75 MHz). Chemical shifts (in ppm) were determined relative to either tetramethylsilane (0 ppm). Mass spectra were acquired on voyager Elite DE-STR mass spectrometer (Perspective Biosystems, Framingham, MA) in reflectron positive mode. Samples were prepared by mixing directly on target 1 μL of SiaNAI solution (1–5 pmol) with 1 μL of 2,5dihydroxybenzoic acid matrix solution (10 mg/mL in CH₃OH/H₂O, 50/50, vol/vol).

Antibodies

Anti-TMEM165 was from Sigma–Aldrich (St Louis, MO, USA). Anti-TGN46 and anti-EEA1 antibodies were from BD Biosciences (Franklin lakes, NJ, USA).

Cell culture

Primary skin fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Lonza), at 37°C in humidity-saturated 5% CO₂ atmosphere. The PMM2-CDG (CDG-Ia) patient was compound heterozygous for the c.422G>A, p.R141H; c.357C>A, p.F119L mutations in
phosphomannomutase 2 PMM2. The ALG11-CDG (CDG-Ip) patient was homozygous for the missense mutation c.479G>T, p.G160V in ALG11 (this study). The COG5-CDG patient was homozygous for a nonsense mutation in COG5 (c.2518G>T; p.E840X). The COG7-CDG patient was homozygous for the IVS1+4 A>C mutation. The MAN1B1-CDG patient was homozygous for the missense mutation p.R334C (submitted paper). The three TMEM165-CDG patients are homozygous for the splice mutation c.792+182G>A (TMEM165 P1), homozygous for the (c.377G >A; p.R126H) (TMEM165 P2) and compound heterozygous for the c.376C>T; p.R126C and c.910G >A; p.G304R (TMEM165 P3) respectively. The ATP6V0A2-CDG patient was homozygous for the mutation c.1324G>A, p.E442X. The CDG-IIx used in this study presents when analyzed by mass spectrometry, a severe abnormal Golgi glycosylation profile.

**Metabolic labeling with alkyne-tagged sialic acid**

Fibroblasts were grown over night on glass cover slips (12mm diameter). Medium was then changed with pre-warmed medium containing 500 µM of alkynyl-modified sugar (SiaNAl). The labeling was stopped at the different time points mentioned by fixing the cells with 4% paraformaldehyde or -20°C methanol. In case of PAF fixation, cells were then permeabilized in 0.5% Triton X-100 for 10 min. Permeabilized cells were then incubated in the click chemistry buffer containing CuSO4, 5H2O-BTAA-Ascorbate-Potassium Phosphate, Azidofluor 545). After 2 h saturation in blocking buffer [0.2% gelatin, 1% BSA and 2% normal goat serum (Invitrogen) in PBS], fixed cells were incubated at RT for 1h with primary antibody diluted at 1:100 in blocking buffer. After washing with PBS, cells were incubated for 1 h with Alexa 488-, Alexa 568- or Alexa 700-conjugated secondary antibodies (Molecular Probes) diluted at 1:600 in blocking buffer. Immunostaining and fluorescent proteins were detected through an inverted Leica TCS-SP5 confocal microscope. Pictures were taken by using the Leica Application Suite Advanced Fluorescence (LAS AF) software (Leica Microsystems Wetzlar, Germany). For comparison purposes, each picture has been taken under the same settings. For the quantification, we used the The Leica TCS-SP5 intensity-plotting tool that provides relative fluorescence intensities in different collection channels over a region of interest (ROI). Plot of fluorescence intensity in a Region of interests (ROI) corresponding to the Golgi region has been done for each cell. For image analysis, three different fields of two independent experiments were examined. A total of around 100 cells have been quantified. The LAS AF pictures were then exported in TIFF format and processed with Adobe Photoshop 7.0.

**Quantification of alkyne-tagged sialic acid**

Control cells, PMM2 and COG7 deficient CDG cells, were seeded onto a T-75 flask and cultivated in a growth medium supplemented with or without 500 µM of alkynyl-modified sialic acid (SiaNAl) during 72 h. Cells were then scrapped with a rubber policeman, washed with Phosphate Buffered Saline (PBS, 3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mMNaCl, pH 7.4) and centrifuged at 3000 rpm. Cellular pellets were dried and sequentially extracted three times by 500 µL of water. Pellets contained membrane glycoproteins and glycolipids whereas supernatant contained soluble glycoproteins and cytosolic sialic acids. Pellet was suspended in MeOH/CHCl3/water (6:2:4, v/v/v) and centrifuged at 13400 rpm to precipitated the
membrane proteins and solubilized the glycolipids. Glycolipids and membrane proteins were separated and dried. Supernatant containing soluble proteins and free sialic acid was dried and suspended with 80 % acetone/water at -20 °C overnight and centrifuged at 13,400 rpm to precipitate soluble proteins. Cytosolic sialic acid and soluble proteins were separated and dried. Intact sialic acids of these different factions were liberated by mild hydrolysis in 0.1 M TFA at 80 °C and coupled to DMB. For DMB derivatization, sialic acids reacted with a volume of DMB reagent at 50 °C for 2.5 h, then separated isocratically on a C18 reverse phase HPLC column (250 × 4.6 mm, 5 μm, Vydac) by a solvent mixture of acetonitrile/methanol/water (10:12:78, v/v/v) and identified by referring to the elution positions of standard Neu5Ac, Neu5Gc and SiaNAI derivatives 3-4.
SUPPLEMENTAL FIGURES

**Fig. S1.** Fibroblasts from healthy individuals were metabolically labelled with 500 µM of SiaNAl for different times (6, 12, 24 and 48h), fixed and stained with azido fluorescent probes for sialic acid into glycoconjugates (in red) or antibody against late Golgi marker (TGN46 in blue).
**Fig. S2.** *In situ* labelling of newly synthesized glycoproteins with alkyne-tagged sialic acid. In order to discriminate free alkylated sialic acids from those associated to glycoconjugates, we compared the fluorescent staining on paraformaldehyde (PAF) fixed versus methanol fixed cells. We can indeed expect that free alkyne-tagged sialic acids should be soluble in methanol and hence the fixation and washing with methanol should remove any labeling induced by free SiaNAl in fluorescence microscopy. No clear differences could be observed into the distribution of fluorescence between both types of fixation. These observations confirm that sialic acid analogues are linked to glycoconjugates in the identified compartments.
Fig. S3. Differential incorporation of alkyne-tagged sialic acid into CDG-I and CDG-II deficient patients. Fibroblasts from healthy individuals and isolated from known CDG-I and II patients were metabolically labeled with 500µM of SiaNAI for 24h, fixed and stained for alkyne tagged sialoconjugates with azidofluor 545 (in red), for late Golgi marker (in blue) and for Eea1 (in green).
LIST OF ABBREVIATIONS

CDG, Congenital Disorders of Glycosylation; Neu5Ac, 5-N-Acetyl-β-Neuraminic acid; SiaNAI, N-5-pentynoyl-β-Neuraminic acid; HPLC, high pressure liquid chromatography; CuAAC, Copper(I)-catalyzed Azide Alkyne Cycloaddition; BTTAA, 2-[4-((bis[(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl]amino)methyl)-1H-1,2,3-triazol-1-yl]acetic acid; TGN, trans Golgi network; BSA, bovine serum albumin; TIEF, transferrin isoelectrofocalisation
SUPPLEMENTAL REFERENCES


