Instytut Immunologii i Terapii Doświadczalnej im. Ludwika Hirszfelda

Polskiej Akademii Nauk

Krzysztof Stefan Mikołajczyk

Rola N-glikozylacji w aktywności ludzkiej syntazy Gb3/CD77

Rozprawa doktorska

Promotor:

Prof. dr hab. Marcin Czerwiński

Laboratorium Glikobiologii

Wrocław 2022

Badania opisane w niniejszej rozprawie doktorskiej zostały wykonane w ramach projektu Diamentowy Grant finansowanego przez Ministerstwo Edukacji i Nauki pt. „**Ludzka syntaza Gb3/CD77: czy N-glikany mogą wpływać na syntezę glikosfingolipidów?**” kierowanego przez mgr Krzysztofa Mikołajczyka (nr grantu 0097/DIA/2017/46).

Składam serdeczne podziękowania

prof. dr hab. Marcinowi Czerwińskiemu, promotorowi niniejszej rozprawy,

za opiekę merytoryczną, cenne uwagi oraz motywowanie do pracy,

współpracownikom i doktorantom,

za codzienną życzliwość i rozmowy, dzielenie się swoim doświadczeniem

oraz okazaną pomoc.

SPIS TREŚCI

STRESZCZENIE

Ludzka syntaza Gb3/CD77 (α1,4-galaktozylotransferaza, syntaza P1/P^k, UDP-galactose: β-D-galactosyl-β1-R 4-α-D-galactosyltransferase; EC 2.4.1.228), kodowana przez gen *A4GALT*, jest glikozylotransferazą należącą do białek transmembranowych typu II. Enzym w zależności od reszty aminokwasowej w pozycji 211 łańcucha polipeptydowego rozpoznaje dwa lub trzy różne akceptory. Głównymi produktami są glikosfingolipidowe antygeny Gb3 (P^k) i P1 (należące do ludzkiego układu grupowego krwi P1PK), zawierające terminalną strukturę Galα1→4Gal. W naszym laboratorium wykazano, że enzym z podstawieniem p.Q211E może syntezować również trzeci produkt nazywany antygenem NOR, zawierający terminalny disacharyd Galα1→4GalNAc; erytrocyty z takimi antygenami są rozpoznawane przez większość ludzkich surowic, a zjawisko to jest nazywane poliaglutynacją NOR. Ostatnie doniesienia wskazują, że ludzka syntaza Gb3/CD77, oprócz akceptorów glikosfingolipidowych, może rozpoznawać także akceptory glikoproteinowe, takie jak kompleksowe N-glikany, w których po dodaniu terminalnej galaktozy powstają terminalne oligosacharydy Galα1→4Galβ1→4GlcNAc-R, nazywane glikotopami P1.

Antygen Gb3 produkowany przez ludzką syntazę Gb3/CD77 może być receptorem dla patogenów, takich jak uropatogenne szczepy *E. coli* czy zoonotyczne szczepy *S. suis*, oraz toksyn Shiga (Stx) produkowanych przez bakterie *S. dysenteriae* (serotyp 1) oraz *E. coli* (szczepy STEC). Zakażenia tymi bakteriami mogą być przyczyną krwotocznego zapalenia okrężnicy oraz zespołu hemolityczno-mocznicowego (HUS). Badania uzyskane w naszym laboratorium wskazują, że oprócz glikosfingolipidu Gb3, będącego głównym funkcjonalnym receptorem dla toksyn Shiga, także glikotop P1 zlokalizowany na glikoproteinach może wiązać i internalizować toksynę Shiga typu 1, wywołując efekt cytotoksyczny.

Syntaza Gb3/CD77 ma dwa potencjalne miejsca N-glikozylacji zlokalizowane na resztach asparaginy w pozycjach 121 (motyw N_{121}AS i) i 203 (N_{203}LT) łańcucha polipeptydowego. W naszym laboratorium wykazano uprzednio, że enzymatyczna deglikozylacja enzymu powoduje utratę jego zdolności katalitycznych, ale podstawy molekularne tego zjawiska pozostawały nieznane. Celem badań zawartych w niniejszej rozprawie doktorskiej było więc określenie roli N-glikozylacji w aktywności ludzkiej syntazy Gb3/CD77, a w szczególności: 1) określenie, które miejsca N-glikozylacji znajdujące się w ludzkiej syntazie Gb3/CD77 mają przyłączone reszty cukrowe (wraz z charakterystyką typu struktur cukrowych); 2) zbadanie wpływu miejsc N-glikozylacji ludzkiej syntazy

Gb3/CD77 na właściwości enzymu (aktywność enzymatyczną, swoistość akceptorową oraz lokalizację wewnątrzkomórkową); 3) analiza wrażliwości komórek CHO-Lec2 transfekowanych genami kodującymi warianty glikozylacyjne ludzkiej syntazy Gb3/CD77 na toksyny Shiga; 4) zbadanie wpływu reszt cukrowych na konformację enzymu i określenie ich potencjalnego wpływu na jego aktywność katalityczną (analizy *in silico*). Wyniki badań zrealizowanych w celu odpowiedzi na wyżej wymienione pytania opublikowano w dwóch publikacjach oryginalnych.

Pierwsza publikacja wchodząca w skład rozprawy doktorskiej stanowi wprowadzenie teoretyczne do części eksperymentalnej. Zostały w niej omówione następujące zagadnienia: rola N-glikanów w aktywności glikozylotransferaz, molekularne mechanizmy leżące u podstaw zmian aktywności zdeglikozylowanych enzymów, oraz metody badania wpływu N-glikanów na aktywność tej grupy enzymów. W publikacji opisałem 34 glikozylotransferaz, dla których istnieją dane literaturowe na temat związku pomiędzy N-glikozylacją i aktywnością.

Badania nad rolą N-glikozylacji w aktywności ludzkiej syntazy Gb3/CD77 zostały podzielone na analizę enzymu o pełnej długości (zlokalizowanego w aparacie Golgiego) oraz analizę jego formy rozpuszczalnej, pozbawionej hydrofobowego fragmentu transmembranowego. W obu podejściach, otrzymałem warianty glikozylacyjne różniące się resztami aminokwasowymi w pozycji trzeciej sekwonów N-glikozylacji (N_{121}AA oraz N_{203}LA). Ponadto, analizowałem warianty glikozylacyjne dwóch wersji syntazy Gb3/CD77: enzym Q zawierający w pozycji 211 łańcucha polipeptydowego resztę glutaminy, oraz enzym E z resztą kwasu glutaminowego w tej samej pozycji.

W drugiej publikacji wchodzącej w skład rozprawy doktorskiej przedstawiłem badania enzymu o pełnej długości przeprowadzone z wykorzystaniem komórek CHO-Lec2. Wykazałem, że oba miejsca N-glikozylacji ludzkiej syntazy Gb3/CD77 mają przyłączone reszty cukrowe. Analiza aktywności enzymatycznej wariantów glikozylacyjnych wykazała, że enzymy z podstawieniami p.S123A mają podobną aktywność enzymatyczną w porównaniu do enzymów w pełni N-glikozylowanych, natomiast aktywność wariantów z podstawieniami p.T205A jest obniżona. Warianty pozbawione N-glikanów (z podstawieniami p.S123A/p.T205A) wykazały szczątkową aktywność enzymatyczną. Wszystkie warianty glikozylacyjne syntezowały terminalne struktury Galα1→4Gal, zarówno na glikosfingolipidach, jak i glikoproteinach, a wszystkie komórki transfekowane wektorami kodującymi glikowarianty były wrażliwe na oba typy holotoksyn Shiga (Stx1 i Stx2).

Spadek żywotności komórek transfekowanych genami kodującymi enzymy pozbawione N-glikanów (a więc o śladowej aktywności) sugeruje, że nawet śladowe ilości Gb3 na glikosfingolipidach i/lub glikotopów P1 na glikoproteinach mogą powodować efekt cytotoksyczny. Wykazałem także, że N-glikan przyłączony do sekwonu N_{203} ma kluczowe znaczenie dla lokalizacji enzymu w aparacie Golgiego, a jedną z przyczyn znacznego obniżenia aktywności zdeglikozylowanej ludzkiej syntazy Gb3/CD77 może być nieprawidłowa lokalizacja enzymu.

W badaniach opisanych w trzeciej publikacji analizowałem warianty glikozylacyjne syntazy Gb3/CD77 pozbawione fragmentów transmembranowych, produkowane w komórkach HEK293E. Masa cząsteczkowa wariantów pozbawionych jednego N-glikanu była obniżona, natomiast warianty całkowicie pozbawione N-glikanów nie były obecne w medium. Można je było natomiast zidentyfikować w lizatach komórek HEK293E (frakcja nierozpuszczalna białka). Najprawdopodobniej brak N-glikanów powodował nieprawidłowe fałdowanie białka i jego zatrzymywanie w siateczce śródplazmatycznej (ER), lub znaczące obniżenie jego rozpuszczalności, co uniemożliwiało ich efektywne wydzielanie poza komórkę. Analiza aktywności *in vitro* form rozpuszczalnych wariantów glikozylacyjnych wykazała, że jedynie warianty pozbawione N-glikanu w pozycji N_{121} są aktywne, natomiast enzymy pozbawione N-glikanu w pozycji N_{203} oraz obu miejsc N-glikozylacji jednocześnie nie wykazują aktywności enzymatycznej.

Charakterystyka struktur cukrowych rozpuszczalnych form ludzkiej syntazy Gb3/CD77 wykonana z użyciem glikozydaz o różnej swoistości wykazała obecność dwóch typów struktur N-glikanowych: enzymy w pełni N-glikozylowane zawierały N-glikany kompleksowe i wielomannozowe, natomiast warianty z podstawieniem w jednym sekwonie zawierają tylko N-glikany typu kompleksowego. Z uwagi na brak znanej struktury przestrzennej ludzkiej syntazy Gb3/CD77, przeprowadzono modelowanie białka *in silico* na modelu enzymu z dołączonymi resztami cukrowymi. Uzyskane dane potwierdziły, że N-glikan przyłączony do sekwonu w pozycji N_{203} znajduje się w pobliżu reszt aminokwasowych wchodzących w skład centrum aktywnego enzymu, a dodatkowo wykazały, że region ten charakteryzuje się dużą stabilnością łańcucha polipeptydowego. Z kolei sekwon N_{121} jest zlokalizowany w obrębie labilnej struktury drugorzędowej białka, z dala od centrum aktywnego, przez co nie powinien wpływać na funkcje katalityczne. Dane te sugerują możliwość allosterycznej regulacji aktywności enzymatycznej ludzkiej syntazy Gb3/CD77 przez N-glikan przyłączony do asparaginy w pozycji N_{203}.

Dalsze badania nad ludzką syntazą Gb3/CD77 powinny skupić się na poznaniu struktury przestrzennej enzymu, co pozwoli ostatecznie określić rolę N-glikanów w kształtowaniu katalitycznych właściwości enzymu, a także wyjaśnić molekularne podstawy rozszerzenia swoistości akceptorowej enzymu z podstawieniem p.Q211E. Badania nad tym enzymem mogą też potencjalne zastosowania aplikacyjne, na przykład w poszukiwaniu nowych metod leczenia nowotworów, ponieważ ludzka syntaza Gb3/CD77 bierze udział w procesie przejścia epitelialno-mezenchymalnego (EMT), lub w próbie zastosowania glikotopów P1 jako receptorów „wabikowych" w leczeniu zakażeń STEC.

ABSTRACT

Human Gb3/CD77 synthase (α1,4-galactosyltransferase, synthase P1/P^k, UDP-galactose: β-D-galactosyl-β1-R 4-α-D-galactosyltransferase; EC 2.4.1.228), encoded by the *A4GALT* gene, is a glycosyltransferase belonging to type II transmembrane proteins. The enzyme recognizes two or three different acceptors depending on the amino acid residue at position 211 of the polypeptide chain. The main products are glycosphingolipid antigens Gb3 (P^k) and P1; both belong to the human P1PK blood group system and contain the terminal structure Galα1→4Gal. It was discovered in our laboratory that the enzyme with the p.Q211E substitution can also synthesize a third product called NOR antigen terminating with disaccharide Galα1→4GalNAc; red blood cells with such antigen are recognized by most of the human sera, which is referred to as NOR polyagglutination. Recent reports indicate that human Gb3/CD77 synthase, in addition to glycosphingolipid acceptors, may also recognize glycoproteins, such as complex N-glycans, in which after the galactose residue attachment, terminal Galα1→4Galβ1→4GlcNAc-R oligosaccharides (called P1 glycotopes) are being synthesized.

The Gb3 antigen produced by human Gb3/CD77 synthase is a receptor for pathogens, such as uropathogenic strains of *E. coli* or zoonotic strains of *S. suis*, and Shiga toxins produced by *S. dysenteriae* (serotype 1) and *E. coli* (STEC strains). Infections with those strains cause hemorrhagic colitis and hemolytic-uremic syndrome (HUS), which can lead to severe complications. Recent data indicate that in addition to Gb3 glycosphingolipid, which is major receptor for Shiga toxins, also P1 glycotopes located on glycoproteins can bind and internalize the Shiga toxin type 1, triggering a cytotoxic effect.

Human Gb3/CD77 synthase contains two potential N-glycosylation sites located on asparagine residues at positions 121 (N_{121}AS) and 203 (N_{203}LT). Recently, in our laboratory, it was shown that enzymatic deglycosylation of recombinant Gb3/CD77 synthase caused abolishing of its activity, but the molecular background of this phenomenon remained unclear. Thus, the main aim of my doctoral dissertation was to determine the role of N-glycosylation in the activity of human Gb3/CD77 synthase, and in particular: 1) to determine which potential N-glycosylation sites in human Gb3/CD77 synthase contain attached oligosaccharide residues, with the initial characterization of the carbohydrate structure types; 2) to investigate the influence of N-glycosylation sites of human Gb3/CD77 synthase on the properties of the enzyme (enzymatic activity, acceptor specificity

and intracellular localization); 3) to evaluate the sensitivity of human Gb3/CD77 synthase glycovariants expressed in CHO-Lec2 cells to Shiga toxins; 4) to examination the effect of oligosaccharide residues on the conformation of the enzyme and determine their potential effect on its catalytic activity (*in silico* analyses). The implementation of these research objectives has been included in two original publications.

The first publication of my doctoral dissertation is a theoretical introduction to the experimental part. It discusses the role of N-glycans in the glycosyltransferases activity, the molecular mechanisms underlying changes in the deglycosylated enzymes activity as well as the methods for examining the role of N-glycans in glycosyltransferases activity. In the publication, I discuss 34 glycosyltransferases, about which the data about N-glycans role in activity regulation has been published.

The studies about the role of N-glycosylation of human Gb3/CD77 synthase have been divided into the analysis of a full-length enzyme located in the Golgi apparatus, and its soluble form, devoid of a hydrophobic transmembrane fragment. In both approaches, glycovariants of human Gb3/CD77 synthase differ by the amino acid residues in the third position of N-glycosylation sites (N_{121}AA and N_{203}LA) were obtained. In addition, I analyzed the glycovariants of two versions of Gb3/CD77 synthase: the Q enzyme containing the glutamine residue at position 211 of the polypeptide chain, and the E enzyme with the glutamic acid residue in the same position.

In the second publication included in my doctoral dissertation, I present studies about the full-length human Gb3/CD77 synthase expressed in CHO-Lec2 cells. I have shown that both N-glycosylation sites are occupied. The analysis of enzymatic activity showed that the glycovariants with p.S123A substitutions showed similar enzymatic activity compared to fully N-glycosylated enzymes, in contrast to the glycovariants with p.T205A substitutions, which activity was markedly reduced. The glycovariants deprived of N-glycans (with p.S123A/p.T205A substitutions) showed only residual activity. All glycovariants synthesize terminal Galα1→4Gal disaccharides both on glycosphingolipids and glycoproteins. Moreover, all analyzed CHO-Lec2 cells transfected with genes encoding glycovariants were sensitive to Shiga holotoxins (Stx1 and Stx2). The decreased viability of CHO-Lec2 cells expressing enzymes devoid of N-glycans (with residual enzymatic activity) suggests that only trace amounts of Gb3 glycosphingolipid and/or P1 glycotopes on glycoproteins may trigger cytotoxic effects. I also showed that N-glycan attached to the N_{203} site is essential for proper subcellular localization of the enzyme in the Golgi

apparatus, suggesting that the mislocalization after N-glycans elimination may be responsible for significantly decreased enzyme activity.

In the third publication, I present the data about the glycovariants of Gb3/CD77 synthase without a transmembrane domain expressed using HEK293E cells. The molecular weight of the glycovariants with one N-glycan eliminated was reduced, while the variants completely unglycosylated were not present in the medium, but I identified them in cell lysates as an insoluble fraction. Most likely, the lack of N-glycans caused misfolding of the enzyme and its retention in the ER or a significant reduction of the glycovariants solubility, which in turn prevented their effective secretion. The analysis of *in vitro* activity of the glycovariants showed that only those without N-glycan at N_{121} site were active, while enzymes lacking N-glycan at N_{203} and both N-glycosylation sites simultaneously showed no enzymatic activity at all.

The evaluation of N-glycan structures attached to the soluble forms of human Gb3/CD77 synthase was performed using glycosidases of different specificity and showed that complex and oligomannose structures are present in fully N-glycosylated enzymes, while complex N-glycans were found in glycovariants with substitution in only one sequon. Since the spatial structure of human Gb3/CD77 synthase is not known, *in silico* protein modeling using an enzyme model with carbohydrate residues attached was performed. The obtained data confirmed that the N-glycan attached to the N_{203} site is located near the amino acid residues and may influence the active site of the enzyme; in addition, this region demonstrated the relatively highly rigid polypeptide chain. In contrast, the N_{121} sequon is located within the labile secondary structure of the protein, relatively far away from the active site, thus N-glycan attached to this site seems to not affect catalytic functions of the enzyme. These data suggest the possibility of allosteric regulation of the enzymatic activity of human Gb3/CD77 synthase by N-glycan attached to asparagine at position N_{203}.

Further studies about human Gb3/CD77 synthase should focus on the evaluation of the spatial structure of the enzyme, which may allow us to finally determine the role of N-glycans in regulating its catalytic properties, and explaining the molecular basis of broadening the acceptor specificity of the enzyme with the p.Q211E substitution. Such studies can also have potential practical applications, e.g. in cancer treatment, because Gb3/CD77 synthase is involved in epithelial-mesenchymal transition EMT, or in attempts to introduce P1 glycotopes as "decoy" receptors in treatment of STEC-caused infections.

LISTA PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

1. **Mikolajczyk K.**, Kaczmarek R., Czerwinski M. How glycosylation affects glycosylation: the role of N-glycans in glycosyltransferase activity. *Glycobiology*. 2020; 12: 941-969. doi: 10.1093/glycob/cwaa041.

2. **Mikolajczyk K.**, Bereznicka A., Szymczak-Kulus K., Haczkiewicz-Lesniak K., Szulc B., Olczak M., Rossowska J., Majorczyk E., Kapczynska K., Bovin N., Lisowska M., Kaczmarek R., Miazek A., Czerwinski M. Missing the sweet spot: one of the two N-glycans on human Gb3/CD77 synthase is expendable. *Glycobiology*. 2021; 9: 1145-1162. doi: 10.1093/glycob/cwab041.

3. **Mikolajczyk K.**, Sikora M., Hanus C., Kaczmarek R., Czerwinski M. One of the two N-glycans on the human Gb3/CD77 synthase is essential for its activity and allosterically regulates its function. *Biochem Biophys Res Commun.* 2022; 617: 36-41. doi: 10.1016/j.bbrc.2022.05.085.

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Oświadczenie

Oświadczam, że mój udział w następującej pracy:

Mikolajczyk K., Kaczmarek R., Czerwinski M*. How glycosylation affects glycosylation: the role of N-glycans in glycosyltransferase activity. *Glycobiology*. 2020; 30: 941-969. doi: 10.1093/glycob/cwaa041. * correspondence author, Impact Factor 2020 = 4,313

jest prawidłowo scharakteryzowany w poniższej tabeli.

Autor	Udział [%]	Opis udziału własnego
Krzysztof Mikolajczyk	90	Sformułowanie problemu badawczego, przygotowanie założeń pracy, zgromadzenie literatury na temat roli N-glikozylacji w aktywności glikozylotransferaz; przygotowanie publikacji: pisanie, przygotowanie table i rycin, recenzja, przygotowanie odpowiedzi dla recenzentów.
Radoslaw Kaczmarek	5	Przygotowanie publikacji: recenzja i edycja.
Marcin Czerwinski	5	Sformułowanie problemu badawczego, przygotowanie publikacji: recenzja i edycja.

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Radoslaw Kaczmarek	5	Przygotowanie publikacji: recenzja i edycja.
Marcin Czerwinski	5	Sformułowanie problemu badawczego, przygotowanie publikacji: recenzja i edycja.

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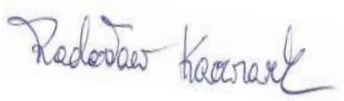

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Oświadczenie

Oświadczam, że mój udział w następującej pracy:

Mikolajczyk K., Bereznicka A., Szymczak-Kulus K., Haczkiewicz-Lesniak K., Szulc B., Olczak M., Rossowska J., Majorczyk E., Kapczynska K., Bovin N., Lisowska M., Kaczmarek R., Miazek A., Czerwinski M*. Missing the sweet spot: one of the two N-glycans on human Gb3/CD77 synthase is expendable. *Glycobiology*. 2021, 31:1145-1162. * autor korespondencyjny, Impact Factor 2021 = 4.313

jest prawidłowo scharakteryzowany w poniższej tabeli.

Autor	Udział [%]	Opis udziału własnego
Krzysztof Mikolajczyk	75	Sformułowanie tez badawczych, zaprojektowanie metodologii badań, przeprowadzenie eksperymentów: klonowanie i sekwencjonowanie plazmidów, qPCR, hodowla komórek HEK293, transfekcja komórek, przygotowanie lizatów komórkowych, trawienie PNGazą F, cytofluorymetria przepływowa, identyfikacja antygenów na powierzchni komórek, testy cytotoksyczności, izolacja i analiza GSLs, spektrometria mas (przygotowanie próbek i analiza wyników), badania immunolokalizacji (przygotowanie próbek), analiza statystyczna, przygotowanie rycin, pisanie publikacji.
Anna Bereznicka	1	Przeprowadzenie eksperymentów: hodowla komórek HEK293, przygotowanie publikacji: recenzja i edycja.
Katarzyna Szymczak-Kulus	1	Przeprowadzenie eksperymentów: analiza monoklonalnego przeciwciała anty-A4GALT, przygotowanie publikacji: recenzja i edycja.
Katarzyna Haczkiewicz -Lesniak	5	Przeprowadzenie eksperymentów: przygotowanie próbek do reakcji immunogold, analizy z wykorzystaniem mikroskopu elektronowego.
Bozena Szulc	5	Przeprowadzenie eksperymentów: mikroskopia konfokalna.
Mariusz Olczak	1	Przygotowanie publikacji: recenzja i edycja.
Joanna Rossowska	2	Przeprowadzenie eksperymentów: sortowanie komórek.
Edyta Majorczyk	1	Przeprowadzenie eksperymentów: qPCR; przygotowanie publikacji: recenzja i edycja.

Katarzyna Kapczynska	1	Przeprowadzenie eksperymentów: spektrometria mas (analiza wyników); przygotowanie publikacji: recenzja i edycja.
Nicolai Bovin	2	Przeprowadzenie eksperymentów: otrzymanie koniugatów oligosacharyd-PAA.
Marta Lisowska	1	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Radoslaw Kaczmarek	1	Przygotowanie publikacji: recenzja i edycja.
Arkadiusz Miazek	2	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Marcin Czerwinski	2	Sformułowanie tez badawczych; przygotowanie publikacji: recenzja i edycja.

Krzysztof Mikołajczyk

Mgr Anna Bereźnicka

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53-114 Wrocław

09.02.2022

Oświadczenie

Oświadczam, że mój udział w następującej pracy:

Mikolajczyk K., Bereznicka A., Szymczak-Kulus K., Haczkiewicz-Lesniak K., Szulc B., Olczak M., Rossowska J., Majorczyk E., Kapczynska K., Bovin N., Lisowska M., Kaczmarek R., Miazek A., Czerwinski M*. Missing the sweet spot: one of the two N-glycans on human Gb3/CD77 synthase is expendable. *Glycobiology*. 2021, 31:1145-1162. * autor korespondencyjny, Impact Factor 2021 = 4.313

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Joanna Rossowska	2	Przeprowadzenie eksperymentów: sortowanie komórek.
Edyta Majorczyk	1	Przeprowadzenie eksperymentów: qPCR; przygotowanie publikacji: recenzja i edycja.

Katarzyna Kapczynska	1	Przeprowadzenie eksperymentów: spektrometria mas (analiza wyników); przygotowanie publikacji: recenzja i edycja.
Nicolai Bovin	2	Przeprowadzenie eksperymentów: otrzymanie koniugatów oligosacharyd-PAA.
Marta Lisowska	1	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Radoslaw Kaczmarek	1	Przygotowanie publikacji: recenzja i edycja.
Arkadiusz Miazek	2	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Marcin Czerwinski	2	Sformułowanie tez badawczych; przygotowanie publikacji: recenzja i edycja.

Anna Borowicka

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28.01.2022

Oświadczenie

Oświadczam, że mój udział w następującej pracy:

Mikolajczyk K., Bereznicka A., Szymczak-Kulus K., Haczkiewicz-Lesniak K., Szulc B., Olczak M., Rossowska J., Majorczyk E., Kapczynska K., Bovin N., Lisowska M., Kaczmarek R., Miazek A., Czerwinski M*. Missing the sweet spot: one of the two N-glycans on human Gb3/CD77 synthase is expendable. *Glycobiology*. 2021, 31:1145-1162. * autor korespondencyjny, Impact Factor 2021 = 4.313

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Edyta Majorczyk	1	Przeprowadzenie eksperymentów: qPCR; przygotowanie publikacji: recenzja i edycja.

Katarzyna Kapczynska	1	Przeprowadzenie eksperymentów: spektrometria mas (analiza wyników); przygotowanie publikacji: recenzja i edycja.
Nicolai Bovin	2	Przeprowadzenie eksperymentów: otrzymanie koniugatów oligosacharyd-PAA.
Marta Lisowska	1	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Radoslaw Kaczmarek	1	Przygotowanie publikacji: recenzja i edycja.
Arkadiusz Miazek	2	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Marcin Czerwinski	2	Sformułowanie tez badawczych; przygotowanie publikacji: recenzja i edycja.

Katarzyna Szmigiel-Kawas

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Oświadczenie

Oświadczam, że mój udział w następującej pracy:

Mikolajczyk K., Bereznicka A., Szymczak-Kulus K., Haczkiewicz-Lesniak K., Szulc B., Olczak M., Rossowska J., Majorczyk E., Kapczynska K., Bovin N., Lisowska M., Kaczmarek R., Miazek A., Czerwinski M*. Missing the sweet spot: one of the two N-glycans on human Gb3/CD77 synthase is expendable. *Glycobiology*. 2021, 31:1145-1162. * autor korespondencyjny, Impact Factor 2021 = 4.313

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Radoslaw Kaczmarek	1	Przygotowanie publikacji: recenzja i edycja.
Arkadiusz Miazek	2	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Marcin Czerwinski	2	Sformułowanie tez badawczych; przygotowanie publikacji: recenzja i edycja.

Katarzyna Haczkiewicz-Leśniak

Dr inż. Bożena Szulc
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ul. Fryderyka Joliot-Curie 14a
50-383 Wrocław

28.01.2022

Oświadczenie

Oświadczam, że mój udział w następującej pracy:

Mikolajczyk K., Bereznicka A., Szymczak-Kulus K., Haczkiewicz-Lesniak K., Szulc B., Olczak M., Rossowska J., Majorczyk E., Kapczynska K., Bovin N., Lisowska M., Kaczmarek R., Miazek A., Czerwinski M*. Missing the sweet spot: one of the two N-glycans on human Gb3/CD77 synthase is expendable. *Glycobiology*. 2021, 31:1145-1162. * autor korespondencyjny, Impact Factor 2021 = 4.313

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Katarzyna Szymczak-Kulus	1	Przeprowadzenie eksperymentów: analiza monoklonalnego przeciwciała anty-A4GALT, przygotowanie publikacji: recenzja i edycja.
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Katarzyna Kapczynska	1	Przeprowadzenie eksperymentów: spektrometria mas (analiza wyników); przygotowanie publikacji: recenzja i edycja.
Nicolai Bovin	2	Przeprowadzenie eksperymentów: otrzymanie koniugatów oligosacharyd-PAA.
Marta Lisowska	1	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Radoslaw Kaczmarek	1	Przygotowanie publikacji: recenzja i edycja.
Arkadiusz Miazek	2	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Marcin Czerwinski	2	Sformułowanie tez badawczych; przygotowanie publikacji: recenzja i edycja.

Sada Bozena

Prof. dr hab. Mariusz Olczak
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28.01.2022

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Arkadiusz Miazek	2	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
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prof. dr hab. Mariusz Olczak
Wydział Biotechnologii
Uniwersytetu Wrocławskiego

Dr hab. Joanna Rossowska

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28.01.2022

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Marta Lisowska	1	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Radoslaw Kaczmarek	1	Przygotowanie publikacji: recenzja i edycja.
Arkadiusz Miazek	2	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Marcin Czerwinski	2	Sformułowanie tez badawczych; przygotowanie publikacji: recenzja i edycja.

joanna Rossowska

Opole, dn. 05.04.2022 r.

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Oświadczam, że mój udział w następującej pracy:

Mikolajczyk K., Bereznicka A., Szymczak-Kulus K., Haczkiewicz-Lesniak K., Szulc B., Olczak M., Rossowska J., Majorczyk E., Kapczynska K., Bovin N., Lisowska M., Kaczmarek R., Miazek A., Czerwinski M*. Missing the sweet spot: one of the two N-glycans on human Gb3/CD77 synthase is expendable. *Glycobiology*. 2021, 31:1145-1162. * autor korespondencyjny, Impact Factor 2021 = 4.313

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Krzysztof Mikolajczyk	75	Sformułowanie tez badawczych, zaprojektowanie metodologii badań, przeprowadzenie eksperymentów: klonowanie i sekwencjonowanie plazmidów, qPCR, hodowla komórek HEK293, transfekcja komórek, przygotowanie lizatów komórkowych, trawienie PNGazą F, cytofluorymetria przeplywowa, identyfikacja antygenów na powierzchni komórek, testy cytotoksyczności, izolacja i analiza GSLs, spektrometria mas (przygotowanie próbek i analiza wyników), badania immunolokalizacji (przygotowanie próbek), analiza statystyczna, przygotowanie rycin, pisanie publikacji.
Anna Bereznicka	1	Przeprowadzenie eksperymentów: hodowla komórek HEK293, przygotowanie publikacji: recenzja i edycja.
Katarzyna Szymczak-Kulus	1	Przeprowadzenie eksperymentów: analiza monoklonalnego przeciwciała anty-A4GALT, przygotowanie publikacji: recenzja i edycja.
Katarzyna Haczkiewicz -Lesniak	5	Przeprowadzenie eksperymentów: przygotowanie próbek do reakcji immunogold, analizy z wykorzystaniem mikroskopu elektronowego.
Bozena Szulc	5	Przeprowadzenie eksperymentów: mikroskopia konfokalna.
Mariusz Olczak	1	Przygotowanie publikacji: recenzja i edycja.
Joanna Rossowska	2	Przeprowadzenie eksperymentów: sortowanie komórek.
Edyta Majorczyk	1	Przeprowadzenie eksperymentów: qPCR; przygotowanie publikacji: recenzja i edycja.

Katarzyna Kapczynska	1	Przeprowadzenie eksperymentów: spektrometria mas (analiza wyników); przygotowanie publikacji: recenzja i edycja.
Nicolai Bovin	2	Przeprowadzenie eksperymentów: otrzymanie koniugatów oligosacharyd-PAA.
Marta Lisowska	1	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Radoslaw Kaczmarek	1	Przygotowanie publikacji: recenzja i edycja.
Arkadiusz Miazek	2	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Marcin Czerwinski	2	Sformułowanie tez badawczych; przygotowanie publikacji: recenzja i edycja.

Edyta Majorczyk

Dr Katarzyna Kapczyńska

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28.01.2022

Oświadczenie

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Mikolajczyk K., Bereznicka A., Szymczak-Kulus K., Haczkiewicz-Lesniak K., Szulc B., Olczak M., Rossowska J., Majorczyk E., Kapczynska K., Bovin N., Lisowska M., Kaczmarek R., Miazek A., Czerwinski M*. Missing the sweet spot: one of the two N-glycans on human Gb3/CD77 synthase is expendable. *Glycobiology*. 2021, 31:1145-1162. * autor korespondencyjny, Impact Factor 2021 = 4.313

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Katarzyna Szymczak-Kulus	1	Przeprowadzenie eksperymentów: analiza monoklonalnego przeciwciała anty-A4GALT, przygotowanie publikacji: recenzja i edycja.
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Bozena Szulc	5	Przeprowadzenie eksperymentów: mikroskopia konfokalna.
Mariusz Olczak	1	Przygotowanie publikacji: recenzja i edycja.
Joanna Rossowska	2	Przeprowadzenie eksperymentów: sortowanie komórek.
Edyta Majorczyk	1	Przeprowadzenie eksperymentów: qPCR; przygotowanie publikacji: recenzja i edycja.

Katarzyna Kapczynska	1	Przeprowadzenie eksperymentów: spektrometria mas (analiza wyników); przygotowanie publikacji: recenzja i edycja.
Nicolai Bovin	2	Przeprowadzenie eksperymentów: otrzymanie koniugatów oligosacharyd-PAA.
Marta Lisowska	1	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Radoslaw Kaczmarek	1	Przygotowanie publikacji: recenzja i edycja.
Arkadiusz Miazek	2	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Marcin Czerwinski	2	Sformułowanie tez badawczych; przygotowanie publikacji: recenzja i edycja.

Katarzyna Kapczyńska

Nicolai Bovin 08.12.2021

Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry

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Declaration

I hereby declare that my contribution to the following manuscript:

Mikolajczyk K., Bereznicka A., Szymczak-Kulus K., Haczkiewicz-Lesniak K., Szulc B., Olczak M., Rossowska J., Majorczyk E., Kapczynska K., Bovin N., Lisowska M., Kaczmarek R., Miazek A., Czerwinski M*. Missing the sweet spot: one of the two N-glycans on human Gb3/CD77 synthase is expendable. *Glycobiology*. 2021, 31:1145-1162. * correspondence author, Impact Factor 2021 = 4.313

is correctly characterized in the table below.

Contributor	Contribution [%]	Description of main tasks
Krzysztof Mikolajczyk	75	Conceptualization of the study, design of methodology and investigation: cloning and sequencing of plasmids, qPCR, culturing of HEK293 cells, transfection of the cells, preparation of the cell lysates, treatment with PNGase F, flow cytometry, identification of cell surface antigens, cytotoxicity tests, GSLs isolation and identification, mass spectrometry (samples preparation and analysis of the results), immunolocalization experiments (samples preparation); statistical analysis, preparation of figures, writing of the article.
Anna Bereznicka	1	Investigation: culturing of HEK293 cells; writing: reviewing and editing
Katarzyna Szymczak-Kulus	1	Investigation: evaluation of the monoclonal anti-A4GALT antibody. Writing: reviewing and editing.
Katarzyna Haczkiewicz -Lesniak	5	Investigation: preparation of the samples for immunogold reaction, electron microscopy studies.
Bozena Szulc	5	Investigation: confocal microscopy.
Mariusz Olczak	1	Writing: reviewing and editing.
Joanna Rossowska	2	Investigation: cell sorting.
Edyta Majorczyk	1	Investigation: qPCR; writing: review and editing.

Katarzyna Kapczynska	1	Investigation: mass spectrometry (analysis of the results); writing: reviewing and editing
Nicolai Bovin	2	Investigation: production of the PAA-oligosaccharide conjugates
Marta Lisowska	1	Investigation: production of the monoclonal anti-A4GALT antibody.
Radoslaw Kaczmarek	1	Writing: reviewing and editing
Arkadiusz Miazek	2	Investigation: production of the monoclonal anti-A4GALT antibody
Marcin Czerwinski	2	Conceptualization of the study, writing: review and editing

Nicolai Bovin, 09.10.2021

Dr inż. Marta Lisowska
11.02.2022
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Oświadczenie

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Anna Bereznicka	1	Przeprowadzenie eksperymentów: hodowla komórek HEK293, przygotowanie publikacji: recenzja i edycja.
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Bozena Szulc	5	Przeprowadzenie eksperymentów: mikroskopia konfokalna.
Mariusz Olczak	1	Przygotowanie publikacji: recenzja i edycja.
Joanna Rossowska	2	Przeprowadzenie eksperymentów: sortowanie komórek.
Edyta	1	Przeprowadzenie eksperymentów: qPCR; przygotowanie publikacji: recenzja i edycja.

Majorczyk		
Katarzyna Kapczynska	1	Przeprowadzenie eksperymentów: spektrometria mas (analiza wyników); przygotowanie publikacji: recenzja i edycja.
Nicolai Bovin	2	Przeprowadzenie eksperymentów: otrzymanie koniugatów oligosacharyd-PAA.
Marta Lisowska	1	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Radoslaw Kaczmarek	1	Przygotowanie publikacji: recenzja i edycja.
Arkadiusz Miazek	2	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Marcin Czerwinski	2	Sformułowanie tez badawczych; przygotowanie publikacji: recenzja i edycja.

Marta
Lisowska

Dr Radosław Kaczmarek
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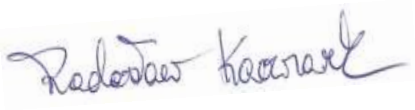
Radoslaw Kaczmarek	1	Przygotowanie publikacji: recenzja i edycja.
Arkadiusz Miazek	2	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Marcin Czerwinski	2	Sformułowanie tez badawczych; przygotowanie publikacji: recenzja i edycja.



Prof. dr hab. Arkadiusz Miążek
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Radoslaw Kaczmarek	1	Przygotowanie publikacji: recenzja i edycja.
Arkadiusz Miazek	2	Przeprowadzenie eksperymentów: otrzymanie monoklonalnego przeciwciała anty-A4GALT.
Marcin Czerwinski	2	Sformułowanie tez badawczych; przygotowanie publikacji: recenzja i edycja.

Prof. dr hab. Marcin Czerwiński

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Katarzyna Szymczak-Kulus	1	Przeprowadzenie eksperymentów: analiza monoklonalnego przeciwciała anty-A4GALT, przygotowanie publikacji: recenzja i edycja.
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Marcin Czerwinski	2	Sformułowanie tez badawczych; przygotowanie publikacji: recenzja i edycja.

Marcin Czerwiński

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20.08.2022

Oświadczenie

Oświadczam, że mój udział w następującej pracy:

Mikolajczyk K.*, Sikora M., Hanus C., Kaczmarek R., Czerwinski M. One of the two N-glycans on the human Gb3/CD77 synthase is essential for its activity and allosterically regulates its function. *Biochem Biophys Res Commun.* 2022; 617: 36-41. doi: 10.1016/j.bbrc.2022.05.085. * correspondence author, Impact Factor 2021 = 3,322

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Autor	Udział [%]	Opis udziału własnego
Krzysztof Mikolajczyk	65	Sformułowanie tez badawczych, zaprojektowanie metodologii badań, przeprowadzenie eksperymentów: klonowanie i sekwencjonowanie plazmidów, hodowla i transfekcja komórek HEK293E, przygotowanie lizatów komórkowych, trawienie PNGazą F i Endo H, analizy statystyczne, przygotowanie rycin, pisanie publikacji.
Mateusz Sikora	20	Przeprowadzenie eksperymentów: modelowanie molekularne ludzkiej syntazy Gb3/CD77 (GlycoSASA, VMD, ANM), obliczenia z wykorzystaniem ProDy; przygotowanie publikacji: recenzja i edycja.
Cyril Hanus	5	Przeprowadzenie eksperymentów: modelowanie molekularne ludzkiej syntazy Gb3/CD77 (GlycoSHIELD). Przygotowanie publikacji: recenzja i edycja.
Radoslaw Kaczmarek	5	Przygotowanie publikacji: recenzja i edycja.
Marcin Czerwinski	5	Sformułowanie tez badawczych, przygotowanie publikacji: recenzja i edycja.

Krzysztof Mikołajczyk

PhD Mateusz Sikora

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20.08.2022

Declaration

I hereby declare that my contribution to the following manuscript:

Mikolajczyk K.*, Sikora M., Hanus C., Kaczmarek R., Czerwinski M. One of the two N-glycans on the human Gb3/CD77 synthase is essential for its activity and allosterically regulates its function. *Biochem Biophys Res Commun*. 2022; 617: 36-41. doi: 10.1016/j.bbrc.2022.05.085.
* correspondence author, Impact Factor 2021 = 3.322

is correctly characterized in the table below.

Contributor	Contribution [%]	Description of main tasks
Krzysztof Mikolajczyk	65	Conceptualization of the study, design of methodology and investigation: cloning and sequencing of plasmids, culturing of HEK293E cells, transfection of the cells, preparation of the cell lysates, treatment with PNGase F and Endo H, statistical analysis, preparation of figures, writing of the article.
Mateusz Sikora	20	Investigation: molecular modeling of the human Gb3/CD77 (GlycoSASA, VMD, ANM), calculations using ProDy implementation; writing: reviewing and editing
Cyril Hanus	5	Investigation: molecular modeling of the human Gb3/CD77 (GlycoSHIELD). Writing: reviewing and editing.
Radoslaw Kaczmarek	5	Writing: reviewing and editing.
Marcin Czerwinski	5	Conceptualization of the study; writing: reviewing and editing

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Paris 2022-07-11

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20.08.2022

Oświadczenie

Oświadczam, że mój udział w następującej pracy:

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jest prawidłowo scharakteryzowany w poniższej tabeli.

Autor	Udział [%]	Opis udziału własnego
Krzysztof Mikolajczyk	65	Sformułowanie tez badawczych, zaprojektowanie metodologii badań, przeprowadzenie eksperymentów: klonowanie i sekwencjonowanie plazmidów, hodowla i transfekcja komórek HEK293E, przygotowanie lizatów komórkowych, trawienie PNGazą F i Endo H, analizy statystyczne, przygotowanie rycin, pisanie publikacji.
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Review

Review

How glycosylation affects glycosylation: the role of N-glycans in glycosyltransferase activity

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Abstract

N-glycosylation is one of the most important posttranslational modifications of proteins. It plays important roles in the biogenesis and functions of proteins by influencing their folding, intracellular localization, stability and solubility. N-glycans are synthesized by glycosyltransferases, a complex group of ubiquitous enzymes that occur in most kingdoms of life. A growing body of evidence shows that N-glycans may influence processing and functions of glycosyltransferases, including their secretion, stability and substrate/acceptor affinity. Changes in these properties may have a profound impact on glycosyltransferase activity. Indeed, some glycosyltransferases have to be glycosylated themselves for full activity. N-glycans and glycosyltransferases play roles in the pathogenesis of many diseases (including cancers), so studies on glycosyltransferases may contribute to the development of new therapy methods and novel glycoengineered enzymes with improved properties. In this review, we focus on the role of N-glycosylation in the activity of glycosyltransferases and attempt to summarize all available data about this phenomenon.

Key words: enzyme activity, glycan, glycosyltransferase, N-glycosylation

Introduction

N-glycosylation is a common and complex posttranslational modification (PTM) of proteins. Glycans have been found in the majority of organisms, as well as in viruses that carry on their envelope oligosaccharide chains originating from the infected host cells (Wacker et al. 2002; Eichler 2013; Nothaft and Szymanski 2013; Samuelson and Robbins 2014; Jarrell et al. 2014; Li, Debowski, Liao, Tang, Nilsson, Marshall, Stubbs, Benghezal 2016a; Schäffer and Messner 2016; Nagashima et al. 2018). N-linkage is one of the six glycosylation types, including O- and S-linked glycosylation, C-linked mannosylation, phospho-glycation and glypiation (Spiro 2002; Reily et al. 2019). However, N- and O-glycans occur most frequently. The ubiquity of N-glycans is believed to be a result of their extreme diversity (made possible by a large number of ways in which sugar residues can be linked), relative hydrophilicity, spatial flexibility of oligosaccharide chains and ability to transfer an electric charge by some sugar moieties (Drickamer and Taylor 1998; Spiro 2002; Moremen et al. 2012).

N-glycans are attached to asparagine within a consensus sequence called sequon, which is usually a canonical Asn–X–Ser/Thr motif, where X is any amino acid except proline. However, N-glycans may also be linked to N–X–C, N–Q–C, N–S–G or Q–G–T sequons (referred to as noncanonical motifs) (Lowenthal et al. 2016). An estimated 70% of N–X–S/T sequons are N-glycosylated (Marshall 1972; Marshall 1974; Spiro 2002; Aebi 2013; Breitling and Aebi 2013; Kim et al. 2015; Tannous et al. 2015), and the rest (approximately 30%) remain unoccupied because of structural constraints imposed by the flanking amino acid sequences (Petrescu et al. 2004; Ben-Dor et al. 2004).

N-glycans may play many different roles at molecular and cellular levels, e.g., in protein folding (Mitra et al. 2006; Lee et al. 2015; Tannous et al. 2015; Jayaprakash and Surolia 2017), secretion (Fiedler

and Simons 1995; Skropeta 2009; Goettig 2016), maturation (Agthe et al. 2018) and intracellular trafficking (Vagin et al. 2009; Niwa et al. 2012; Moharir et al. 2013; Rosnoblet et al. 2013); communication and cell–cell interactions (Skropeta 2009; Dennis et al. 2009; Yasuda et al. 2015; Zhuo et al. 2016; Varki 2017; Cui et al. 2018; Huang et al. 2018; Neerincx and Boyle 2019); immune responses (Skropeta 2009; Varki 2017; Ryan and Cobb 2012); and diseases (Leroy 2006; Kizuka et al. 2017; Lazniewska and Weiss 2017; Sato and Hane 2018), including cancer (Vajaria and Patel 2017), and may mediate in host cell entry of viruses, such as SARS-CoV-2 (Watanabe et al. 2020). Additionally, N-glycosylation may regulate brain development and functions (Handa-Narumi et al. 2018). The role of N-glycans in protein biogenesis derives from their influence on the secondary structure and physicochemical properties of proteins (Zhu et al. 2014; Jayaprakash and Surolia 2017). N-glycans may also contribute to conformational distortion, altering the thermodynamic stability of proteins, as in the case of MM1 (an artificially modified Gc-MAF serum factor protein) (Gavrilov et al. 2015) or the WW domain of the human Pin 1 protein (Price et al. 2010).

In this review, we summarize the present-day knowledge about the N-glycosylation of glycosyltransferases (GTs) and its role in shaping their activities. One overarching shortcoming to the studies completed to date is that they mostly analyzed recombinant forms of GTs, missing a deeper look at the *in vivo* aspects of glycosylations. Future studies will benefit from new developments in glycoproteomics, which provides new perspectives on N- and O-glycosylation, and their tissue-, cell- and site-specific heterogeneity (Losfeld et al. 2017; Arigoni-Affolter et al. 2019). Nevertheless, several lines of evidence confirm that the presence of oligosaccharide chains on GTs may be required for their proper folding, stability, solubility, intracellular localization and catalytic function. Conversely, modifying N-glycosylation may help generate enzymes with improved properties (Liu et al. 2014), while N-glycosylation inhibitors may have clinical applications, including therapies for cancers and infectious diseases (Dwek et al. 2002; Brown et al. 2007; Tyrrell et al. 2017). Thus, studies about the relationship between N-glycans on glycosyltransferases and their functions are gaining traction in industrial biotechnology and may bolster drug discovery.

Glycosyltransferase activity can depend on N-glycosylation

Glycosyltransferases catalyze the transfer of carbohydrate moieties from an activated nucleotide sugar donor to an acceptor molecule and retain or invert the stereochemistry of the anomeric center (in comparison with the donor substrate stereochemistry). Most GTs are type II transmembrane proteins consisting of a cytoplasmic N-terminal stem region, a C-terminal catalytic domain located in the lumen of the Golgi or endoplasmic reticulum (ER) and a lipid bilayer-embedded transmembrane domain (Lairson et al. 2008). Glycosyltransferases may adopt one of the four structural folds, called GT-A, GT-B, GT-C and GT-D, which differ in the topology and type of donor used in the catalysis (Lairson et al. 2008; Gloster 2014; Albesa-Jové et al. 2014; Zhang et al. 2015; Albuquerque-Wendt et al. 2019). Many GTs (nearly all from the GT-A superfamily) require a divalent metal ion (typically Mn^{2+}) for activity. These enzymes contain the D-X-D motif (Asp-any residue-Asp), which coordinates the metal ion. GT structures and mechanisms of action have been extensively discussed in several reviews (Lairson et al. 2008; Gloster 2014; Albesa-Jové et al. 2014; Rini and Esko 2017; Moremen and Haltiwanger 2019).

N-glycosylation may affect the enzyme activity in several ways: (1) by facilitating the folding of the polypeptide chain (N-glycans are attached cotranslationally and may stabilize proper protein fold), (2) by ensuring the correct subcellular localization of the protein (which may otherwise accumulate in the ER and degrade) and (3) by preventing protein aggregation (Figure 1) (Ryšlavá et al. 2013). These processes are largely intertwined insofar with ER retention, protein aggregation and/or poor stability, which may result from protein misfolding. In addition, N-glycans may have a more direct influence on catalytic functions by improving the thermal and kinetic stability of enzymes (Price et al. 2010; Jayaprakash and Surolia 2017). The importance of individual N-glycan chains for enzymatic activity may depend on their size and the sequon that they link up to (Skropeta 2009; Goettig 2016).

Glycosyltransferase activity: how to evaluate the potential influence of N-glycosylation

Three general approaches may be used to study the role of N-glycosylation in enzyme activity: (1) removing N-glycans from native proteins using exo- or endoglycosidases (Mulloy et al. 2017), preventing the attachment of glycans to the nascent protein by (2) site-directed mutagenesis (Kasturi et al. 1997) or (3) using N-glycosylation inhibitors (Figure 2) (Esko et al. 2017). Outcomes of experiments that involve eliminating N-glycosylation sites by mutagenesis must be interpreted with a caveat that the method inherently changes also the protein part of the molecule, which is impossible to control. Thus, any changes in enzyme activity may result from other effects of deglycosylation such as protein misfolding and/or aggregation and ER retention (stalled transport to the Golgi) and degradation (Skropeta 2009), not be directly related with impact on catalytic functions of enzyme. To avoid false conclusions, outcomes of mutagenesis studies should be validated using other techniques.

N-glycosylation inhibitors may block N-glycosylation altogether (tunicamycin) or inhibit glycosidases (α-glucosidase and α-mannosidase) that are involved in the maturation of N-glycans. Tunicamycin inhibits the GlcNAc phosphotransferase (GPT), thus disabling the transfer of *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) from UDP-GlcNAc to dolichol-P, which produces dolichol-PP-GlcNAc, a key substrate in the initiation of N-glycan. On the other hand, glycosidase inhibitors prevent the trimming of $Glc_3Man_9GlcNAc_2$ after its transfer to a glycoprotein. Such immature oligosaccharides cannot be properly processed by downstream enzymes, so the emerging N-glycans lack the characteristic terminal sugars. Castanospermine (α-glucosidase I and II inhibitors) and australine (which inhibits α-glucosidase I) cause accumulation of fully glucosylated chains, whereas 1-deoxynojirimycin (α-glucosidase II inhibitor) treatment results in chains containing one to two glucose residues. Swainsonine inhibits α-mannosidase II, causing the accumulation of paucimannose oligosaccharides ($Man_4GlcNAc_2$ and $Man_5GlcNAc_2$) and hybrid-type chains at the cost of complex oligosaccharides. Other mannosidase inhibitors, such as 1-deoxymannojirimycin and kifunensine, which selectively inhibit α-mannosidase I, result in accumulation of $Man_{7\text{-}9}GlcNAc_2$ oligosaccharides on glycoproteins (Esko et al. 2017). These alterations of N-glycans may markedly impact protein functions, such as enzyme activity, so their employment in N-glycosylation studies may increase our knowledge about the role of N-glycans in activity of GTs.

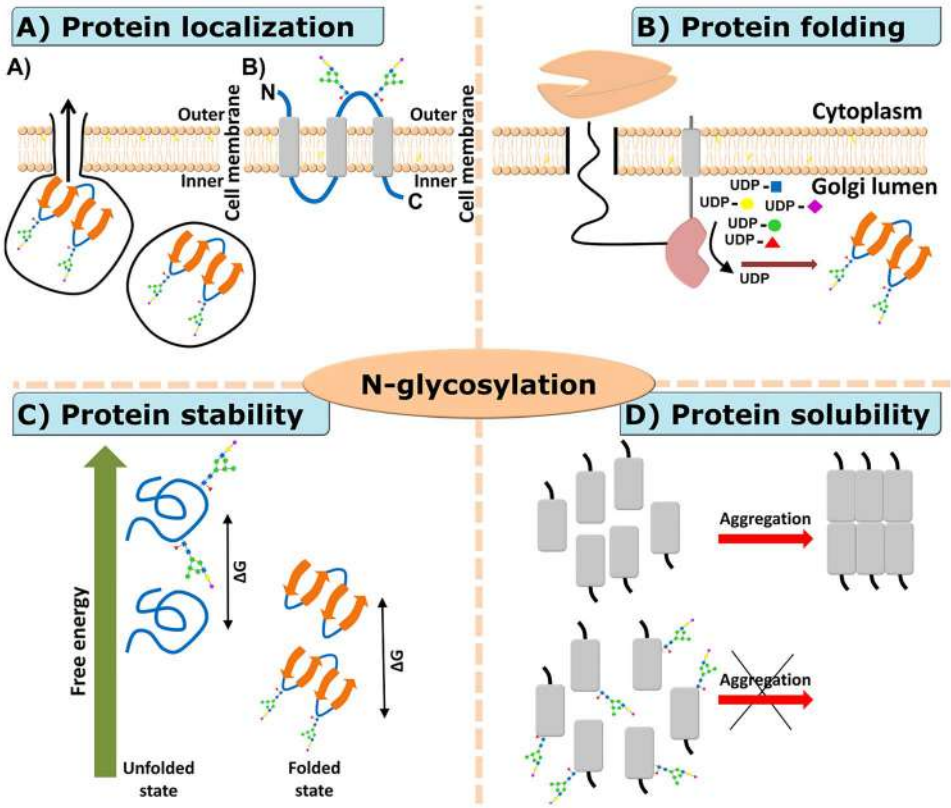

Fig. 1. Functions of N-glycans that may be associated with enzymatic activity. (**A**) N-linked oligosaccharides form hydrophilic moieties on secreted proteins, or extracellular fragments of membrane proteins enhance their stability and solubility. (**B, C**) N-glycosylation influences protein stability by providing folding dynamics associated with the free energy levels in the folding process. (**D**) When N-linked glycans are absent, the exposed hydrophobic amino acid residues may drive aggregation or oligomerization of the protein. Protein oligomerization may cause a decrease in glycosyltransferase activity (Kellokumpu et al. 2016).

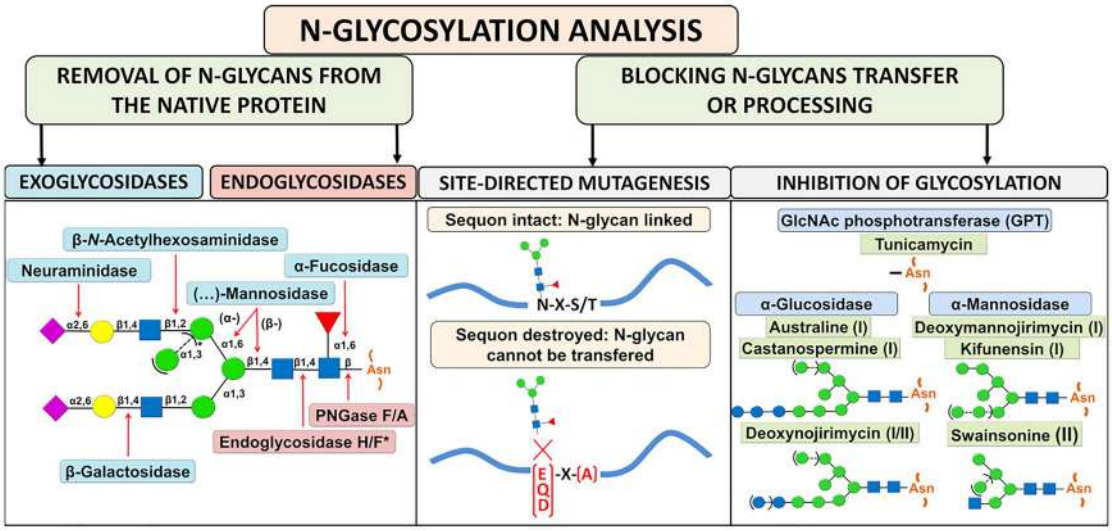

Fig. 2. Methods of studying N-glycosylation and its influence on protein activity. (**A**) Exo- and endoglycosidases remove nonreducing terminal carbohydrate moieties and cleave inner parts of oligosaccharide chains, respectively. N-glycanase (PNGase) cleaves the GlcNAc-N bond, releasing the entire N-glycan. PNGase F cannot remove N-glycans with an α1,3-fucosylated core, in contrast to PNGase A. Endoglycosidases H and F cleave between two GlcNAc residues in oligomannose or hybrid N-glycans and oligomannose or biantennary N-glycans, respectively. (**B**) In site-directed mutagenesis, the N-glycosylation sequon is modified to prevent the transfer of N-glycan to asparagine. (**C**) Inhibition of enzymes that process N-glycans (α-glucosidases and α-mannosidases) results in the synthesis of modified N-glycans or the blocking of N-glycan synthesis altogether (tunicamycin inhibitor).

The selection of N-glycan elimination strategy is pivotal because each method has inherent drawbacks. Using glycosidases to determine the role of N-glycans in the enzyme activity entails exposure to solvents, which may denature the evaluated protein (Mulloy et al. 2017). On the other hand, substitution of amino acids within N-glycosylation sequons (site-directed mutagenesis method) may trigger unfavorable changes in the tertiary structure of the protein. Inhibitors of N-glycosylation also cause side effects, e.g., by inducing ER stress due to the accumulation of misfolded proteins, ultimately leading to apoptosis (Esko et al. 2017; Abdullahi et al. 2017). Thus, a combination of different approaches is usually the best way to study the role of individual N-glycosylation sites. Indeed, the potential number of different mechanisms that may underlie the altered activity of GT calls for a comprehensive approach in the design of studies that involve removal of N-glycans or blocking N-glycosylation.

Role of N-glycosylation in the activity of selected glycosyltransferases

Sialyltransferases

Sialyltransferases (STs) transfer sialic acid from an activated sugar donor to glycosphingolipid or glycoprotein acceptors (Bhide and Colley 2016). Eukaryotic sialyltransferases are type II transmembrane proteins localized in the Golgi. STs usually contain sialyl motifs, which are conservative protein sequences required for full activity, and N-glycosylation sequons are often located within these motifs (El-Battari et al. 2003; Noel et al. 2017).

Rat ST6Gal-I exists in two membrane-associated isoforms: one (called STtyr) is cleaved and secreted, while the other (STcys) remains in the Golgi. Chen and Colley (2000) analyzed the STtyr isoform and soluble forms of the STtyr that lack the transmembrane domain and cytoplasmic tail (Table I-1A) (Chen and Colley 2000). Upon eliminating N-glycans from the full-length membrane-associated STtyr isoform expressed in CHO cells, the enzyme showed activity *in vivo*, but it was not cleaved and accumulated in the Golgi (as the STcys form). In addition, the expression level of the p.N146A/p.N158Q variant was lower than that of the wild-type control. Moreover, a variant with the p.N158Q substitution was not able to exit the ER, indicating that the N158 site is required for the enzyme to traverse the Golgi. In contrast, the p.N158Q and p.N146A/p.N158Q variants of STtyr were not active at all *in vitro*, and the p.N146S variant was the only one to show partial activity (Table II-1A) (Chen and Colley 2000). Thus, the lack of *in vitro* activity of the full-length STtyr could be a result of aggregation in cell lysates which were used for analysis. Indeed, the analysis of truncated enzyme forms (with deletions of 64–403 and 97–403 amino acids) with the p.N146A/p.N158Q substitution showed that the enzyme was not secreted and exhibited no *in vivo* activity, indicating that N-glycans play an essential role in the folding of the enzyme. Importantly, it should be pointed out that the activity assays were used for the analysis of different enzyme forms and the conditions by which the enzymes were analyzed (in living cells and cell lysates) may also influence the results (Chen and Colley 2000). The analysis of the soluble forms of STtyr showed that the presence of N-glycans is a prerequisite for activity in both *in vivo* and *in vitro* models. These enzyme variants were active only when fully glycosylated, and eliminating N-glycans led to ER retention and inactivation. Thus, it seems that N-glycans are required for proper folding and solubility of the enzyme, because the unglycosylated STtyr form self-associates or oligomerizes (Chen and Colley 2000). In addition, the presence of N-glycans with terminal GlcNAc is crucial for the ST6Gal-I activity, whereas N-glycans with terminal sialic acid or galactose are of lesser importance. Thus, the terminal sugar may be important for enzyme activity (Fast et al. 1993).

The human ST6Gal-I homolog is one of the few glycosyltransferases the structure of which was determined with its intrinsic N-glycosylation pattern partially preserved. One sequon at N149 was N-glycosylated while the other one (N161) presumably lost its N-glycan during crystallization (Table I-1A) (Kuhn et al. 2013). The overexpression of an N-terminally truncated (Δ108) variant of ST6Gal-I in *Pichia pastoris* (Legaigneur et al. 2001; Luley-Goedl et al. 2016), insect (Kim et al. 2003) and HEK293 (Kuhn et al. 2013) cells yielded an active enzyme, while the enzyme obtained in *Saccharomyces cerevisiae* was inactive (Malissard et al. 1999). It was shown that N-glycans from the *P. pastoris*-derived enzyme contain short and unbranched high-mannose structures, in contrast to the long and branched high-mannose N-glycans identified in the ST6Gal-I expressed in *S. cerevisiae*. The enzyme obtained in insect and HEK293 cells contained paucimannose N-glycans and human-like N-glycans, respectively. Taken together, these data suggest that the type of N-glycan chains may dramatically influence catalytic activity; therefore, selecting the right host seems to be of key importance for obtaining an active protein. On the other hand, the expression of human ST6Gal-I in the *Escherichia coli* clone pSTMX in low-temperature cultivation condition (Hidari et al. 2005) and strain pGro7/BL21 (in both approaches, the produced enzymes were increased solubility) yielded an active enzyme (Watanabe et al. 2012), suggesting that expression systems designed to increase protein solubility can compensate for the lack of eukaryotic N-glycosylation in bacterial systems.

The human ST3Gal-II contains two predicted N-glycosylation sites at N92 and N211 (Table I-1B), but only the N211 site is N-glycosylated while the N92 site remains unglycosylated, probably due to steric hindrance created by a nearby proline (Ruggiero et al. 2015). The p.N92Q variant of ST3Gal-II expressed by CHO-K1 cells showed an increased and a decreased *in vitro* activity in the presence of a glycolipid and glycoprotein acceptor, respectively (Table II-1B) (Ruggiero et al. 2015). In contrast, variants with the p.N211Q and p.N92Q/p.N211Q substitutions showed markedly reduced activities *in vitro*, regardless of the acceptor type (Table II-1B). When the N-glycan at the N211 site was missing, the enzyme misfolded, stalled in the ER and showed no activity (the activity assay was used cell lysate). The N211 site is conserved in many vertebrates and located between two ST3Gal family motifs (Ruggiero et al. 2015). Thus, the N-glycan at N211 seems to influence either the recognition of the substrate or the enzyme folding, both of which are necessary for proper enzyme activity. In contrast, since the N92 site is not glycosylated, the observed change in substrate affinity arises probably due to substitution of glutamine for asparagine in the variant enzyme, which was used to evaluate the role of this site in the ST3Gal-II activity (Ruggiero et al. 2015).

Insect α2,6-sialyltransferase from *Bombyx mori* (BmST) contains three potential N-glycosylation sites: N274, N318 and N367 (Table I-1C) (Kajiura et al. 2015). When the enzyme was expressed in *E. coli*, its activity was significantly reduced, probably due to a decreased solubility, misfolding and/or aggregation. Further studies, which used soluble enzyme variants (expressed in Sf9 insect cells), demonstrated that the deletion of the N-glycan attached to N274, which is localized in the functionally important C-terminal L-motif, caused an increase in sugar donor affinity. However, the roles of sequons N318 and N367 were not examined (Kajiura et al. 2015). In addition, the effects of changes at the N274 site varied depending on which amino acid was substituted for N after site-directed

Table I. Glycosyltransferases that require N-glycans for enzymatic activity

Lp.	Enzyme	Species	Uniprot accession number	Functions	Putative N-glycosylation sites	References
1. Sialyltransferases						
A	α2,6-Sialyltransferase-I, ST6Gal-I (EC:2.4.99.1)	*Homo sapiens*	P15907	Cell differentiation, sialylation of E-selectin, ICAM-1 and VCAM-1, carcinogenesis, immune system regulation	*H. sapiens*: N149[a], N161[a]	(Fast et al. 1993; Chen and Colley 2000; Dall'Olio 2000; Wang et al. 2015; Luley-Goedl et al. 2016; Venturi et al. 2019)
		Rattus norvegicus	P13721		*R. norvegicus*: N146[a], N168[a], N285	
B	α2,3-Sialyltransferase-II, ST3Gal-II (EC:2.4.99.4)	*H. sapiens*	Q16842	Ganglioside synthesis, late-onset obesity and insulin resistance progress	N92, N211[a]	(Takashima and Tsuji 2000; Vallejo-Ruiz et al. 2001; Saito et al. 2003; Ruggiero et al. 2015; Taniguchi 2008; Hatano et al. 2011; Teppa et al. 2016; Lopez et al. 2017)
C	α2,6-Sialyltransferase, ST6Gal (EC:2.4.99.x)	*B. mori*	ND	Unknown	N274[a], N318, N367	(Kajiura et al. 2015)
D	α2,3-Sialyltransferase-V, ST3Gal-V (EC:2.4.99.9)	*Mus musculus*	O88829	GM3 synthesis, epilepsy pathogenesis	N180[a], N224[a], N334[a]	(Fragaki et al. 2013; Uemura et al. 2006)
E	α2,3-Sialyltransferase-I, ST3Gal-I (EC:2.4.99.4)	*H. sapiens*	Q11201	GM1b ganglioside and O-mucin-type glycoprotein synthesis, immune system regulation, carcinogenesis	N27 (according to (Jeanneau et al. 2004)), N79, N114, N201, N323	(Priatel et al. 2000; Grabie et al. 2002; Jeanneau et al. 2004; Kao et al. 2006; Van Dyken et al. 2007; Solatycka et al. 2012; Sproviero et al. 2012; Bai et al. 2015; Wen et al. 2017; Yeo et al. 2019)
F	Poly-α2,8-sialyltransferase 8B, ST8Sia-II (EC:2.4.99.x)	*M. musculus*	O35696	Polysialylation of NCAM molecule, brain functions, immune system regulation, mental disorder pathogenesis, carcinogenesis, cell proliferation and motility	N60, N72, N89[a], N134, N219[a], N234[a]	(Mühlenhoff et al. 2001; Angata et al. 2004; Weinhold et al. 2005; Stoenica et al. 2006; Drake et al. 2008; Oltmann-Norden et al. 2008; Schreiber et al. 2008; Kröcher et al. 2013; Guan et al. 2015; Wang et al. 2016; Gong et al. 2017; Mori et al. 2017; Werneburg *et al*. 2017; Sato and Hane 2018)

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Table I. Continue

Lp.	Enzyme	Species	Uniprot accession number	Functions	Putative N-glycosylation sites	References
G	Poly-α-2,8-sialyltransferase, ST8Sia-IV (EC:2.4.99.x)	*Cricetulus griseus* (Chinese hamster)	Q64690	Polysialylation of NCAM molecule	N50, N74[a], N119, N204, N219	(Mühlenhoff et al. 2001; Mori et al. 2017)
H	α2,8-Sialyltransferase-I, ST8Sia-I, GD3 synthase (EC:2.4.99.x)	*Gallus gallus*	P79783	GD3 ganglioside synthesis, development of the retina and brain in chicks	N57, N105, N200	(Martina et al. 1998; Bieberich et al. 2000)
		M. musculus	Q64687	GD3 ganglioside synthesis	N70, N118, N213, N244 (Uniprot)	
2. Fucosyltransferases						
A	α1,3-fucosyltransferase-VII, FUT7 (EC:2.4.1. x)	*H. sapiens*	Q11130	Sialyl Lewis^{x} (sLe^{x}) synthesis, carcinogenesis, embryo implantation, immune system regulation, integrin expression, cell adhesion, signaling pathway	N81[a], N291[a]	(Prorok-Hamon et al. 2005; Wang, Wang, Zhang, Shen, Chen 2007a; Wang, Wang, Zhang, Shen, Chen 2007b; Wang et al. 2008; Zhang et al. 2009; Wan et al. 2013; Pink et al. 2016; Zhang et al. 2018; Jassam et al. 2019)
B	α1,3/4-Fucosyltransferase-III, FUT3 (EC:2.4.1.65)	*H. sapiens*	P21217	Lewis blood group antigen synthesis (Le^{a} and Le^{b}) and E-selectin ligand sLe^{a}, cell–pathogen interactions, carcinogenesis	N154[a], N185[a]	(Kukowska-Latallo et al. 1990; Koszdin and Bowen 1992; Legault et al. 1995; Nguyen et al. 1998; ChristenChristensen, Jensen, Bross, Orntoft 2000; Nordén et al. 2009; Padró et al. 2011; do Nascimento *et al*. 2015; Cai et al. 2016; Varki 2017; Nakashima *et al*. 2019)
C	α1,3/4-Fucosyltransferase-V, FUT5 (EC:2.4.1.65)	*H. sapiens*	Q11128	Le^{x}/SSEA-1 and sLe^{x} synthesis, carcinogenesis, spermatogenesis, cell–pathogen interactions	N60, N105, N167[a], N198[a]	(Kukowska-Latallo et al. 1990; Legault et al. 1995; Christensen, Jensen, Bross, Orntoft 2000; Chiu et al. 2007; Nordén et al. 2009; Padró et al. 2011)
D	α1,3/4-Fucosyltransferase-VI, FUT6 (EC:2.4.1.65)	*H. sapiens*	P51993	E-selectin ligand and sLe^{x} synthesis, carcinogenesis, diabetes pathogenesis, cell–pathogen interactions	N46, N91, N153[a], N184[a]	(Legault et al. 1995; Christensen, Jensen, Bross, Orntoft 2000; Nordén et al. 2009; Padró et al. 2011; Li et al. 2016; Guo et al. 2012; Zhao et al. 2016)

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Table I. Continue

Lp.	Enzyme	Species	Uniprot accession number	Functions	Putative N-glycosylation sites	References
E	α1,3-Fucosyltransferase-IV, FUT4 (EC:2.4.1.x)	*R. norvegicus*	Q62994	CD15 molecule synthesis, cell adhesion, immune system regulation, embryonic development	N117[a], N218[a]	(Baboval et al. 2000; Othman et al. 2018; Brito et al. 2009)
F	α1,3-Fucosyltransferase-IX, FUT9 (EC:2.4.1.x)	*H. sapiens*	Q9Y231	sLe^x synthesis in the brain, carcinogenesis, immune system regulation, cell adhesion, nervous system physiology, cell–pathogen interactions	N62[a], N101[a], N153[a]	(Nishihara et al. 2003; Bogoevska et al. 2006; Kudo et al. 2007; Sikora et al. 2009; Gouveia et al. 2012; Seelhorst et al. 2013; Kashiwazaki et al. 2014; Auslander et al. 2017; Blanas et al. 2019)
3. *N*-Acetylglucosaminyltransferases/*N*-Acetylgalactosaminyltransferases						
A	β1,3-*N*-Acetylglucosaminyltransferase-II, B3GnT2 (EC:2.4.1.149)	*H. sapiens*	Q9NY97	Poly-*N*-acetyllactosamine synthesis, cancer treatment, immune system physiology	N79[a], N89[a], N127[a], N173, N219[a]	(Kato et al. 2005; Togayachi et al. 2007; Togayachi et al. 2010; Kudo et al. 2019)
B	β1,4-*N*-Acetylgalactosaminyltransferase-I, GalNAc-T, GM2/GD2/GA2 synthase (EC:2.4.1.92)	*H. sapiens*	Q00973	GM2, GD2 and GA2 synthesis, neurodegenerative disease pathogenesis, carcinogenesis, immune system physiology, hereditary spastic paraplegia pathogenesis	N79[a], N179[a], N274[a]	(Lutz et al. 1994; Haraguchi et al. 1995; Furukawa et al. 2002; Nagafuku et al. 2012; Harlalka et al. 2013; Sha et al. 2014; Wakil et al. 2014; Kwon et al. 2017; Ledeen and Wu 2018)
C	β1,4-*N*-Acetylglucosaminyltransferase-III, GnT-III (EC:2.4.1.144)	*R. norvegicus*	Q02527	Bisecting GlcNAc synthesis, carcinogenesis	N243[a], N261[a], N399[a] (according to (Nagai et al. 1997)) N245, N263, N401 (Uniprot)	(Nishikawa et al. 1992; Nagai et al. 1997)
D	β1,4-*N*-Acetylglucosaminyltransferase-IV, GnT-IVa (EC:2.4.1.145)	*Bos taurus* (bovine)	O77836	Multiantennary N-glycan synthesis	N5, N77[a], N458[a]	(Oguri et al. 1997; Minowa et al. 1998)
E	β1,2-*N*-Acetylglucosaminyltransferase-II, GnTII (EC:2.4.1.143)	*H. sapiens*	Q10469	Complex N-glycan synthesis, congenital disorder disease pathogenesis	N69, N86	(Wang et al. 2002; Kadirvelraj et al. 2018; Miyazaki et al. 2018)

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Table I. Continue

Lp.	Enzyme	Species	Uniprot accession number	Functions	Putative N-glycosylation sites	References
		B. mori	(only GenBank accession number: BBD84862.1)	N-glycan synthesis	N45, N69, N73, N82, N110, N245, N319, N384, N441	(Miyazaki, Miyashita, Mori et al. 2019a; Miyazaki, Miyashita, Nakamura et al. 2019b)
4. Galactosyltransferases						
A	β1,3-Galactosyltransferase-IV, B3Gal-T4, GM1/GD1b/GA1 synthase (EC:2.4.1.62)	*M. musculus*	Q9Z0F0	Ganglioside synthesis, carcinogenesis, signal pathway regulation	N143[a]	(Daniotti et al. 1999; Martina et al. 2000; Nishio et al. 2004; Dong et al. 2010)
B	β1,4-Galactosyltransferase-I, B4Gal-T1 (EC:2.4.1.x)	*H. sapiens*	P15291	Complex N-glycan synthesis, carcinogenesis, nervous system physiology, cell adhesion, fertilization, inflammatory response, cell–cell and cell–matrix interactions, embryonal development, immune system physiology	N69 (according to (Malissard et al. 1996)), N113 (Uniprot)	(Malissard et al. 1996; Fukuta et al. 2001; Steffgen et al. 2002; Zhang et al. 2003; Zhu et al. 2005; Cheng et al. 2010; Xu et al. 2011; Zhu et al. 2012; Tang et al. 2013; Sun et *al.* 2014; Gu et al. 2015; Wei et al. 2019)
5. Glucuronyltransferases						
A	UDP-glucuronosyltransferase-1 (EC:2.4.1.17)	*H. sapiens*	UGT1.1 (Uniprot, P22309) UGT1.4 (Uniprot, P22310) UGT1.6 (Uniprot, P19224) UGT1.9 (Uniprot, O60656)	Elimination of xenobiotics and endogenous compounds, cancer treatment	UGT1.1: N102, N295, N347; UGT1.4: N119, N142, N189 (according to (Nakajima et al. 2010)), N296, N348; UGT1.6: N294 (N293 in (Nakajima et al. 2010)), N346; UGT1.9: N71[a], N99 (Uniprot), N292[a], N344[a];	(Antonio et al. 2003; Krishnaswamy et al. 2004; Ouzzine et al. 2006; Thibaudeau et al. 2006; Udomuksorn et al. 2007; Girard et al. 2008; Pritchett et al. 2008; Justenhoven et al. 2010; Nakajima et al. 2010; Yu et al. 2010; de Almagro et al. 2011; Al Saabi et al. 2013; Kato et al. 2013)

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Table I. Continue

Lp.	Enzyme	Species	Uniprot accession number	Functions	Putative N-glycosylation sites	References
B	UDP-glucuronosyltransferase 2B (EC:2.4.1.17)	*H. sapiens*	UGT2B7 (Uniprot: P16662) UGT2B15 (Uniprot: P54855) UGT2B17 (Uniprot: O75795)	Elimination of xenobiotics and endogenous compounds, carcinogenesis, cancer treatment	UGT2B7: N67, N68, N315; UGT2B15: N65[a], N316[a], N483[a]; UGT2B17: N65, N316, N483	(Barbier et al. 2000; Antonio et al. 2003; Staines et al. 2004; Thibaudeau et al. 2006; Barre *et al.* 2007; Chouinard et al. 2007; Mano et al. 2007; Sun and Di Rienzo 2009; Chanawong et al. 2015; Hu et al. 2015; Kallionpää et al. 2015; Romero-Lorca et al. 2015; Hu et al. 2016; Yang et al. 2017; Shen et al. 2019)
		Macaca fascicularis	UGT2B19 (Uniprot: Q9XT55) UGT2B20 (Uniprot: O77649)		UGT2B19: N315; UGT2B20: N65[a], N103, N316, N483;	(Barbier et al. 1999; Bélanger et al. 1999; Barbier et al. 2000; Tukey and Strassburg 2000)
C	UDP-glucuronosyltransferase 2B (EC:2.4.1.17)	*R. norvegicus*	UGT2B1 (Uniprot: P09875) UGT2B2 (Uniprot: P08541)	Elimination of xenobiotics and endogenous compounds, UGT2B2 is specific for endogenous lipids	UGT2B1: N134, N316[a]; UGT2B2: N316[a]	(Mackenzie 1990; Yamashita et al. 1995; Daidoji et al. 2005)
D	UDP-glucuronosyltransferase 1.6 (EC:2.4.1.17)	*R. norvegicus*	UGT1.6 (Uniprot: P08430)	Elimination of xenobiotics and endogenous compounds, specific for phenolic molecules	UGT1.6: N281, N291, N429	(Green and Tephly 1989; Harding et al. 1989; Kessler et al. 2002; Daidoji et al. 2005)
		Oryctolagus cuniculus (rabbit)	UGT1.6 (Uniprot: Q28611)		UGT1.6: N294	(Lamb et al. 1994; Green and Tephly 1989; Harding et al. 1989)
E	UDP-glucuronosyltransferase 2B13, UGT2B13 (EC:2.4.1.17)	*O. cuniculus* (rabbit)	P36512	Elimination of xenobiotics and endogenous compounds, specific for phenolic and gallate derivatives	UGT2B13: N69, N101, N317	(Green and Tephly 1989; Li et al. 1997)
6. Other classes of glycosyltransferases						
A	Oligosaccharyltransferase STT3 subunit (EC:2.4.99.18)	*S. cerevisiae* (strain ATCC 204508/S288c)	P39007	Transfers *en bloc* a defined oligosaccharide to a nascent protein chain	N60[a], N535, N539[a]	(Li et al. 2005; Mohorko et al. 2011; Braunger et al. 2018; Wild et al. 2018)
B	β1,2-Xylotransferase, AtXYLT (EC:2.4.2.38)	*A. thaliana*	Q9LDH0	N-glycan xylosylation	N51[a], N301[a], N478 (according to (Pagny et al. 2003)), N479 (Uniprot)	(Faye and Chrispeels 1989; Pagny et al. 2003; Bencúr et al. 2005; Kajiura et al. 2012)

Table II. Influence of N-glycans on activities of glycosyltransferases

N-glycosidases	Inhibitors	Activity	Protein substitutions	Activity	References
1. Sialyltransferases					
A) Rat α2,6-sialyltransferase-I, ST6Gal-I Rat					
PNGase F: sensitive, activity abolished	ND	ND	Cell lysates	*In vitro*	(Fast et al. 1993; Chen and Colley 2000; Luley-Goedl et al. 2016)
			N146S	Decreased	
			N158Q	Abolished	
Endo H: retained activity;			N146S/N158Q	Abolished	
Endo F: decreased activity			Enzyme in cells	*In vivo*	
			N146S	Retained	
			N158Q	Retained	
Neuraminidase and galactosidase: decreased activity			N146S/N158Q	Decreased	
Neuraminidase, galactosidase and *N*-acetylhexosaminidase: decreased activity			Truncated soluble enzymes[b]	*In vivo*	
			N146S/N158Q	Abolished	
Human					
ND	ND	ND	ND	ND	(Kuhn et al. 2013; Luley-Goedl et al. 2016)
B) Human α2,3-sialyltransferase-II, ST3Gal-II					
PNGase F: sensitive	Tunicamycin	ND	Cell lysates	*In vitro*	(Ruggiero et al. 2015)
Endo H: sensitive	Castanospermine		N92Q	- Glycolipid acceptor: increased; glycoprotein acceptor: decreased	
			N211Q	- Glycolipid acceptor: decreased; glycoprotein acceptor: decreased	
			N92Q/N211Q	- Glycolipid acceptor: decreased; glycoprotein acceptor: decreased	
C) Silkworm α2,6-sialyltransferase, ST6Gal					
PNGase F: sensitive	ND	ND	Soluble enzymes	*In vitro*	(Kajiura et al. 2015)
			N274A	Decreased	

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Table II. Continue

N-glycosidases	Inhibitors	Activity	Protein substitutions	Activity	References
			N274Q	Decreased	
D) Murine α2,3-sialyltransferase-V, ST3Gal-V					
PNGase F: sensitive	Castanospermine	Retained	Cell lysates	*In vitro*	(Uemura et al. 2006; Uemura et al. 2015)
Endo H: sensitive	Kifunensine	Retained	N224K	Retained	
	Tunicamycin	Abolished	N180Q	Decreased	
			N180K	Decreased	
			N180S	Decreased	
			N224Q	Decreased	
			N224D	Decreased	
			N334Q	Decreased	
			N334K	Decreased	
			N180Q/N224Q/N334Q	Abolished	
E) Human α2,3-sialyltransferase-I, ST3Gal-I					
ND	ND	ND	Soluble enzymes	*In vitro*	(Jeanneau et al. 2004)
			N79D	Retained	
			N323H	Retained	
			N79D/N323H	Retained	
			N114S/N201Q	Retained	
			N201Q	Retained	
			N323H	Retained	
			N79D/N114S/N201Q	Decreased	
			N79D/N114S/N201Q/ N323H	Decreased	
F) Murine poly-α2,8-sialyltransferase 8B, ST8Sia-II					
PNGase F: sensitive	ND	ND	Soluble enzymes	*In vivo*	(Mühlenhoff et al. 2001; Mori et al. 2017)
			N60Q	Retained	
			N72Q	Retained	
			N89Q	Retained	
			N134Q	Retained	
			N219Q	Retained	
			N234Q	Retained	
			N72Q/N89Q	Retained	
			N72Q/N134Q	Retained	
			N60/N89Q	Retained	
			N89Q/N134Q	Retained	
			N89Q/N234Q	Retained	
			N134Q/N219Q	Retained	
			N134Q/N234Q	Retained	
			N60Q/N72Q/N134Q	Retained	

Continued

Table II. Continue

N-glycosidases	Inhibitors	Activity	Protein substitutions	Activity	References
			N134Q/N219Q/N234Q	Retained	
			N60Q/N72Q/N89Q/N134Q	Retained	
			N60Q/N72Q/N134Q/N219Q	Retained	
			N60Q/N72Q/N134Q/N219Q/N234Q	Decreased	
			N60Q/N72Q/N89Q/N134Q/N234Q	Decreased	
			N89Q/N219Q	Abolished	
			N89Q/N219Q/N234Q	Abolished	
			N60Q/N89Q/N219Q/N234Q	Abolished	
			N72Q/N89Q/N219Q/N234Q	Abolished	
			N89Q/N134Q/N219Q/N234Q	Abolished	
			N72Q/N89Q/N134Q/N219Q/N234Q	Abolished	
			N60Q/N89Q/N134Q/N219Q/N234Q	Abolished	
			N60Q/N72Q/N89Q/N219Q/N234Q	Abolished	
			N60Q/N72Q/N89Q/N134Q/N219Q	Abolished	
			N60Q/N72Q/N89Q/N134Q/N234Q	Abolished	
			Soluble enzymes	*In vitro*	
			N60Q	Retained	
			N72Q	Retained	
			N134Q	Retained	
			N72Q/N134Q	Retained	
			N60Q/N72Q/N134Q	Retained	
			N134Q/N219Q	Retained	
			N219Q	Decreased	
			N234Q	Decreased	
			N134Q/N234Q	Decreased	
			N60Q/N72Q/N134Q/N219Q	Decreased	
			N60Q/N72Q/N134Q/N219Q/N234Q	Decreased	
			N89Q/N219Q/N234Q	Abolished	
			N134Q/N219Q/N234Q	Abolished	
			N60Q/N72Q/N89Q/N134Q	Abolished	
			N60Q/N89Q/N219Q/N234Q	Abolished	
			N72Q/N89Q/N219Q/N234Q	Abolished	
			N89Q/N134Q/N219Q/N234Q	Abolished	
			N72Q/N89Q/N134Q//N219Q/N234Q	Abolished	
			N60Q/N89Q/N134Q/N219Q/N234Q	Abolished	
			N60Q/N72Q/N134Q/N219Q/N234Q	Abolished	
			N60Q/N72Q/N89Q/N219Q/N234Q	Abolished	
			N60Q/N72Q/N89Q/N134Q/N219Q	Abolished	
			N60Q/N72Q/N89Q/N134Q/N234Q	Abolished	
			N60Q/N72Q/N89Q/N134Q/N219Q/N234Q	Abolished	

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Table II. Continue

N-glycosidases	Inhibitors	Activity	Protein substitutions	Activity	References
			N72Q/N89Q	Abolished	
			N89Q	Abolished	
			N60/N89Q	Abolished	
			N89Q/N134Q	Abolished	
			N89Q/N219Q	Abolished	
			N89Q/N234Q	Abolished	
G) Hamster poly-α-2,8-sialyltransferase, ST8Sia-IV					
PNGase F: sensitive	ND	ND	Soluble enzymes	*In vivo*	(Mühlenhoff et al. 2001; Mori et al. 2017)
			N50Q	Retained	
			N119Q	Retained	
			N204Q	Retained	
			N219Q	Retained	
			N119Q/N204Q	Retained	
			N204Q/N219Q	Retained	
			N119Q/N204Q/N219Q	Retained	
			N50Q/N119Q/N204Q/N219Q	Retained	
			N74Q	Decreased	
			N50Q/N74Q	Abolished	
			N50Q/N74Q/N119Q	Abolished	
			N74Q/N204Q/N219Q	Abolished	
			N74Q/N119Q/N204Q/N219Q	Abolished	
			N50Q/N74Q/N204Q//N219Q	Abolished	
			N50Q/N74Q/N119Q/N219Q	Abolished	
			N50Q/N74Q/N119Q/N204Q	Abolished	
			N50Q/N74Q/N119Q/N204Q/N219Q	Abolished	
			Soluble enzymes	*In vitro*	
			N50Q	Retained	
			N119Q	Retained	
			N204Q	Retained	
			N219Q	Retained	
			N119Q/N204Q	Retained	
			N204Q/N219Q	Retained	
			N119Q/N204Q/N219Q	Retained	
			N50Q/N119Q/N204Q/N219Q	Retained	
			N74Q	Abolished	
			N50Q/N74Q	Abolished	
			N50Q/N74Q/N119Q	Abolished	
			N74Q/N204Q/N219Q	Abolished	

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Table II. Continue

N-glycosidases	Inhibitors	Activity	Protein substitutions	Activity	References
			N74Q/N119Q/N204Q/N219Q	Abolished	
			N50Q/N74Q/N204Q//N219Q	Abolished	
			N50Q/N74Q/N119Q/N219Q	Abolished	
			N50Q/N74Q/N119Q/N204Q	Abolished	
			N50Q/N74Q/N119Q/N204Q/N219Q	Abolished	
H) Chicken and mouse α2,8-sialyltransferase-I, ST8Sia-I, GD3 synthase					
Chicken					
PNGase F: sensitive, abolished activity	1-Deoxymannojirimycin	Retained	ND	ND	(Martina et al. 1998)
	Swainsonine	Retained			
	Castanospermine	Decreased			
	1-Deoxynojirimycin	Decreased			
	Tunicamycin	Abolished			
Mouse					
Endo H: sensitive	Castanospermine	Decreased	ND	ND	(Bieberich et al. 2000)
2. Fucosyltransferases					
A) Human α1,3-fucosyltransferase-VII, FUT7					
PNGase F: sensitive	Tunicamycin	ND	Cell lysates	*In vitro*	(Prorok-Hamon et al. 2005)
			N81Q	Abolished	
			N291Q	Decreased	
			N81Q/N291Q	Abolished	
B) Human α1,3fucosyltransferase-III, FUT3					
Endo H: sensitive	Tunicamycin	Abolished	Cell lysates	*In vitro*	(Kukowska-Latallo et al. 1990; Legault et al. 1995; Nguyen et al. 1998; Christensen, Jensen, Bross, Orntoft 2000)
	Castanospermine	Decreased	N154Q	Abolished	
			N185Q	Decreased	
			N154Q/N185Q	Abolished	
C) Human α1,3-fucosyltransferase-V, FUT5					
ND	ND	ND	Cell lysates	*In vitro*	(Christensen, Jensen, Bross, Orntoft 2000; Legault et al. 1995)
			N167Q	Abolished	
			N198Q	Decreased	
			N167Q/N198Q	Abolished	
D) Human α1,3-fucosyltransferase-VI, FUT6					
ND	ND	ND	Cell lysates	*In vitro*	(Christensen, Jensen, Bross, Orntoft 2000; Legault et al. 1995)
			N153Q	Decreased	
			N184Q	Decreased	

Continued

Table II. Continue

N-glycosidases	Inhibitors	Activity	Protein substitutions	Activity	References
			N153Q/N184Q	Decreased	
E) Rat α1,3-fucosyltransferase-IV, FUT4					
ND	ND	ND	Cell lysates	*In vitro*	(Baboval et al. 2000)
			N117G	Decreased	
			N218G	Abolished	
			N220A	Abolished	
			N117G/N220A	Abolished	
F) Human α1,3-fucosyltransferase-IX, FUT9					
ND	Tunicamycin	ND	Soluble enzymes	*In vitro*	(Seelhorst et al. 2013)
			N62Q	Decreased	
			N101Q	Decreased	
			N153Q	Abolished	
			N62Q/N101Q	Abolished	
			N62Q/N153Q	Abolished	
			N101Q/N153Q	Abolished	
3. *N*-Acetylglucosaminyltransferases/*N*-Acetylglucosaminyltransferases					
A) Human β1,3-*N*-Acetylglucosaminyltransferase-II, BGnT2					
PNGase F: sensitive,	Tunicamycin	Abolished	Soluble enzymes	*In vitro*	(Kato et al. 2005)
decreased activity	Castanospermine	Retained	N79Q	Decreased	
			N89Q	Decreased	
			N127Q	Decreased[c]	
			N173Q	–[d]	
			N219Q	Abolished[c]	
B) Human and mouse β1,4-*N*-Acetylgalactosaminyltransferase, GM2/GD2/GA2 synthase					
Human					
ND	ND	ND	Cell lysates	*In vitro*	(Haraguchi et al. 1995)
			N79Q	Decreased	
			N179Q	Decreased	
			N274Q	Decreased	
			N179Q/N274Q	Decreased	
			N79Q/N179Q/N274Q	Decreased	
			Transfected cells	*In vivo*	
			N79Q	Retained	
			N179Q	Retained	
			N274Q	Retained	
			N179Q/N274Q	Retained	
			N79Q/N179Q/N274Q	Retained	
Mouse					
Endo H: sensitive	Castanospermine	Decreased	ND	ND	(Bieberich et al. 2000)
C) Rat β1,4-*N*-Acetylglucosaminyltransferase-III					
ND	Tunicamycin	Abolished	Cell lysates	*In vitro*	(Nagai et al. 1997)

Continued

Table II. Continue

N-glycosidases	Inhibitors	Activity	Protein substitutions	Activity	References
	Castanospermine	Retained	N243Q	Decreased	
			N261Q	Decreased	
			N399Q	Decreased	
			N243Q/N281Q	Decreased	
			N243Q/N399Q	Decreased	
			N281Q/N399Q	Decreased	
			N243Q/N281Q/N399Q	Abolished	
D) Bovine β1,4-*N*-Acetylglucosaminyltransferase-IV, GnT-IVa					
PNGase F: sensitive	ND	ND	Cell lysates	*In vitro*	(Minowa et al. 1998)
			S79A	Retained	
			T460A	Retained	
E) Human and silkworm β1,2-*N*-Acetylglucosaminyltransferase-II, GnTII					
Human					
PNGase F: sensitive, retained activity; endo H, not sensitive	ND	ND	ND	ND	(Miyazaki et al. 2018)
Silkworm					
PNGase F: sensitive, retained activity	ND	ND	ND	ND	(Miyazaki, Miyashita, Nakamura et al. 2019b)
4. Galactosyltransferases					
A) Murine β1,3-Galactosyltransferase-IV, B3Gal-T4, GM1/GD1B/GA1 synthase					
PNGase F: sensitive;	Tunicamycin	Abolished	Cell lysates	*In vitro*	(Martina et al. 2000)
Endo H: sensitive	Castanospermine	Decreased	N143Q	Abolished	
B) Human β1,4-Galactosyltransferase-I, B4Gal-T1					
Endo H: sensitive, retained activity	ND	ND	Soluble enzyme	*In vitro*	(Malissard et al. 1996)
			N69D	Retained	
5. Glucuronyltransferases					
A) Human UDP-Glucuronosyltransferase 1 (UGT1A1, UGT1A4, UGT1A6, UGT1A9)					
Endo H: UGT1A1, sensitive;	**UGTA9**		**UGTA9**		(Nakajima et al. 2010)
UGT1A4, sensitive;	Tunicamycin	Abolished	Cell lysates	*In vitro*	
UGT1A6, sensitive;	Castanospermine	Decreased	N71Q	Decreased	
UGT1A9, sensitive, retained activity	1-Deoxynojirimycin	Decreased	N292Q	Decreased	
			N344Q	Decreased	
			N71Q/N292Q	Decreased	
			N292Q/N344Q	Decreased	
			N71Q/N344Q	Abolished	
			N71Q/N292Q/N344Q	Abolished	
B) Human (UGT2B7, UGT2B15, UGT2B17) and simian (UGT2B19, UGT2B20) UDP-Glucuronosyltransferase 2B					
Human					

Continued

Table II. Continue

N-glycosidases	Inhibitors	Activity	Protein substitutions	Activity	References
Endo H: UGT2B7, sensitive; UGT2B15, sensitive, decreased activity; UGT2B17, sensitive	UGT2B15 Tunicamycin	ND	UGT2B15 Transfected cells S316E N483D N65D	*In vitro* Decreased Decreased Abolished	(Barbier et al. 2000)
Simian					
Endo H: UGT2B20, sensitive, decreased activity; UGT2B19, retained activity	UGT2B20 Tunicamycin	ND	UGT2B20 Transfected cells N103K S316E N483D N65D	*In vitro* Retained Retained Retained Abolished	(Barbier et al. 2000)
C) Rat (UGT2B1, UGT2B2) UDP-Glucuronosyltransferase 2B					
UGT2B2: Endo H, sensitive; endo D, not sensitive; PNGase A, sensitive	Tunicamycin	Decreased	ND	ND	(Mackenzie 1990)
D) Rat and rabbit UDP-Glucuronosyltransferase UGT1A6					
Rabbit					
Endo H: sensitive, retained activity; PNGase A, sensitive	ND	ND	ND	ND	(Green and Tephly 1989)
Rat					
Endo H: sensitive, retained activity; PNGase A, sensitive	Tunicamycin	ND	ND	ND	(Green and Tephly 1989; Harding et al. 1989)
E) Rabbit UDP-Glucuronosyltransferase UGT2B13					
Endo H: sensitive, retained activity; PNGase A, sensitive	ND	ND	ND	ND	(Green and Tephly 1989)
6. Other classes of glycosyltransferases					
A) Yeast oligosaccharyltransferase STT3 subunit					
PNGase F: sensitive; endo H, sensitive	ND	ND	Transfected cells N60Q T541A Cell lysates N60Q T541A	*In vivo* Retained Decreased *In vitro* Retained Decreased	(Wild et al. 2018; Li et al. 2005)
B) Plant β1,2-Xylotransferase, AtXYLT					
PNGase F: sensitive	Tunicamycin	Abolished	Soluble enzymes S53A T303A T480A S53A/T303A	*In vitro* Retained Decreased Retained Abolished	(Pagny et al. 2003)

[a]N-glycosylation site necessary for enzyme activity.
[b]Two enzymes without 64–403 and 97–403 amino acids deleted were analyzed.
[c]Low protein yield.
[d]No protein obtained.

mutagenesis; K_m of the p.N274A variant was higher than that of the p.N274Q (Table II-1C). These results suggest that the change of *in vitro* activity upon eliminating the N-glycan at N274 is not solely related to the loss of N-glycan but may well be caused by the presence of different amino acid side chains within the sequon (as a result of site-directed mutagenesis used in this study) (Kajiura et al. 2015).

Murine ST3Gal-V contains three predicted N-glycosylation sites at N180, N224 and N334 (Table I-1D). When the enzyme was expressed in CHO (activity evaluation) and HEK293 (secretion analysis) cells, the variant with all sequons modified completely lost its enzymatic activity; however, single variants (p.N180Q, p.N224Q and p.N334Q) showed some residual activity (Table II-1D) (Uemura et al. 2006). Activities of the evaluated variants mostly correlated with their secretion efficiency which was the most evident in the case of variants with substitutions at the N180 site, suggesting that a decrease or loss of activity may have resulted from a reduction of solubility and/or misfolding (in particular in the case of p.N180Q variant). Moreover, the variant with p.N180Q substitution was not cleaved or secreted in comparison with wild-type enzyme and other variants, indicating that this variant was unable to exit the ER (Uemura et al. 2006). The same study demonstrated that substitutions of amino acids located near the N-glycosylation sites that have been modified in ST3Gal-V may rescue its activity (referred to by the authors as the SUNGA method) (Table II-1D) (Uemura et al. 2006). Indeed, in some enzymes, substituting the asparagine at an N-glycosylation site (or residues in its immediate vicinity) with selected amino acids may impact on protein the same (or similarly) as an N-glycan and replace some of its functions. The amino acids for the SUNGA mutagenesis of ST3Gal-V were selected by comparing its sequence at the relevant positions to other sialyltransferases. On the other hand, CHO cells expressing ST3Gal-V treated with tunicamycin did not show any enzymatic activity, whereas kifunensine and castanospermine treatment showed no effects (Table II-1D) (Uemura et al. 2006). These findings underscore the importance of N-glycans (especially the high-mannose core glycan) for the ST3Gal-V activity, but the mechanism of this phenomenon remains unknown.

The human ST3Gal-I contains five predicted N-glycosylation sites at N27, N79, N114, N201 and N323 (Table I-1E) and was obtained as a soluble form using insect cell expression system. Removal of individual N-glycans from the enzyme did not affect its activity, but a modification of more than two sequons at a time (p.N79D/p.N114S/p.N201Q and p.N79D/p.N114S/p.N201Q/p.N323H variants) reduced the enzyme activity (Table II-1E). The triple variant exhibited decreased expression levels (Table II-1E) (Jeanneau et al. 2004). Thus, altered activities of the underglycosylated ST3Gal-I variants could be caused by their misfolding and/or aggregation. Indeed, the soluble enzyme variants without N-glycans were not correctly secreted and processed, suggesting that its folding and intracellular trafficking depend on N-glycosylation and that the decreased enzyme activity after eliminating N-glycans may be a secondary effect (Jeanneau et al. 2004).

Polysialyltransferases catalyze the attachment of poly-α2,8-sialic acid to the terminal sialic acid residues of glycoproteins (Huang et al. 2017). Murine ST8Sia-II contains six potential N-glycosylation sites, N60, N72, N89, N134, N219 and N234 (Table I-1F), while hamster ST8Sia-IV has five sequons: N50, N74, N119, N204 and N219 (Table I-1G) (Mühlenhoff et al. 2001). Enzymatic activities of their variants with substitutions at sequons were evaluated in CHO cells. ST8Sia-II variants with single deletions of any N-glycosylation sequon revealed an activity similar to the fully glycosylated enzyme, but only when tested *in vivo*. The variants p.N89Q, p.N219Q and p.N234Q showed a decreased *in vitro* polysialylation capability, but the largest decline was found in the case of p.N89Q variant (Table II-1F). In contrast to the murine enzyme, hamster ST8Sia-IV required only the N74 sequon for full *in vitro* and *in vivo* activity, while the other N-glycosylation sites had only a small influence (Mühlenhoff et al. 2001) (Table II-1G). The N89 site in ST8Sia-II and N74 in ST8Sia-IV are conserved in all cloned mammalian α2,8-sialyltransferases which may explain the marked decrease in activity after eliminating N-glycans. In another study, the p.N74S and p.N119S variants of ST8Sia-IV showed marked decreases in activity (p.N74S) or were completely inactive (p.N119S) (Close et al. 2000). However, in a more recent study, Mühlenhoff et al. (2001) demonstrated no influence of the N119 site on catalytic functions, whereas the combined elimination of the N74-linked and other glycans yielded an inactive enzyme. Notably, asparagine at position 119 was substituted by serine in one study and by glutamine in another study, so the discrepancy between results may have been caused by the different influence of serine and glutamine side chains on folding and/or solubility. Interestingly, the results of the *in vivo* and *in vitro* activity measurements performed on ST8Sia-IV were consistent, while for ST8Sia-II the *in vivo* and *in vitro* activities were different (Table II-1F, -1G) (Mühlenhoff et al. 2001). A similar phenomenon was found in the case of rat ST6Gal-I, which required N-glycans only for the *in vivo* activity (Chen and Colley 2000). These results highlight the point that in order to draw accurate conclusions, both the *in vitro* and *in vivo* enzyme activities should be evaluated.

When COS-7 cells expressing chicken ST8Sia-I (which contains three predicted N-glycosylation sites: N57, N105 and N200) (Table I-1H) were treated with tunicamycin, the enzyme exhibited no activity, in contrast to the 1-deoxymannojirimycin- and swainsonine-treated cells, which yielded an active enzyme, and castanospermine- and 1-deoxynojirimycin-treated cells, which yielded an enzyme with a decreased activity (Table II-1H) (Martina et al. 1998). When active, the enzyme normally exited the ER and trafficked to the Golgi while its lack of activity correlated with accumulation in the ER. In addition, the N-glycan trimming in the ER did not influence the enzyme activity, but is necessary for its trafficking to the Golgi from the ER. Similarly, a neuroblastoma cell line transfected with murine ST8Sia-I (contained four potential N-glycosylation sites at N70, N118, N213 and N244) (Table I-1H) and treated with castanospermine, the enzyme had a decreased activity and accumulated in the ER (Table II-1H) (Bieberich et al. 2000). These results highlight the crucial role of N-glycans in proper intracellular localization and/or folding which are necessary for full activity of ST8Sia-I (Martina et al. 1998; Bieberich et al. 2000).

Fucosyltransferases

Fucosyltransferases (FUTs) catalyze the attachment of fucose to glycoconjugates. FUTs contain conserved motifs that are necessary for their catalytic activity, similarly to sialyltransferases (de Vries et al. 2001). The human α1,3-fucosyltransferase-VII (FUT7), which synthesizes sialyl Lewis^x (sLe^x) on selectin ligands (mainly PSGL-1), contains two N-glycosylation sites: N81 and N291 (Table I-2A). Removal of N-glycans from any one or both of these two sequons caused a significant decrease in FUT7 activity *in vitro* (studied in CHO cells), with the p.N81Q substitution causing a complete loss of the activity (Table II-2A) (Prorok-Hamon et al. 2005). Surprisingly, in the presence of core2-modified PSGL-1 acceptor, underglycosylated FUT7 (p.N81Q, p.N291Q and p.N81Q/p.N291Q variants) were able to generate P- and L-selectins, but only *in vivo* (Knibbs et al.

1998; Prorok-Hamon et al. 2005). Taken together, N-glycosylation of human FUT7 seems to be necessary for its activity *in vitro* but not *in vivo*, and underglycosylation does not impede intracellular localization because the trafficking of variant enzymes was unaffected. The *in vivo* activity was evaluated using a complementation method in which binding of the selectin-IgM chimeras to the CHO/F7P1 cells, which expressed FUT7 and PSGL-1, was investigated. The selectin did not bind to the cells unless they expressed core2-modified PSGL-1 molecules carrying C2GnT-I-branched O-glycans that were fucosylated by FucT-VII. In contrast, the *in vitro* activity studies were carried out in the cell lysates using a standard sialyltransferase assay. Thus, the assays used for the evaluation of enzymatic activity were different, and this may have a considerable impact on the results (Prorok-Hamon et al. 2005). It is noteworthy that the core2 β1,6-*N*-acetylglucosaminyltransferase-I (C2GnT-I), which synthesizes core2 O-glycan (the precursor for Le^x synthesis), also required N-glycans for full activity (Prorok-Hamon et al. 2005; Toki et al. 1997).

Christensen, Jensen, Bross et al. (2000) expressed human α1,3/4-fucosyltransferase-III (FUT3; Table I-2B), α1,3/4-fucosyltransferase-V (FUT5; Table I-2C) and α1,3/4-fucosyltransferase-VI (FUT6; Table I-2D) in COS-7 cells and demonstrated that their N-glycosylation sites are essential for full activity (Christensen, Jensen, Bross, Orntoft 2000). The elimination of any sequon from FUT3 (Table II-2B), FUT4 (Table II-2B) and FUT5 (Table II-2B) caused decreases in activity. Eliminating both N-glycosylation sites had the biggest impact. In the case of variants, substitutions of N154 (FUT3), N167 (FUT5) and N153 (FUT6) markedly decreased activities (Christensen, Jensen, Bross, Orntoft 2000; Christensen, Jensen, Bross, Orntoft 2000; Morais et al. 2003). The kinetic parameters of a FUT3 variant with the p.N185Q substitution were unchanged, so the decreased activity may have been caused by misfolding rather than the direct impact of N-glycans on catalytic properties. In addition, tunicamycin treatment of COS-7 cells expressing FUT3 caused a complete loss of catalytic function, whereas castanospermine caused only a small decrease in activity (Table II-2B) (Skropeta 2009). These findings suggest that FUT3 required core glycosylation and glucose trimming for full activity. In summary, N-glycosylation of FUT3, FUT4 and FUT5 is necessary for proper enzyme folding, and thereby it may influence the activity.

The rat α1,3-fucosyltransferase-IV (FUT4), which contains two potential N-glycosylation sites at N117 and N218 (Table I-2E), was expressed in COS-1 cells (Baboval et al. 2000). Substitution at the N218 site caused a larger decrease in *in vitro* activity than the p.N117G replacement (Table II-2E), while the double variant with the p.N117G/p.N220A substitutions revealed no *in vitro* activity at all (Table II-2E). The N218 sequon is conserved in many fucosyltransferases (FUT3–FUT7); hence, probably any change in its glycosylation can dramatically affect the enzyme activity. Notably, intracellular trafficking of the enzyme variants was unaltered. However, the authors failed to evaluate the influence of N-glycans on the enzyme folding. Thus, while the evidence that eliminating N-glycans from the rat FUT4 caused a change in activity is strong, the mechanism was not pinpointed. In addition, in the same study, expression of a truncated soluble FUT4 form in *E. coli* yielded a completely inactive enzyme, suggesting that N-glycosylation (and/or other PTMs) of FUT4 plays a key role in its activity. The lack of activity of the *E. coli*-derived FUT4 could have resulted from misfolding (Baboval et al. 2000).

The human α1,3-fucosyltransferase-IX (FUT9) contains three predicted N-glycosylation sites: N62, N101 and N153 (Table I-2F). When a soluble extracellular catalytic domain of the enzyme was expressed in Sf9 cells, modification of two or more sequons resulted in a complete loss of activity (Table II-2F). Moreover, the N-glycan at N153 appeared to be more important than the other two (N62 and N101), because eliminating it caused a loss of activity (Table II-2F) (Seelhorst et al. 2013). Kinetic studies revealed that substitutions of N62 and N101 caused only small decreases in K_m, with a slight decrease and increase in the acceptor and the donor sugar affinity, respectively. It is quite likely that the N62 and N101 sites are located in the conserved motifs I and II, respectively, while N153 resides in motif IV, which is more important for the catalytic activity (Seelhorst et al. 2013). Similarly to the human FUT3, FUT5 and FUT6 (Christensen, Bross, Ørntoft 2000; Christensen, Jensen, Bross, Orntoft 2000), all N-glycosylation sites in FUT9 lie within the catalytic domain, so eliminating N-glycans may impact catalytic functions (Seelhorst et al. 2013).

N-acetylglucosaminyltransferases and *N*-acetylgalactosaminyltransferases

N-acetylglucosaminyltransferases and *N*-acetylgalactosaminyltransferases are Golgi-resident enzymes that catalyze the attachment of GlcNAc or GalNAc, respectively, to an oligomannose core structure and initiate the synthesis of hybrid and complex N-glycans. The human β1,3-*N*-acetylglucosaminyltransferase-II (BGnT2) contains five potential N-glycosylation sites: N79, N89, N127, N173 and N219 (Table I-3A). When BGnT2 was expressed in insect cells (as a soluble GFP_{uv} fusion protein with a deletion of the first 25 amino acids), the N219 site was found to be necessary for the *in vitro* activity and efficient secretion of the enzyme (the N127 site was also needed for secretion) (Table II-3A) (Kato et al. 2005). The BGnT2 variant with the substitutions p.N79Q, p.N89Q and p.N127Q accumulated in the cells and little was secreted, in contrast to the variants with the single substitutions p.N79Q or p.N89Q, which were detected in the culture medium. These results confirmed that N-glycosylation played a role in folding and/or affected solubility of the enzyme. The enzyme produced in insect cells treated with tunicamycin was inactive and accumulated in the ER, while castanospermine did not affect its activity and subcellular trafficking. Thus, it may be concluded that the enzyme requires N-glycans for its folding and stability (Table II-3A) (Kato et al. 2005).

The human β1,4-*N*-acetylgalactosaminyltransferase (GM2/GD2/GA2 synthase) contains three N-glycosylation sites at N79, N179 and N274 (Table I-3B). Their importance was evaluated using two approaches: measuring cell surface GM2 expression in transfected cell lines (mouse melanoma KF3027 and CHO cells) and measuring enzyme activity in membrane fractions of KF3027 cells. The surface GM2 levels were not affected by substitutions at N-glycosylation sites, in *in vitro* assays variants of the enzyme showed varying degrees of a decreased activity with each single-mutated variants (p.N79Q, p.N174Q or p.N274Q) showing a lesser decline in activity than the double variants (p.N174Q/p.N274Q); the triple-substituted variant (p.N79Q/p.N174Q/p.N274Q) showed residual activity (Table II-3B) (Haraguchi et al. 1995). Kinetic studies of these enzyme variants showed that only V_{max} has changed, with no significant change in K_m; therefore, the affinity for substrates seems to be unaffected after eliminating N-glycans. In addition, immunostaining of the human GM2/GD2/GA2 synthase in human melanoma MeWo cells showed no differences in intracellular localization between variants and the wild-type enzyme (Haraguchi et al. 1995). The castanospermine treatment of F-11 neuroblastoma cells transfected with murine GM2/GD2/GA2 synthase caused a slight decrease in activity (Table II-3B) (Bieberich et al. 2000). Thus,

it seems that the decreases in activity of underglycosylated β1,4-*N*-acetylgalactosaminyltransferases variants result from impaired enzyme stability and there is no evidence that N-glycosylation directly influences catalytic functions of the enzyme (Haraguchi et al. 1995).

Rat β1,4-*N*-acetylglucosaminyltransferase-III (Table I-3C) requires all three N-glycosylation sites (N243, N261 and N399) for full activity. The analysis of enzyme activity in COS-1 cells expressing rat β1,4-*N*-acetylglucosaminyltransferase-III showed that substitutions of N243 and N261 sequons caused more profound decreases in activity than substituting N399 sequon (Table II-3C) (Nagai et al. 1997). The triple-mutated variant (p.N243Q/p.N261Q/p.N399Q) showed no activity at all (Table II-3C). None of the variants localized correctly in the Golgi, so the reduced activities of the variants may have been confounded by partial misfolding leading to the emergence of two pools of the enzyme, one active and the other inactive. The same transfected COS-1 cells treated with tunicamycin or castanospermine exhibited abolished and unchanged activity, respectively. Although the enzyme after castanospermine treatment was not retained in the Golgi, it was still active, suggesting that the presence of N-glycans at all three N-glycosylation sites is primarily important for Golgi retention and, to a lesser extent, for activity. Kinetic evaluation of enzyme variants with single substitutions at N-glycosylation sequons revealed small decreases in the donor substrate affinity of the p.N243Q and p.N261Q variants, with no influence on the acceptor affinity (neither was altered for the p.N399Q variant). These results suggest that the N243 and N261 sites may affect the kinetic properties of the enzyme while N399 site may be important for its stability. In summary, the decreases in enzyme activity of underglycosylated variants may be caused by impact on the enzyme kinetics and stability (Nagai et al. 1997).

Bovine β1,4-*N*-acetylglucosaminyltransferase-IV (GnT-IV) contains three N-glycosylation sites: N5, N77 and N458 (Table I-3D) (Oguri et al. 1997; Minowa et al. 1998). The activity of variants was evaluated in COS-7 cells transfected with constructs encoding enzymes with substitutions at N-glycosylation sites: p.S79A and p.T460A. No change in enzyme activity of these variants was found (Table II-3D), suggesting that N-glycosylation of GnT-IV is not necessary for its activity. The N5 site is probably not glycosylated because of its location within the cytoplasmic domain (Minowa et al. 1998).

Recently, Miyazaki, Miyashita, Mori et al. 2019 analyzed silkworm *B. mori* (Miyazaki *et al.* 2019) and human β1,2-*N*-acetylglucosaminyltransferase-II (Miyazaki et al. 2018) which contain nine (N45, N69, N73, N82, N110, N245, N319, N384 and N441) and two (N69 and N86) predicted N-glycosylation sites, respectively. When these enzymes were treated with PNGase F, they remained active, suggesting that N-glycans are not important for their activity (Tables I-3E and II-3E). However, the available data are limited to enzymatic deglycosylation studies.

Galactosyltransferases

Galactosyltransferases synthesize galactose-containing glycoconjugates by transferring galactose from UDP-Gal to specific acceptors (Hennet 2002). Murine β1,3-galactosyltransferase-IV contains one conserved N-glycosylation sequon at N143 (Table I-4A). When expressed in CHO-K1 cells treated with N-glycosylation inhibitors, the enzyme completely (tunicamycin) or partially (castanospermine) lost activity (Table II-4A). Moreover, the castanospermine treatment led to a marked increase of enzyme affinity and decrease V_{max} for the substrate and, to a lesser extent, for the GM2 acceptor (Martina et al. 2000). Notably, the enzyme accumulated in the ER, which may have resulted from misfolding, so the reduced kinetic parameters may have been confounded by a pool of inactive enzymes rather than represent a decreased turnover rate. The enzyme with the p.N143Q substitution did not show any activity (Table II-4A) and accumulated in the ER, indicating that the N143-linked glycan is important for its transport to the Golgi. Thus, the N-glycan may be involved in both folding and intracellular trafficking, and both processes are important for enzyme activity (Martina et al. 2000).

The soluble form of human β1,4-galactosyltransferase-I expressed in *S. cerevisiae* strain BT 150 also contains one N-glycan at N69 (Table I-4B), but it is not required for its activity (Table II-4B) (Malissard et al. 1996).

UDP-glucuronosyltransferases

UDP-glucuronosyltransferases (UGTs) catalyze the transfer of a glucuronosyl moiety to lipophilic substrates, converting them into glucuronides, which are later transported to excretory organs and eliminated from the cells. Generally, UGTs are responsible for the metabolism and detoxification of phenols, bile acids, bilirubin, steroid hormones, pollutants and other exogenous compounds (Hu et al. 2019). UGTs are type I ER membrane-associated proteins with a single C-terminal transmembrane domain, and the catalytic domain is located on the luminal side. In mammals, UGTs have been categorized into four major families: UGT1 (divided into two groups: UGT1A and UGT1B), UGT2 (subcategorized to UGT2A and UGT2B), UGT3 and UGT8 (Meech et al. 2019; Tukey and Strassburg 2000).

Several UDP-glucuronosyltransferase isoforms of UGT1A (Table I-5A) and UGT2B (Table I-5B) from various species are N-glycosylated (Mackenzie 1990; Barbier et al. 2000; Ouzzine et al. 2006). Green and Tephly (1989) reported that rat UGT1A6 (Table I-5D) and UGT2B2 (Table I-5C) and rabbit liver-derived UGT1A6 (Table I-5D) and UGT2B13 (Table I-5E) contain high-mannose N-glycans. These enzymes did not show any change in activity after the elimination of N-glycans using endo H (Table II-5C–E) (Green and Tephly 1989). Rat UGT1A6 expressed in COS-7 cells revealed a decreased molecular mass after treatment with tunicamycin, but its influence on the activity was not evaluated (Table II-5D) (Harding et al. 1989). Other studies showed that tunicamycin-treated COS-7 cells transfected with rat UGT2B1 and UGT2B2 (Table I-5C) produced enzymes with decreased activity (Table II-5C), but there were no further studies on the molecular background of this change (Mackenzie 1990).

Human UGT1A9 (Table I-5A) which contains three predicted N-glycosylation sites at N71, N292 and N344 was expressed in HEK293 cells (Nakajima et al. 2010). Treatment with tunicamycin caused a loss of enzyme activity (Table II-5A), while castanospermine and 1-deoxynojirimycin-treated cells produced an enzyme with only a slightly decreased activity. Treatment with endo H glycosidase had no effect (Table II-5A). The kinetic parameters of the endo H-treated enzyme were the same as those of the wild-type control. In the site-directed mutagenesis studies, the double-mutated variants p.N71Q/p.N292Q and p.N292Q/p.N344Q showed much lower activity in comparison with that of the wild-type control, while the p.N71Q/p.N344Q variant and the triple-mutated variant p.N71Q/p.N292Q/p.N344Q were inactive (Table II-5A) (Nakajima et al. 2010). In summary, N-glycans in human UGT1A9 seem to be crucial for its catalytic functions; presumably, they facilitate proper folding. Additionally, the presence of N-glycans improved the thermal

stability of this enzyme, so the changes in the activity of the enzyme variants may be associated with destabilization of UGT1A9 (Nakajima et al. 2010).

In 2000, Barbier *et al*. explored the role of N-glycosylation in the activity of the human UGT2B15, as well as the simian UGTB19 and UGT2B20 (Table I-5B) which were expressed in HEK293 cells (Barbier et al. 2000). All these enzymes lost much of their activities upon eliminating the first N-glycosylation site (Table II-5B). The p.S316E and p.N483D substitutions in UGTB15 revealed a decreased activity; in contrast, the p.N103K, p.S316E and p.N483D substitutions in UGTB20 had no effect (Table II-5B). Kinetic analysis of UGT2B15 and UGTB20 enzymes with substituted the first N-glycosylation site revealed no significant differences in K_m in comparison with the wild-type enzyme. Moreover, the treatment of UGT2B15 and UGT2B20 with endo H caused a decrease in activity, while the activity of UGT2B19 was unaffected (Table II-5B) (Barbier et al. 1999; Barbier et al. 2000). Taken together, the decreases in enzyme activity of UGT2B15 and UGT2B20 after eliminating their N-glycans resulted from deterioration in stability.

Other classes of glycosyltransferases

Oligosaccharyltransferase (OST) is one of the major ER-localized enzymes in the N-glycosylation pathway, expressed across all kingdoms of life (Matsumoto et al. 2013; Ollis et al. 2015; Kohda 2018). It initiates the synthesis of N-glycans by transferring *en bloc* an oligosaccharide from dolichol-pyrophosphate-activated donor to asparagine at the N-glycosylation sequon. OSTs differ in structures between phyla, and in mammals, they may have eight to nine subunits (Lu et al. 2018). The yeast enzyme contains five subunits (STT3, SWP1, WBP1, OST1 and OST2), while OSTs in bacteria, archaea and protozoans consist of a single subunit (Wild et al. 2018). There are seven N-glycosylation sites in the yeast OST, five of which contain N-glycans (N539 in STT3, N336 and N400 in OST1 and N60 and N332 in WBP1 subunits). The most conserved STT3 subunit contains two N-glycosylation sites (N60 and N539); only N539 is glycosylated (Table I-6A). The presence of N-glycan at the N539 site may be involved in the interaction with amino acids crucial for catalytic processes in the catalytic site; in addition, it seems that the N-glycan at N539 participates in interactions with other subunits, facilitating retention of the enzyme in the ER, and engages in the oligosaccharide binding process (Wild et al. 2018). Indeed, cells expressing enzyme variants without N-glycan at this site showed an inhibited cell growth or eventually died, suggesting that N-glycans are required for the production of active OST (Table II-6A) (Li et al. 2005).

Plant β1,2-xylotransferase from *Arabidopsis thaliana* (AtXylT) catalyzes the synthesis of N-glycan-containing α1,2-xylose, a glycan moiety found only in plants. The enzyme contains three potential N-glycosylation sites at N51, N301 and N478 (Table I-6B) (Pagny et al. 2003). It was expressed as a soluble ΔN31 variant (with a deleted N-terminal transmembrane domain, but with all three sequons present) in insect cells Sf9; treatment of these cells with tunicamycin yielded an enzyme with no catalytic activity (detected in cell lysates and culture medium) (Table II-6B). In addition, site-directed mutagenesis of N-glycosylation sequons showed that the N-glycan attached at N301 site is necessary for the activity and stability of the enzyme. Substitutions of the N51 or N478 sequon had no influence on the activity, but a combined modification of N51 and N301 glycosylation sites caused a complete loss of activity (Table II-6B). The activity of enzyme variants was evaluated in cell lysates and culture medium (Pagny et al. 2003). It seems that the altered activity of enzyme after elimination of N-glycans is caused by ER retention leading to its degradation (Pagny et al. 2003).

Conclusions

The role of the glycome (especially N- and O-linked glycans) in homeostasis is undisputed because glycans participate in many key life processes, such as development, growth, immune response and cell–pathogen interactions (Varki 2017). Genes encoding glycosyltransferases account for 1–2% of bacterial, archaeal and eukaryotic protein-coding genes (Lairson et al. 2008). N-glycans play a key role in the quality control of nascent glycoproteins, but more intriguingly, they are also important for the activity of the very enzymes that synthesize them, i.e., glycosyltransferases.

Demonstrating a clear link between altered N-glycosylation and changed activity of a GT is difficult because the methods used to study glycovariants produce confounding side effects, some of which are impossible to fully control. N-glycosylation is a cotranslational modification that can impact protein folding, so enzyme variants that lack N-glycans can misfold and/or aggregate and stall in the ER. These effects represent a serious limitation of studies that involve activity assays in cell lysates, which can misguide the investigators to believe that they have found a loss of enzyme catalytic activity when in fact the decline reflects partial misfolding and/or a reduced solubility of the enzyme. *In vitro* assays using purified recombinant variants of GTs suffer from the same problems. Also, using site-directed mutagenesis to remove N-glycosylation sequons introduces another potential confounder because changes in the sequence of amino acids can cause folding/trafficking problems and/or a loss of activity in their own right. Such limitations must be acknowledged and authors should strive to validate results when possible. GTs tolerate fusion with other proteins, so one way to check if variants of a GT localize correctly in the Golgi is to fuse them with a fluorescent reporter (Hassinen et al. 2010). Also, expression of GTs in prokaryotic systems, such as *E. coli*, which are not able to carry out eukaryotic PTM, can offer a glimpse into how important glycosylation is for their activity. Examples of active mammalian GTs expressed in *E. coli*, such as human ST6Gal-I, are known (Watanabe et al. 2012). Another limitation in many studies is the lack of data about the activity of unglycosylated GT variants measured in native conditions. This is an important issue because glycosyltransferases are Golgi-resident enzymes and the cellular environment may provide necessary conditions to fulfill their catalytic functions. Thus, data obtained with recombinant forms of GTs may be misleading. In addition, selecting the right cell type is very important; for example, a human glycosyltransferase expressed in insect cells may have different activity in comparison with the same enzyme expressed in human cells.

Some N-glycosylation sites in proteins remain unglycosylated. For example, human ST6Gal-I contains three predicted N-glycosylation sites, but only two are occupied (Chen and Colley 2000). Similarly, three of the four sequons in murine ST8Sia-I are occupied by N-glycans (Martina et al. 1998). Among GTs discussed in this work, approximately 82% of human and 93% of murine sequons had N-glycans attached. The location of N-glycosylation sites in the protein sequence determines its utilization; e.g., transmembrane fragments of enzymes are not N-glycosylated due to steric hindrance. On the other hand, N-glycans of GTs are not all equally important for their functions. Nonetheless, few GTs (human α2,3-sialyltransferase ST3Gal-I, bovine β1,4-*N*-acetylglucosaminyltransferase-IV and human

β1,4-galactosyltransferase-1) showed no changes in activity after their N-glycans were altered. In the case of rat α2,6-sialyltransferase-I ST6Gal-I, human and silkworm β1,2-*N*-acetylglucosaminyltransferase GnTII, chicken and mouse α2,8-sialyltransferase ST8Sia-I, mouse β1,4-*N*-acetylgalactosaminyltransferase (GM2/GD2/GA2 synthase), rat UDP-glucuronosyltransferase 2B (UGT2B1 and UGT2B2), rabbit and rat UDP-glucuronosyltransferase UGT1A6 and rabbit UDP-glucuronosyltransferase UGT2B13, the activity studies were limited to enzymatic deglycosylation of the enzymes, so their results must be interpreted with caution. Only a comprehensive approach, combining an array of different methods, may provide a reliable picture of how N-glycosylation (as well as other PTM) influences enzyme activity.

Studies on N-glycosylation may help to develop novel glycoproteins with improved secretion, activity and thermostability. This potential was well studied in a yeast protein expression system and reviewed in (Ge et al. 2018). Such proteins (usually enzymes) are sought after in many science and technology fields, energy, agriculture, pharma and cleaning agents (Hyun et al. 2018), and they can have clinical importance (Van Beilen and Li 2002). One example is adeno-associated virus (AAV) vector-based gene therapy for the inherited bleeding disorder hemophilia A, which is currently being evaluated in clinical trials (Perrin et al. 2019). Coagulation factor FVIII, a deficiency of which underlies hemophilia A, is a large glycoprotein with 25 N-glycosylation sites, 19 of which lie within the B domain. The limited size of the genetic payload that AAV vectors can accommodate and ferry to the target cells necessitated ridding FVIII transgenes of the B domain, which is dispensable for its procoagulant function but important for secretion. This prompted attempts to replace the B domain with a short spacer that retains six N-glycosylation sites, which improves the FVIII expression 3-fold in mice compared with a B domain-deleted FVIII (McIntosh et al. 2013).

Using N-glycosylation as a druggable target in infectious diseases and cancers has recently emerged as a new field in clinical studies. To date, three N-glycosylation inhibitors, castanospermine, swainsonine and 1-deoxynojirimycin, have been considered as drug candidates (Wu et al. 2002; Brown et al. 2007). Additionally, 6-O-butanoyl-castanospermine (Celgosivir) was tested as an antiviral agent against HIV (Bridges et al. 1994), hepatitis C virus (HCV) (Whitby et al. 2004), Ebola virus (Dowall et al. 2016), herpes simplex virus type-1 (HSV-1) (Bridges et al. 1995) and acute dengue fever viruses (Watanabe et al. 2018), as well as in breast cancer (Gueder et al. 2017). Moreover, an antiviral activity against dengue fever and Japanese encephalitis virus (JEV) was demonstrated for *N*-nonyl-deoxynojirimycin (*N*N-DNJ) (Wu et al. 2002). Swainsonine, as a hydrochloride salt derivative, was tested in the treatment of glioma (Sun et al. 2009) as well as gastric (Sun et al. 2007), hepatocellular (You et al. 2012) and renal carcinomas (Shaheen et al. 2005). None of the N-glycosylation inhibitors have been licensed as a drug to date.

Efforts are afoot to use glycosyltransferases in clinical applications. A perennial interest in transfusion medicine has been to streamline blood banking by enzymatic modification of blood group antigens on red cells, many of which are carbohydrates (Olsson and Clausen 2008; Kaczmarek et al. 2017; Clausen and Olsson 2019). In preclinical studies, recombinant FUT6 and FUT7 were successfully employed to custom-modify glycans N-linked to homing receptors on stem cells *ex vivo*, which primed these cells to enter the target tissues upon systemic administration (Sackstein 2016). These developments show that after decades of maturation, glycobiotechnology is an idea whose time has come.

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Conflict of interest statement

None declared.

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Original Article

Glycan Synthesis

Missing the sweet spot: one of the two N-glycans on human Gb3/CD77 synthase is expendable

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Abstract

N-glycosylation is a ubiquitous posttranslational modification that may influence folding, subcellular localization, secretion, solubility and oligomerization of proteins. In this study, we examined the effects of N-glycans on the activity of human Gb3/CD77 synthase, which catalyzes the synthesis of glycosphingolipids with terminal Galα1$\rightarrow$4Gal (Gb3 and the P1 antigen) and Galα1$\rightarrow$4GalNAc disaccharides (the NOR antigen). The human Gb3/CD77 synthase contains two occupied N-glycosylation sites at positions N_{121} and N_{203}. Intriguingly, we found that while the N-glycan at N_{203} is essential for activity and correct subcellular localization, the N-glycan at N_{121} is dispensable and its absence did not reduce, but, surprisingly, even increased the activity of the enzyme. The fully N-glycosylated human Gb3/CD77 synthase and its glycoform missing the N_{121} glycan correctly localized in the Golgi, whereas a glycoform without the N_{203} site partially mislocalized in the endoplasmic reticulum. A double mutein missing both N-glycans was inactive and accumulated in the endoplasmic reticulum. Our results suggest that the decreased specific activity of human Gb3/CD77 synthase glycovariants resulted from their improper subcellular localization and, to a smaller degree, a decrease in enzyme solubility. Taken together, our findings show that the two N-glycans of human Gb3/CD77 synthase have opposing effects on its properties, revealing a dual nature of N-glycosylation and potentially a novel regulatory mechanism controlling the biological activity of proteins.

Key words: A4GALT, activity, glycosphingolipid, glycosyltransferase, Shiga toxin

Introduction

Glycosyltransferases (GTs) constitute a large group of enzymes catalyzing transfer of sugar residues from carbohydrate donors (*e.g.* UDP-Gal, CMP-NeuNAc) to diverse acceptor molecules, forming glycosidic bonds with retention or inversion of the attached sugar configuration (Breton et al. 2012). On the basis of structural analysis, most GTs are classified into five overall folds: GT-A (Taujale et al. 2020), GT-B (Albesa-Jové et al. 2014), GT-C (Bohl et al. 2021), GT-D (Zhang et al. 2016) and GT-E (Kattke et al. 2019).

N-glycosylation is a ubiquitous posttranslational modification (PTM) of proteins; an estimated 50% may be N-glycosylated (Goettig 2016). N-glycans are attached to a protein by asparagine within a canonical N-X-S/T motif called a sequon (where X is any amino acid residue except proline); however, other sequons, such as N-X-C, N-Q-C, N-S-G or Q-G-T sequons (referred to as noncanonical) may also be utilized (Lowenthal et al. 2016). In eukaryotes, biosynthesis of N-glycans occurs in two cellular compartments: (1) the endoplasmic reticulum (ER), in which the dolichol-P-linked oligosaccharide substrate is attached to the asparagine and (2) the Golgi apparatus, which contains glycan-processing enzymes involved in trimming and maturation of N-glycans (Stanley et al. 2017). Glycosylation may play a major role in many cellular processes, such as protein folding (Jayaprakash and Surolia 2017), maturation (Agthe et al. 2018), secretion (Fiedler and Simons 1995), intracellular trafficking (Rosnoblet et al. 2013), cell–cell interactions (Varki 2017), immune responses (Ryan and Cobb 2012) and disease progression (Kizuka et al. 2017; Vajaria and Patel 2017).

Human Gb3/CD77 synthase (α1,4-galactosyltransferase, P1/P^k synthase, EC 2.4.1.228) encoded by the *A4GALT* gene, is a type II transmembrane GT with C-terminal globular catalytic domain facing the Golgi lumen and N-terminal cytoplasmic domain; it belongs to the CAZy glycosyltransferase family 32 (Carbohydrate Active Enzymes database, CAZy, http://www.cazy.org/) (Lombard et al. 2014). The enzyme retains the donor's anomeric carbon stereochemistry after glycosidic bond formation (Okuda et al. 2006; Furukawa et al. 2014). *In silico* analysis predicted that human Gb3/CD77 synthase adopts the GT-A fold structure (CAZy, http://www.cazy.org/). Members of the GT-A superfamily typically require a divalent metal ion (usually Mn^{2+}) at the catalytic center, which is coordinated by two aspartic acid residues, creating a DXD motif (D_{192}TD in human Gb3/CD77 synthase according to UniProt Q9NPC4, https://www.uniprot.org/).

Previously, we found that in addition to the high-frequency *A4GALT* gene variant (GenBank nucleotide sequence databases with accession number NG_007495.2, National Center for Biotechnology Information, NCBI, https://www.ncbi.nlm.nih.gov/), there is another variant with a point mutation c.631C > G (rs397514502, GenBank nucleotide sequence databases with accession number NG_007495.2, NCBI, https://www.ncbi.nlm.nih.gov/) giving rise to the protein with p.Q211E substitution (hereafter referred to as E or mutein). The enzyme encoded by the high-frequency gene (here designated as Q) catalyzes the transfer of galactose from UDP-galactose to lactosylceramide or paragloboside, producing globotriaosylceramide (Gb3, P^k, CD77) and the P1 antigen, respectively. Both Gb3 and P1 terminate with a Galα1→4Gal moiety. It was recently shown that Gb3/CD77 synthase can also add galactose to Galβ1→Cer, creating galabiosylceramide (Galα1→4Galβ1-Cer) (Akiyama et al. 2021). In addition, the mutein can transfer galactose to a terminal GalNAc, giving rise to the rare NOR antigen (carried by NOR1 and NOR2 glycosphingolipids), which terminates with Galα1→4GalNAc structures (Suchanowska et al. 2012). The ability of human Gb3/CD77 synthase to use two different acceptors is a unique case of glycosyltransferase promiscuity (Kaczmarek et al. 2016a). All these antigens (Gb3, P1 and NOR) belong to the human P1PK blood group system: the presence or absence of P1 on red blood cells (RBCs) determines the P_1 or P_2 blood group phenotypes, respectively. In the rare p phenotype, which may be caused by null mutations in *A4GALT*, the P1PK antigens are not detected on RBCs. The presence of NOR antigens results in the rare NOR phenotype (Kaczmarek et al. 2014; Kaczmarek et al. 2018).

Gb3 is present in human RBCs and lymphocytes, heart, lung, kidney, smooth muscle and epithelium of gastrointestinal tract (Cooling 2015). Several reports showed that elevated expression of Gb3 was found in colorectal, gastric and ovarian cancers (Kovbasnjuk et al. 2005; Geyer et al. 2016). In Fabry disease, which is an X-linked lysosomal storage disorder caused by deficiency of α-galactosidase (OMIM 301500), Gb3 accumulates in organs throughout the body (Miller et al. 2020). Expression of the P1 antigen seems to be limited to the erythroid lineage (Cooling 2015), but it was also detected on ovarian cancer cells, where it was designated as cancer-associated antigen (Jacob et al. 2014).

Galα1→4Gal disaccharide-containing glycosphingolipids (GSLs), such as Gb3 are targeted by bacterial adhesins, including PapG of uropathogenic *Escherichia coli*, and viruses (Cooling 2015). Gb3 is also the main receptor for Shiga toxins (Stxs) secreted by Shiga toxin-producing *E. coli* (STEC) (Lee and Tesh 2019). Humans counteract STEC infections by ingestion of contaminated food or water; every year STEC cause an estimated 2.8 million severe illnesses worldwide (Majowicz et al. 2014). Shiga toxins can cause hemorrhagic colitis, which may progress to hemolytic-uremic syndrome (HUS), an acute and often fatal complication (Bruyand et al. 2018; Cody and Dixon 2019).

Several studies showed that elimination of N-glycans from GTs may affect their activity (Mikolajczyk et al. 2020). The protein sequence of human Gb3/CD77 synthase includes two potential N-glycosylation sites at positions N_{121} and N_{203} (UniProt Q9NPC4, https://www.uniprot.org/). Using site-directed mutagenesis, we generated six N-glycosylation variants of the human Gb3/CD77 synthase (three for Gb3/CD77 synthase and three for the mutein) with substituted N-glycosylation sequons, and analyzed their activity, subcellular trafficking and secretion in CHO-Lec2 cells transfected with vectors encoding the glycovariants. Finally, we evaluated the sensitivities of CHO-Lec2 cells expressing different glycovariants to Shiga toxins.

Results

Gb3/CD77 synthase contains two occupied N-glycosylation sites

Human Gb3/CD77 synthase contains two potential N-glycosylation sites: N_{121}-A-S and N_{203}-L-T (UniProt Q9NPC4, https://www.uniprot.org/) (Figure 1). Our preliminary studies suggested that treatment of the Q enzyme with peptide-N-glycosidase F (PNGase

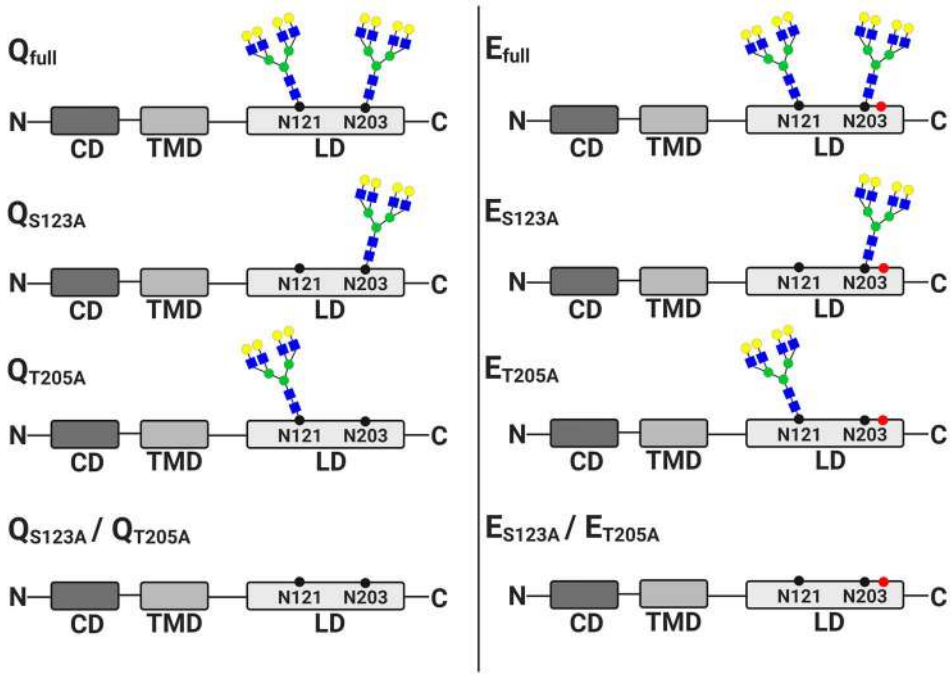


Fig. 1. Schematic representation of human Gb3/CD77 synthase and its mutein glycovariants with N-glycosylation sites occupation. The enzyme contains cytoplasmic domain (CD, encompasses 1–22 amino acids residues), transmembrane domain (TMD, encompasses 23–43 amino acids residues) which resides the enzyme in Golgi apparatus membrane and lumenal domain (LD, encompasses 44–353 amino acids residues), containing catalytic site (the sequences of the enzyme domains according with UniProt Q9NPC4, https://www.uniprot.org/.). The human Gb3/CD77 synthase contains Q at position 211 in contrast to E enzyme with p.Q211E substitution (red dot). Both Gb3/CD77 synthase forms contain two N-glycosylation sites at N_{121} and N_{203} (black dot). Q_{full}, fully N-glycosylated Gb3/CD77 synthase; E_{full}, fully N-glycosylated mutein Gb3/CD77 synthase; Q_{S123A}, Gb3/CD77 synthase with p.S123A substitution; E_{S123A}, mutein Gb3/CD77 synthase with p.S123A substitution; Q_{T205A}, Gb3/CD77 synthase with p.T205A substitution; E_{T205A}, mutein Gb3/CD77 synthase with p.T205A substitution; Q_{S123A}/Q_{T205A}, Gb3/CD77 synthase with p.S123A/p.T205A substitutions; E_{S123A}/E_{T205A}, mutein Gb3/CD77 synthase with p.S123A/p.T205A substitutions [Figure was created with BioRender.com].

F) abolished its *in vitro* activity (Figure S1). Six glycovariants were generated: four single-mutants with p.S123A substitution (Q_{S123A} and E_{S123A} for Gb3/CD77 synthase and its mutein, respectively) and p.T205A substitution (Q_{T205A} and E_{T205A} for Gb3/CD77 synthase and its mutein, respectively), as well as two double-mutants (Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A}) (Figure 1). The enzymes without any substitutions at N-glycosylation sites (fully N-glycosylated) are referred to as "Q_{full}" for the Gb3/CD77 synthase and "E_{full}" for the mutein.

In order to check whether the targeted sequons are N-glycosylated, CHO-Lec2 cells expressing the glycovariants were lysed and analyzed by western blotting. The human Gb3/CD77 synthase was detected using the mouse monoclonal anti-A4GALT antibody (clone 5C7). The occupancy of N-glycosylation sites was examined by comparing the electrophoretic mobility of the glycovariants with the fully N-glycosylated enzyme forms (Q_{full} and E_{full}). The mobility of single-mutants Q_{S123A}, E_{S123A}, Q_{T205A} and E_{T205A} was almost the same, and their apparent molecular weight (MW) was lower (about 37 kDa) than their fully N-glycosylated Q_{full} and E_{full} counterparts (39 kDa) (Figure 2A and B). The double-mutant glycovariants Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A} revealed the lowest apparent MW (32 kDa) of all evaluated proteins. The extra bands recognized by anti-A4GALT antibody (clone 5C7) may have come from proteolytic degradation of the Gb3/CD77 synthase during the cell lysis, whereas the high-molecular weight bands may be enzyme aggregates. In summary, these findings suggest that both N-glycosylation sites of human Gb3/CD77 synthase are occupied (Figure 2A and B).

To corroborate these results, CHO-Lec2 cells transfected with vectors encoding the glycovariants and fully N-glycosylated enzymes were lysed and treated with PNGase F, which removes N-glycans from proteins. This generated products whose electrophoretic mobility corresponded with the glycanless glycovariants Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A} (Figure 2C and D). Additionally, CHO-Lec2 cells expressing the fully N-glycosylated enzymes (Q_{full} and E_{full}) were grown in the presence of tunicamycin, which completely inhibits N-glycosylation (Esko et al. 2017). Both proteins showed similar apparent MW as double-mutants Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A}, showing that elimination of N-glycans from these two sites strips the enzyme of all glycans (Figure 2E and F). In summary, our results indicate that both N-glycosylation sites of human Gb3/CD77 synthase are occupied by N-glycans.

N_{121} and N_{203} sites have opposing effects on the Gb3/CD77 synthase activity

To examine the activity of human Gb3/CD77 synthase glycovariants in CHO-Lec2 cells, we evaluated the expression of P1PK antigens (Gb3 and P1 for Gb3/CD77 synthase; Gb3, P1 and NOR for its mutein) using flow cytometry (Figure 3). Two anti-P1 antibodies were used: (1) anti-P1 (clone 650) that reacts with Gb3 and P1 and (2) anti-P1 (clone P3NIL100) that binds only to P1 (Tables SI and SII). For detection of the NOR antigen, we used mouse monoclonal anti-NOR antibody (clone nor118) (Tables SI and SII) (Duk et al. 2005). Despite being a quantitative assay, flow cytometry cannot discriminate between GSLs and glycoproteins, nor can it discriminate

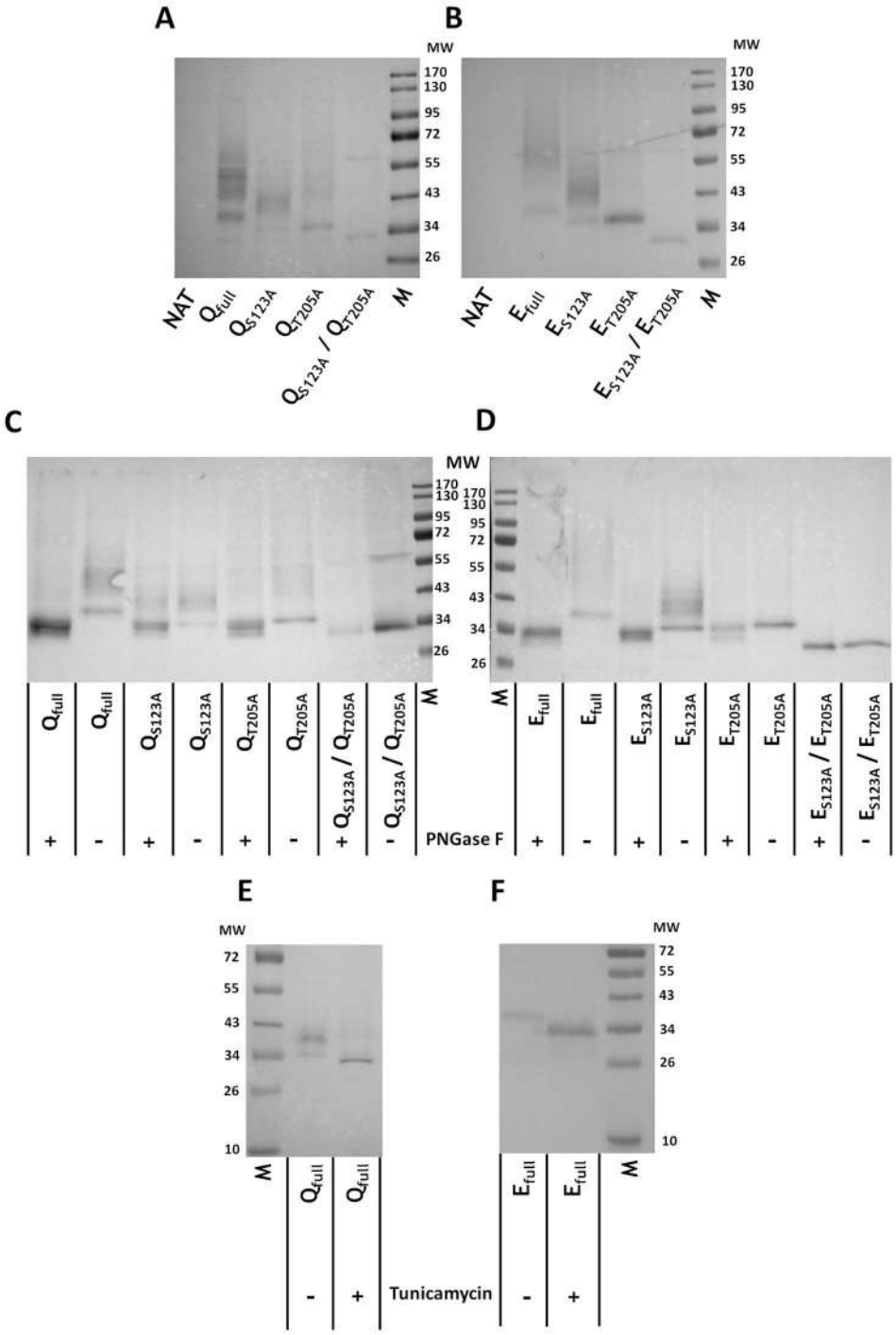


Fig. 2. Glycosylation patterns of human Gb3/CD77 synthase and its mutein glycovariants. Western blotting analysis of lysates prepared from non-transfected CHO-Lec2 cells (NAT) and CHO-Lec2 cells expressing fully N-glycosylated enzymes (Q_{full}, E_{full}) as well as human Gb3/CD77 synthase (Q_{S123A}, Q_{T205A}, Q_{S123A}/Q_{T205A}) and its mutein (E_{S123A}, E_{T205A}, E_{S123A}/E_{T205A}) glycovariants. Human Gb3/CD77 synthase was stained using anti-A4GALT monoclonal antibody (clone 5C7). Molecular weights of (**A**) human Gb3/CD77 synthase and its (**B**) mutein glycovariants were evaluated. (**C**) Human Gb3/CD77 synthase and its (**D**) mutein glycovariants treated (+) or not (−) with PNGase F. CHO-Lec2 cells transfected with vector encoding (**E**) Q_{full} and (**F**) E_{full} were treated (+) or not (−) with tunicamycin. Molecular weight of the bands are presented as a kDa.

between Gb3 and P1 when using the anti-P1 antibody (clone 650). CHO-Lec2 cells expressing glycovariants showed different quantities of Gb3, P1 and NOR antigens (determined by estimating antibody binding capacities) (Figure 4A and B). The highest antibody binding capacities (ABCs) for anti-P1 (650) antibody were found for Q_{S123A} and E_{full} clones, while anti-P1 (P3NIL100) showed highest ABC for

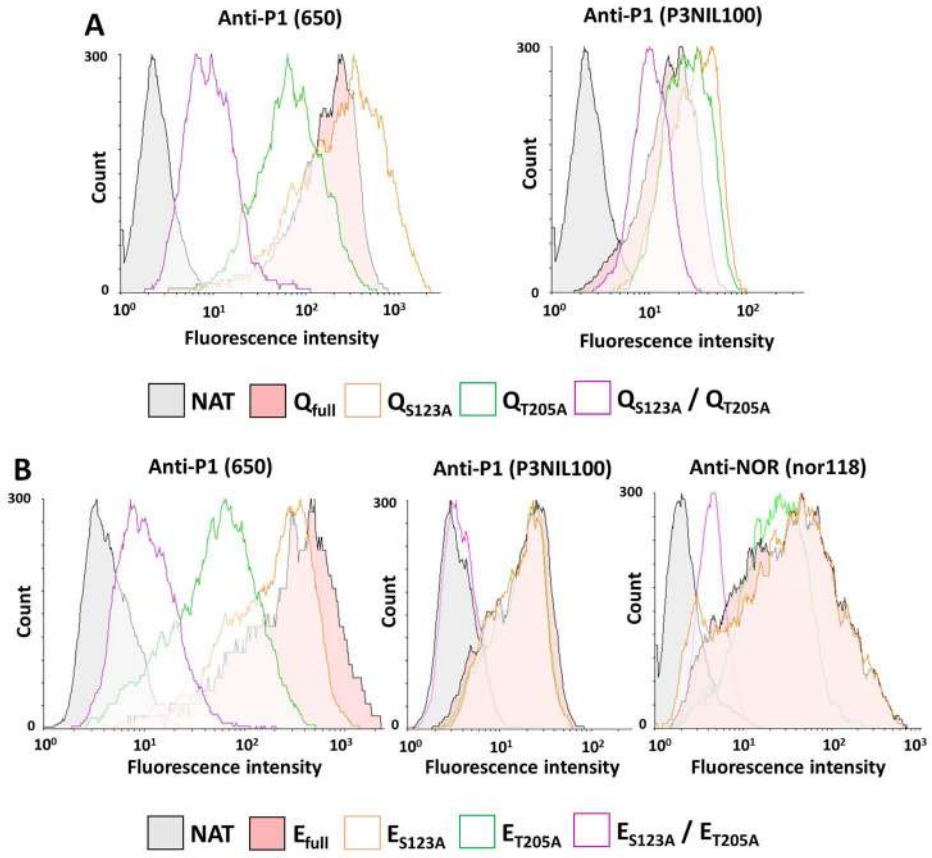


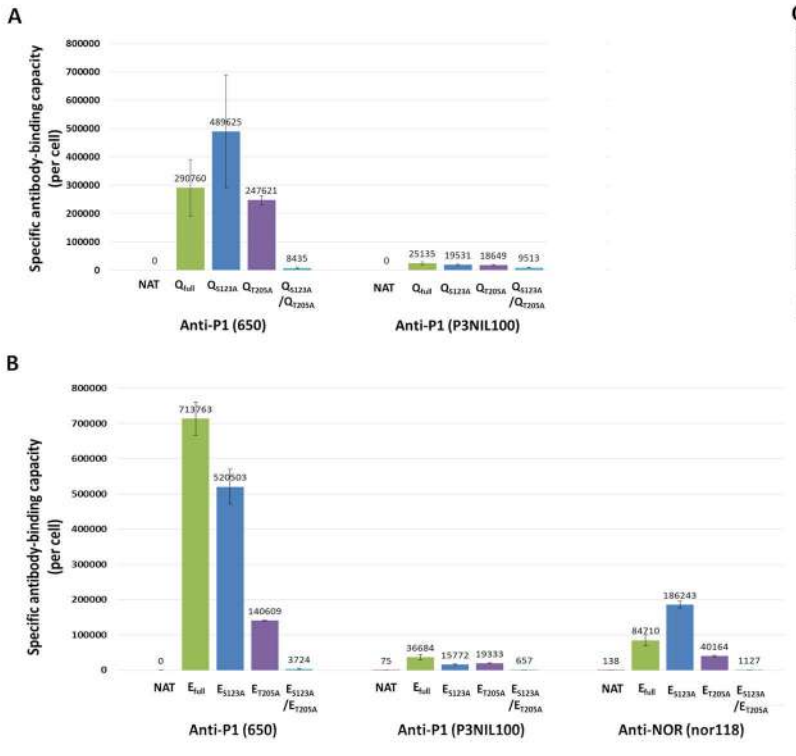
Fig. 3. Flow cytometry analysis of CHO-Lec2 cells. The cells were transfected with *A4GALT* genes encoding (**A**) human Gb3/CD77 synthase and its (**B**) mutein glycovariants. The binding of anti-P1 (clones 650 and P3NIL100) and anti-NOR (clone nor118) antibodies to CHO-Lec2 cells was evaluated. Q_{full}, fully N-glycosylated Gb3/CD77 synthase; E_{full}, fully N-glycosylated mutein Gb3/CD77 synthase; Q_{S123A}, Gb3/CD77 synthase with p.S123A substitution; E_{S123A}, mutein Gb3/CD77 synthase with p.S123A substitution; Q_{T205A}, Gb3/CD77 synthase with p.T205A substitution; E_{T205A}, mutein Gb3/CD77 synthase with p.T205A substitution; Q_{S123A}/Q_{T205A}, Gb3/CD77 synthase with p.S123A/p.T205A substitutions; E_{S123A}/E_{T205A}, mutein Gb3/CD77 synthase with p.S123A/p.T205A substitutions.

Q_{full} and E_{full}. The ABC values for anti-P1 (650 and P3NIL100) were the lowest for the double-mutant glycovariants Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A}, and partially reduced for E_{T205A} and, to a lesser extent, Q_{T205A} (Figure 4C and D). These results suggest that the human Gb3/CD77 synthase requires the N_{203}-linked glycan for enzyme activity while the N_{121}-glycan is dispensable and may even curtail the activity.

To evaluate the activity of human Gb3/CD77 synthase *in vitro*, we used lysates prepared from CHO-Lec2 cells transfected with vectors encoding glycovariants in an enzyme assay using oligosaccharide-polyacrylamide (PAA) conjugates as acceptors. Q_{full} and E_{full} showed the highest activity (Figure 5A–C). Activities of the single-mutant glycovariants were reduced (except for Q_{S123A} with the P1 precursor nLc4-PAA, whose activity was the same as that of Q_{full}'s and for E_{S123A}, which showed increased activity toward the Gb4-PAA which is a precursor of NOR antigen), while the double-mutant glycovariants Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A} were inactive (Figure 5A–C). Generally, these results suggested that a lack of N-glycan at N_{121} had no significantly effect on the enzyme activity, in contrast to missing the N-glycan at N_{203}, when the enzyme shows decreased activity. Finally, the N-glycanless enzymes were completely inactive *in vitro*.

Since P1PK GSLs are the main products of human Gb3/CD77 synthase, we analyzed neutral glycosphingolipids isolated from CHO-Lec2 cells transfected with vectors encoding glycovariants with high-performance thin-layer chromatography (HPTLC, Figure 6A and B). In orcinol staining, we found that Gb3Cer and Gb4Cer were the predominant GSLs in all samples. Neither Q- nor E-derived glycovariants produced GSLs detectable with anti-P1 (P3NIL100) antibody (Figure 6A and B), which suggests that human Gb3/CD77 synthase is unable to synthesize the P1 antigen in CHO-Lec2 cells, most probably due to the lack of the acceptor (nLc4).

The identities of glycosphingolipids derived from CHO-Lec2 transfected cells were confirmed using MALDI-TOF mass spectrometry (Figures S2 and S3). The reflectron-positive mode spectrum showed several clusters of ions corresponding to glucosylceramide (GlcCer), lactosylceramide (LacCer) and certain globo-series GSLs (e.g. Gb3, NOR1). The mass differences between the ions (mostly ~28 Da, which is the molecular mass of two methylene groups) corresponded to isoforms with ceramides having acyl groups of different lengths (*e.g.* 16:0, 18:0, 20:0, 22:0) but the same long-chain base (d18:1 sphingosine). No P1 antigen-corresponding structures were identified (Figures S2 and S3).



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Antibody	Q_{full}	Q_{S123A}	Q_{T205A}	Q_{S123A} / Q_{T205A}
Anti-P1 (650)	290760 ± 91893	489625 ± 179537	247621 ± 12960	8435 ± 2746
Anti-P1 (P3NIL100)	25135 ± 4623	19531 ± 2839	18649 ± 1401	9513 ± 1126
Anti-NOR (nor118)	-	-	-	-
Antibody	**E_{full}**	**E_{S123A}**	**E_{T205A}**	**E_{S123A} / E_{T205A}**
Anti-P1 (650)	713763 ± 44309	520503 ± 40406	140609 ± 1427	3724 ± 1281
Anti-P1 (P3NIL100)	36684 ± 6760	15772 ± 2013	19333 ± 1532	657 ± 285
Anti-NOR (nor118)	84710 ± 13952	186243 ± 9443	40164 ± 1989	1127 ± 52

D

Anti-P1 (650)	
Gb3/CD77 synthase	**Mutein**
$Q_{S123A} > Q_{full} > Q_{T205A} > Q_{S123A}$ / Q_{T205A}	$E_{full} > E_{S123A} > E_{T205A} > E_{S123A}$ / E_{T205A}
Anti-P1 (P3NIL100)	
Gb3/CD77 synthase	**Mutein**
$Q_{full} > Q_{S123A} > Q_{T205A} > Q_{S123A}$ / Q_{T205A}	$E_{full} > E_{T205A} > E_{S123A} > E_{S123A}$ / E_{T205A}
Anti-NOR (nor118)	
Gb3/CD77 synthase	**Mutein**
-	$E_{S123A} > E_{full} > E_{T205A} > E_{S123A}$ / E_{T205A}

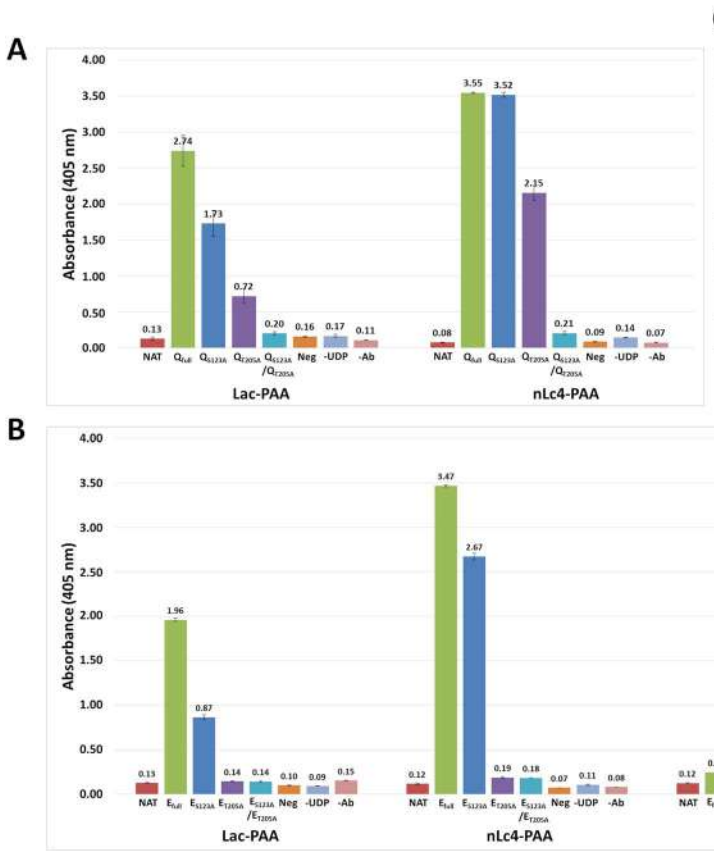
Fig. 4. Quantitative flow cytometry analysis of Gb3, P1 and NOR antigens expressed on CHO-Lec2 cells. ABC per CHO-Lec2 cells was calculated for anti-P1 (clones 650 and P3NIL100) and anti-NOR (clone nor118) antibodies. (**A**) Human Gb3/CD77 and its (**B**) mutein glycovariants (median; error bars represent interquartile ranges). (**C**) ABCs calculated for human Gb3/CD77 and its mutein glycovariants. (**D**) The order of activity level based on the ABC values measured for Gb3/CD77 synthase and its mutein glycovariants. Q_{full}, fully N-glycosylated Gb3/CD77 synthase; E_{full}, fully N-glycosylated mutein Gb3/CD77 synthase; Q_{S123A}, Gb3/CD77 synthase with p.S123A substitution; E_{S123A}, mutein Gb3/CD77 synthase with p.S123A substitution; Q_{T205A}, Gb3/CD77 synthase with p.T205A substitution; E_{T205A}, mutein Gb3/CD77 synthase with p.T205A substitution; Q_{S123A}/Q_{T205A}, Gb3/CD77 synthase with p.S123A/p.T205A substitutions; E_{S123A}/E_{T205A}, mutein Gb3/CD77 synthase with p.S123A/p.T205A substitutions.

Since levels of the P1PK antigens may correlate with the levels of *A4GALT* transcripts in the cells, we examined the expression of genes encoding glycovariants of Q and E enzymes in CHO-Lec2 cells using qPCR. The levels of all transcripts were upregulated in the transfected cells in comparison with the non-transfected CHO-Lec2 (data not shown). The mean threshold cycle (C_T) revealed no significant differences in transcript levels between cells transfected with different glycovariants (Figure S4).

There is a general agreement that GSLs are the main acceptors for human Gb3/CD77 synthase, although it was shown recently that the enzyme can also use glycoproteins (Stenfelt et al. 2019; Morimoto et al. 2020; Szymczak-Kulus et al. 2021). To find out if the glycovariants differ in acceptor preferences, we analyzed the presence of P1 glycotope on glycoproteins derived from CHO-Lec2 cells transfected with vectors encoding different glycovariants. Using western blotting and anti-P1 antibodies, we found that lysates of cells expressing the Q_{full} and E_{S123A} enzymes revealed the strongest binding (Figure 7A and B), with the E_{S123A} reaction being markedly stronger than its fully glycosylated counterpart (E_{full}). Lysates from the Q_{T205A}- and E_{T205A}-expressing cells produced weakly recognized glycoproteins, with a slightly stronger reaction in the case of E enzyme (Figure 7A and B). No binding of anti-P1 (650) antibody was detected in the case of double-mutant glycovariants Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A}, although anti-P1 (P3NIL100) weakly recognized a few bands (Figure 7A and B). These data may suggest that the fully N-glycosylated human Gb3/CD77 synthase and the majority of its glycovariants can efficiently use glycoproteins as acceptors. Moreover, the E_{S123A} enzyme seems to have a higher affinity to glycoprotein acceptors than the other glycovariants.

Cells expressing Gb3/CD77 synthase glycovariants show sensitivity to Shiga toxins

Since the main receptor for Shiga toxins 1 and 2 is Gb3, we evaluated the sensitivity of CHO-Lec2 cells transfected with vectors encoding glycovariants of human Gb3/CD77 synthase and its mutein to these toxins. We found that sensitivity of the cells expressing single-mutant glycovariants of Q and E treated with Stx1 and Stx2 holotoxins was similar or higher than that of the cells expressing fully N-glycosylated enzymes (Figure 8A and B). The CHO-Lec2 cells expressing double-mutant glycovariants (Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A}) were still more sensitive to both Stxs than non-transfected CHO-Lec2 cells, which do not express receptors for Shiga toxins. Viability of Q_{S123A}/Q_{T205A} cells was 34% and 35% for Stx1 and Stx2, respectively (Figure 8A); viability of E_{S123A}/E_{T205A} cells was 37% for Stx1 and 39% for Stx2 (Figure 8B). Cells expressing Q_{full} and single-mutant Q glycovariants were 34–42% and 25–44% viable after treatment with Stx1 and Stx2, respectively; viability of cells expressing E_{full} and the single-mutant E glycovariants was 23–24% and 22–24% upon exposure to Stx1 and Stx2, respectively (Figure 8A and B). The viability correlated with Gb3 levels on the CHO-Lec2 cells expressing the studied glycovariants. Interestingly,



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Anti-P1 (650)	
Gb3/CD77 synthase	Mutein
$Q_{full} > Q_{S123A} > Q_{T205A} > Q_{S123A}/Q_{T205A}$	$E_{full} > E_{S123A} > E_{T205A} > E_{S123A}/E_{T205A}$
Anti-P1 (P3NIL100)	
Gb3/CD77 synthase	Mutein
$Q_{full} = Q_{S123A} > Q_{T205A} > Q_{S123A}/Q_{T205A}$	$E_{full} > E_{S123A} > E_{T205A} > E_{S123A}/E_{T205A}$
Anti-NOR (nor118)	
Gb3/CD77 synthase	Mutein
-	$E_{S123A} > E_{full} > E_{T205A} > E_{S123A}/E_{T205A}$

Fig. 5. Enzymatic activity of human Gb3/CD77 synthase and its mutein glycovariants in CHO-Lec2 cells lysates. Cell lysates prepared from CHO-Lec2 cells transfected with vectors encoding (**A**) human Gb3/CD77 and its (**B**) mutein glycovariants according to (Cheng et al. 2016) protocol. (**C**) The order of *in vitro* activity level measured using PAA-conjugates for Gb3/CD77 synthase and its mutein glycovariants. Lac-PAA acceptor is a precursor of Gb3; nLc4-PAA acceptor is a precursor of P1 antigen; Gb4-PAA acceptor is a precursor of NOR antigen. NAT, non-transfected CHO-Lec2 cells; Q_{full}, fully N-glycosylated Gb3/CD77 synthase; E_{full}, fully N-glycosylated mutein Gb3/CD77 synthase; Q_{S123A}, Gb3/CD77 synthase with p.S123A substitution; E_{S123A}, mutein Gb3/CD77 synthase with p.S123A substitution; Q_{T205A}, Gb3/CD77 synthase with p.T205A substitution; E_{T205A}, mutein Gb3/CD77 synthase with p.T205A substitution; Q_{S123A}/Q_{T205A}, Gb3/CD77 synthase with p.S123A/p.T205A substitutions; E_{S123A}/E_{T205A}, mutein Gb3/CD77 synthase with p.S123A/p.T205A substitutions; Neg, control without the enzyme containing lysates added to reaction; -UDP, control without UDP-Gal donor added to reaction; −Ab, control without added primary antibodies.

the residual amounts of Gb3 produced by double-mutant glycovariants were sufficient to mediate cytotoxicity.

The N_{203} glycan in Gb3/CD77 synthase determines its subcellular localization

To determine whether N-glycosylation affects trafficking and localization of human Gb3/CD77 synthase and its mutein, we evaluated the CHO-Lec2 cells transfected with vectors encoding glycovariants using anti-A4GALT monoclonal antibody (clone 5C7) by immunofluorescence microscopy. It is generally assumed that the human Gb3/CD77 synthase, similarly to other GTs belonging to the glycosyltransferase family 32, is a Golgi-resident enzyme (www.cazy.org); according to Yamaji T. et al. and D'Angelo G. et al., it localizes in *trans*-Golgi network (TGN) (D'Angelo et al. 2013; Yamaji et al. 2019). Co-immunostaining of Q_{full}, E_{full}, Q_{S123A}, E_{S123A}, Q_{T205A} and E_{T205A} glycovariants with anti-A4GALT antibody and anti-syntaxin 16 as a marker of the *trans*-Golgi cisternae revealed that all these enzymes localized in *trans*-Golgi (Figures 9 and 10). Q_{T205A}, E_{T205A}, Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A} glycovariants co-localized with anti-calnexin antibody, which recognizes ER-resident protein (Figures 9 and 10). Moreover, the glycovariants Q_{T205A} and E_{T205A} co-localized with both anti-syntaxin 16 and anti-calnexin antibodies, revealing that these enzymes localized both in the *trans*-Golgi and the ER. In contrast, the double-mutant glycovariants Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A} accumulated in the ER only (Figures 9 and 10). The residual signals derived from anti-LAMP1 (marker of lysosomes) and anti-A4GALT antibodies were found only in the case of Q_{S123A}/Q_{T205A} glycovariant (Figure 9). Thus, it may be concluded that the fully N-glycosylated enzymes (Q_{full} and E_{full}), as well as Q_{S123A} and E_{S123A} glycovariants exit the ER properly and localize in the Golgi. In contrast, the glycovariants with substituted N_{203} site (Q_{T205A} and E_{T205A}) partially fail to leave the ER. The glycanless variants (Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A}) were found only in the ER. Thus, these findings show that N-glycan at position N_{203} plays a crucial role in trafficking and proper subcellular localization of the human Gb3/CD77 synthase.

All glycovariants of human Gb3/CD77 synthase were further evaluated using immunogold reaction with anti-A4GALT monoclonal antibody (clone 5C7). We found that the glycovariants localized in the ER and/or Golgi (Figure S5 and S6). In addition, we observed increased numbers of gold nanoparticles corresponding to fully N-glycosylated Q_{full} and E_{full} enzymes as well as Q_{S123A}

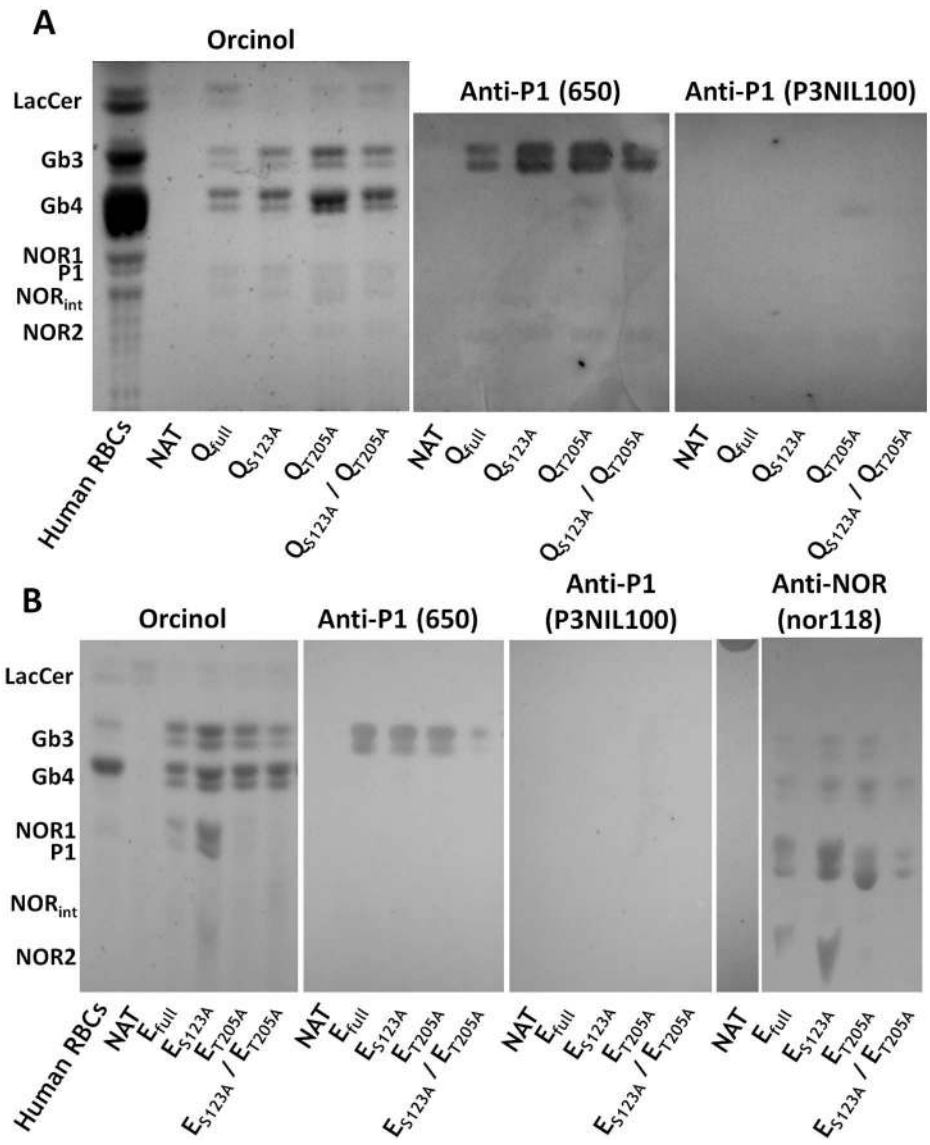

Fig. 6. HPTLC analysis of neutral glycosphingolipids extracted from CHO-Lec2 cells. (**A**) Gb3/CD77 synthase and its (**B**) mutein glycovariants analyzed by orcinol staining or by overlaying with anti-P1 (650 and P3NIL100) and anti-NOR (nor118) antibodies. Human RBCs, the GSLs samples of $P^{1NOR}P^{1}$ human RBCs; NAT, non-transfected CHO-Lec2 cells; Q_{full}, fully N-glycosylated Gb3/CD77 synthase; E_{full}, fully N-glycosylated mutein Gb3/CD77 synthase; Q_{S123A}, Gb3/CD77 synthase with p.S123A substitution; E_{S123A}, mutein Gb3/CD77 synthase with p.S123A substitution; Q_{T205A}, Gb3/CD77 synthase with p.T205A substitution; E_{T205A}, mutein Gb3/CD77 synthase with p.T205A substitution; Q_{S123A}/Q_{T205A}, Gb3/CD77 synthase with p.S123A/p.T205A substitutions; E_{S123A}/E_{T205A}, mutein Gb3/CD77 synthase with p.S123A/p.T205A substitutions.

and E_{S123A} glycovariants, when comparing to glycovariants with p.T205A substitution (Figures S5 and S6). In contrast, the double-mutant Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A} glycovariants showed decreased relative numbers of gold nanoparticles in comparison to other glycovariants (Figures S5 and S6). These data suggest that glycovariants missing the N_{203}-linked glycan were synthesized less efficiently, in contrast to the N_{121} single-mutants, which seemed unaffected by the lack of N-glycan.

Discussion

N-glycosylation of proteins has important and well-defined functions, but one somewhat overlooked function is regulation of glycosyltransferase activity. The available data on that role were recently reviewed in (Mikolajczyk et al. 2020). Elimination of N-glycans usually does not directly impact catalytic functions, but it may affect the enzyme stability, subcellular localization, secretion and ability to oligomerize, which may influence the enzyme activity. In most cases, altered activity of a non-glycosylated or underglycosylated enzyme is caused by misfolding and accumulation in the ER, which prevents transport to the proper cellular compartment and/or its degradation caused by enhanced aggregation (Skropeta 2009; Mikolajczyk et al. 2020).

Contrary to the old "one enzyme - one linkage" rule, some GTs are promiscuous (Biswas and Thattai 2020). One example is human Gb3/CD77 synthase, which can recognize two different acceptors, giving rise to Gb3 and the P1 antigen (Kaczmarek et al. 2016a). A

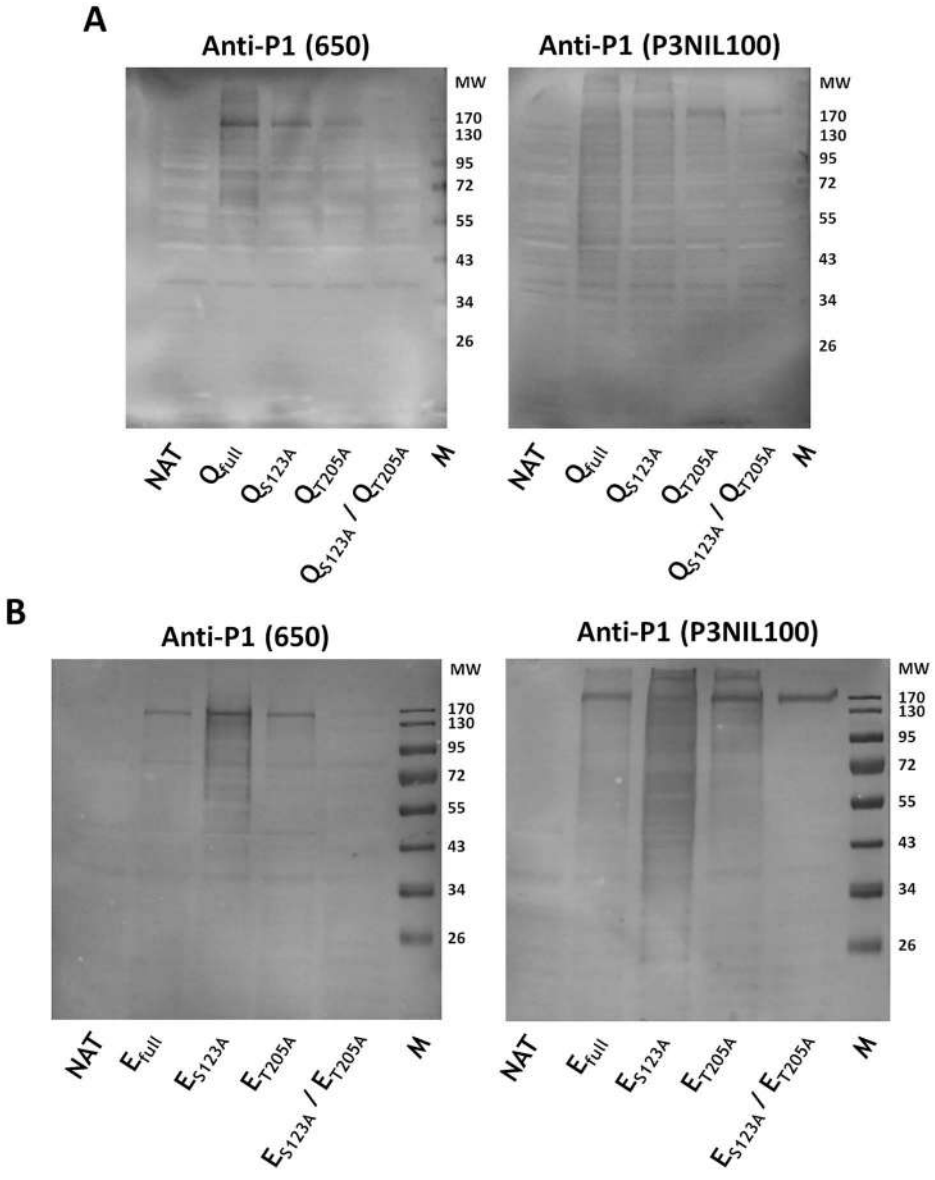

Fig. 7. Western blotting analysis of CHO-Lec2 cell lysates stained with anti-P1 (650 and P3NIL100) antibodies. CHO-Lec2 cells transfected with vectors encoding (**A**) Gb3/CD77 synthase and its (**B**) mutein glycovariants. Q_{full}, fully N-glycosylated Gb3/CD77 synthase; E_{full}, fully N-glycosylated mutein Gb3/CD77 synthase; Q_{S123A}, Gb3/CD77 synthase with p.S123A substitution; E_{S123A}, mutein Gb3/CD77 synthase with p.S123A substitution; Q_{T205A}, Gb3/CD77 synthase with p.T205A substitution; E_{T205A}, mutein Gb3/CD77 synthase with p.T205A substitution; Q_{S123A}/Q_{T205A}, Gb3/CD77 synthase with p.S123A/p.T205A substitutions; E_{S123A}/E_{T205A}, mutein Gb3/CD77 synthase with p.S123A/p.T205A substitutions. Molecular weights of the bands are presented as a kDa.

single amino acid substitution p.Q211E, which enables attachment of galactose to another terminal monosaccharide, GalNAc, creating Galα1→4GalNAc structure, makes the enzyme even more promiscuous. Extension of acceptor specificity via a single amino acid substitution is a rare phenomenon compared to the more common donor specificity changes. For example, the donor specificity of the ABO transferase depends on two amino acids: the enzyme with c.796C>A (p.L266M) and c.803G>C (p.G268A) substitutions attaches galactose instead of *N*-acetylgalactosamine to the acceptor. Moreover, the cisAB enzyme (with p.L266G substitution) can use either donor substrate, producing both the A and B antigens (Ramakrishnan and Qasba 2002; Wagner et al. 2015).

To date, no studies have fully investigated the influence of N-glycosylation on human Gb3/CD77 synthase activity. Human Gb3/CD77 synthase contains two N-glycosylation sites (N_{121} and N_{203}). Previously, we showed that deglycosylated recombinant catalytic domain of Gb3/CD77 synthase expressed in insect cells (Kaczmarek et al. 2016a) is inactive. That result prompted us to comprehensively evaluate the influence of N-glycosylation on the Gb3/CD77 synthase and its mutein activity using full-length enzyme expressed in CHO-Lec2 cells, which were selected because they do not express an endogenous Gb3/CD77 synthase and are deficient in CMP-sialic acid transporter (Stanley 1983; Patnaik and Stanley 2006). Thus, they are incapable of sialylation, leaving a

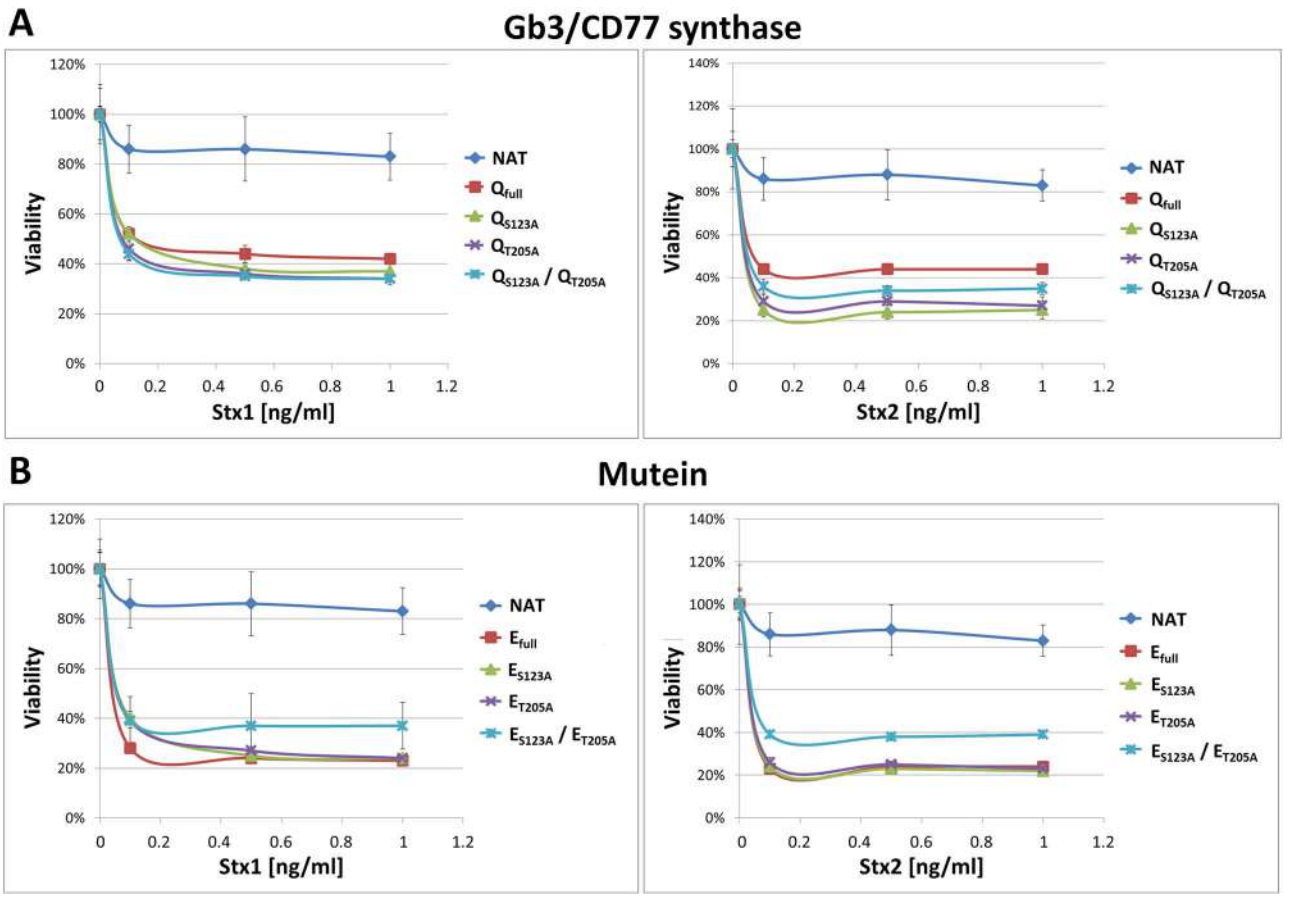

Fig. 8. Shiga toxins cytotoxicity analysis. Viability of CHO-Lec2 cells transfected with vectors encoding (**A**) Gb3/CD77 synthase and its (**B**) mutein glycovariants treated with Stx1 and Stx2 holotoxins were evaluated (at least three independent experiments were conducted, each with three technical replicates; error bars are standard deviations; statistical significance when $p < 0.05$ according to the Kruskal–Wallis ANOVA test). Q_{full}, fully N-glycosylated Gb3/CD77 synthase; E_{full}, fully N-glycosylated mutein Gb3/CD77 synthase; Q_{S123A}, Gb3/CD77 synthase with p.S123A substitution; E_{S123A}, mutein Gb3/CD77 synthase with p.S123A substitution; Q_{T205A}, Gb3/CD77 synthase with p.T205A substitution; E_{T205A}, mutein Gb3/CD77 synthase with p.T205A substitution; Q_{S123A}/Q_{T205A}, Gb3/CD77 synthase with p.S123A/p.T205A substitutions; E_{S123A}/E_{T205A}, mutein Gb3/CD77 synthase with p.S123A/p.T205A substitutions.

large proportion of acceptors available for α-galactosylation, which otherwise could be consumed by sialyltransferases. In this study, we tried to answer the question whether the two N-glycosylation sites are occupied by N-glycans, and how each of these N-glycans or their lack affects the enzyme activity. To that end, we used site-directed mutagenesis to replace the third amino acid at one or both of the two canonical N-glycosylation sequons with alanine to generate constructs encoding glycovariants (Leong et al. 1994; Czerwinski et al. 2007; Kaczmarek et al. 2016b). We found that elimination of N-glycans from the enzyme affects its enzymatic activity and that disruption of each N-glycosylation site produces different effects. In flow cytometry, the Q_{S123A} glycovariant, which lacks the N-glycan at N_{121}, showed similar or increased activity in comparison with the fully N-glycosylated enzyme, but this result was not consistent with *in vitro* activity assays, in which the Q_{S123A} glycovariant revealed decreased activity in comparison with the Q_{full}. Moreover, E_{S123A} exhibited reduced activity compared to the E_{full} enzyme in both flow cytometry and *in vitro* assays (although the NOR synthesis was consistently increased). In microscopy, both glycovariants with substituted N_{121} site localized in the Golgi, similarly to the fully N-glycosylated enzymes. Altogether, these findings at the very least show that Gb3/CD77 synthase does not require the N-glycan linked to N_{121} for activity and/or correct subcellular localization, and suggest that it may be more active without it.

In contrast, we found that the N-glycan at position N_{203} in Gb3/CD77 synthase plays an important role in its activity. Flow cytometry and *in vitro* assays revealed reduced quantities of Gb3 and P1 antigen produced by CHO-Lec2 cells expressing the Q_{T205A} and E_{T205A} glycovariants in comparison with the E_{full} enzyme. Immunofluorescence analysis showed that both Q_{T205A} and E_{T205A} localized in two distinct compartments of the cell: in the ER and the *trans*-Golgi. The ER portions of the enzymes were likely inactive. Thus, partial mislocalization in the ER may affect the overall measurable enzyme activity, because only the Golgi-resident enzyme is capable to perform its catalytic functions. Moreover, the decreased enzyme activity of the glycoforms lacking the N_{203} glycan may be associated with reduced enzyme solubility, because the Q_{T205A} and E_{T205A} glycovariants were not detected in culture media (data not shown), suggesting their poor secretion. In addition, such glycovariants did not exhibit any activity in cell lysates, which may suggest that they aggregate. These results suggest that the N-glycan at position N_{203} site plays a crucial role in subcellular localization, solubility and secretion of the enzyme, thus affecting the specific enzyme activity.

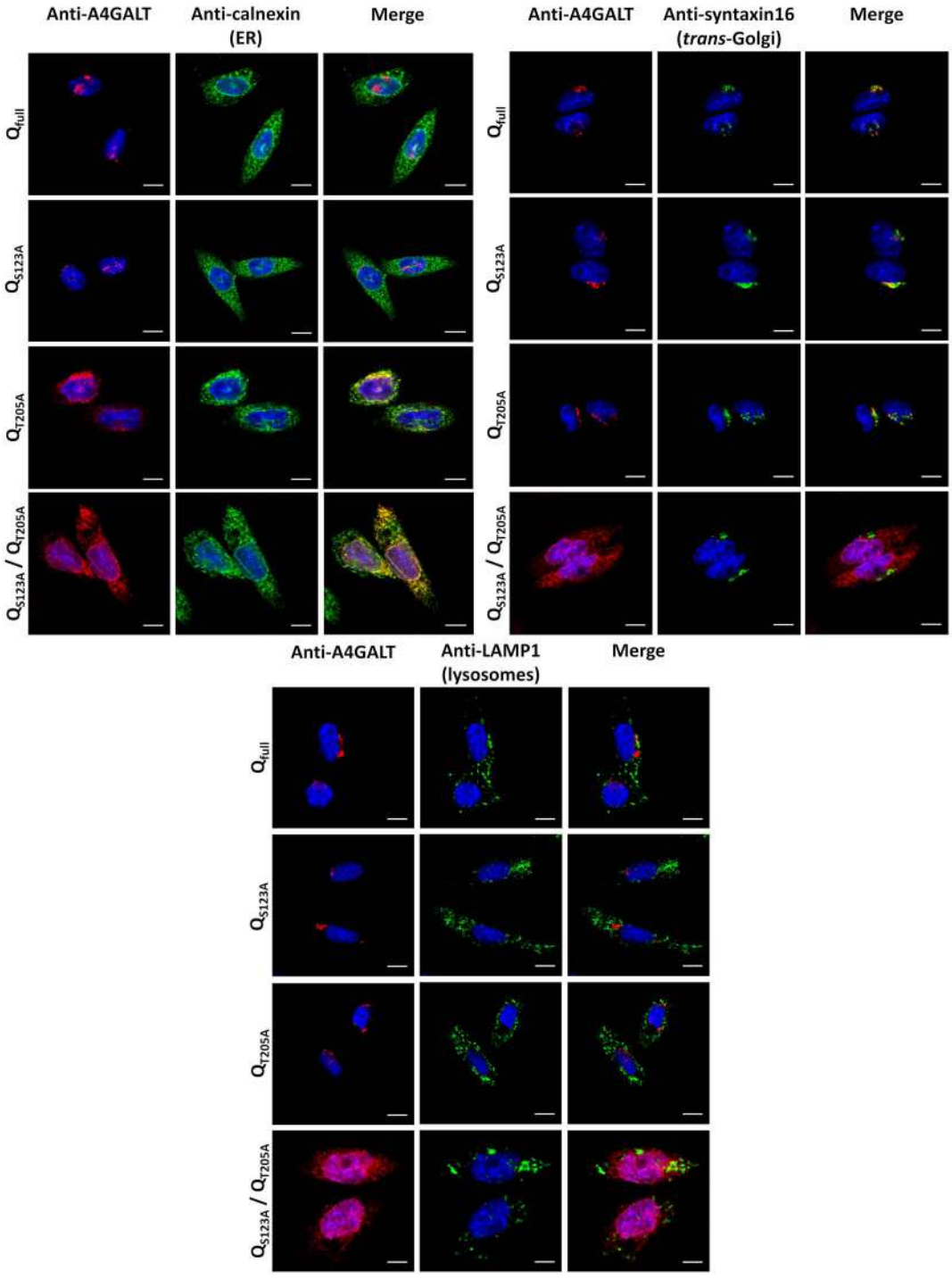

Fig. 9. Subcellular localization of human Gb3/CD77 synthase glycovariants in CHO-Lec2 cells using immunofluorescence. The glycovariants were visulized using anti-A4GALT monoclonal antibody (clone 5C7) (red). The cellular organelles, such as Golgi apparatus, endoplasmic reticulum and lysosomes were immunostained by specific antibodies recognizing organellum-specific markers (green). Cell nuclei were counterstained with DAPI (blue). Q_{full}, fully N-glycosylated Gb3/CD77 synthase; Q_{S123A}, Gb3/CD77 synthase with p.S123A substitution; Q_{T205A}, Gb3/CD77 synthase with p.T205A substitution; Q_{S123A}/Q_{T205A}, Gb3/CD77 synthase with p.S123A/p.T205A substitutions. Scale bar - 10 μm for fully N-glycosylated and single-mutant glycovariants. Scale bar—5 μm for double-mutant glycovariants.

The double-mutant glycovariants (Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A}) showed only residual activity. The loss of activity was probably caused by altered subcellular localization because both enzymes were found only in the ER. Also, we did not detect any enzymatic activity of these glycovariants in cell lysates, which may have been caused by a decrease in solubility; they were not detected

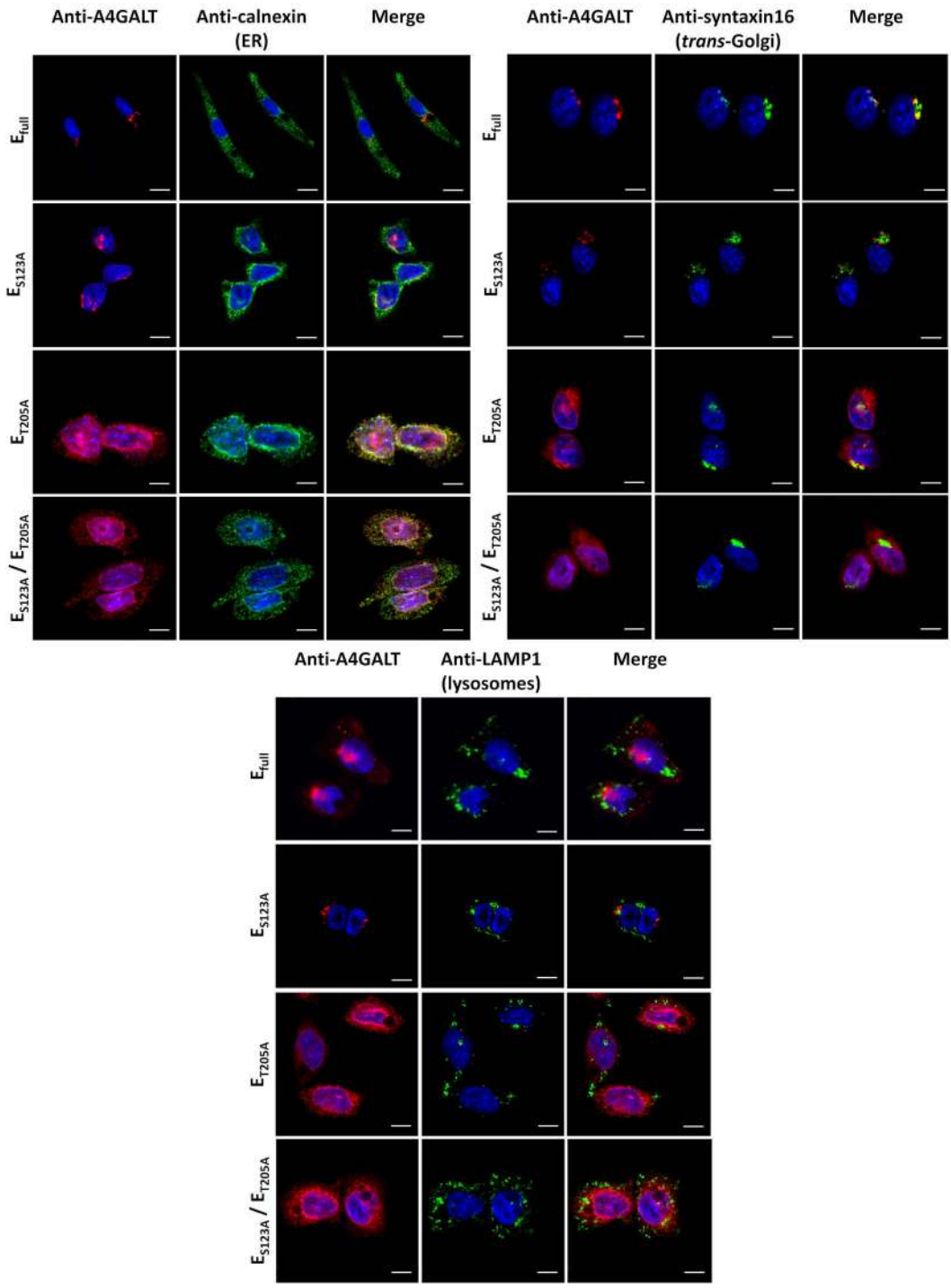

Fig. 10. Subcellular localization of mutein glycovariants in CHO-Lec2 cells using immunofluorescence. The glycovariants were visualized using anti-A4GALT monoclonal antibody (clone 5C7) (red). The cellular organelles, such as Golgi apparatus, endoplasmic reticulum and lysosomes were immunostained by specific antibodies recognizing organellum-specific markers (green). Cell nuclei were counterstained with DAPI (blue). E_{full}, fully N-glycosylated mutein Gb3/CD77 synthase; E_{S123A}, mutein Gb3/CD77 synthase with p.S123A substitution; E_{T205A}, mutein Gb3/CD77 synthase with p.T205A substitution; E_{S123A}/E_{T205A}, mutein Gb3/CD77 synthase with p.S123A/p.T205A substitutions. Scale bar - 10 μm for fully N-glycosylated and single-mutant glycovariants. Scale bar - 5 μm for double-mutant glycovariants.

in the culture medium either (data not shown). Overall, these results show that N-glycosylation of human Gb3/CD77 synthase affects its trafficking, solubility and secretion, but the two N-glycans play vastly different roles.

Several studies showed that removal of N-glycans from GTs may cause a decrease in activity (human α1,3/4-fucosyltransferase-III (Christensen et al. 2000)) and/or its complete loss (murine β1,3-galactosyltransferase-IV (Martina et al. 2000)). The molecular

background of activity change may be related to (1) enzyme misfolding, like for human α2,3-sialyltransferase-II (Ruggiero et al. 2015), (2) altered subcellular localization of the enzyme, *e.g.* human β1,4-galactosyltransferase-IV (Shauchuk et al. 2020), (3) changed kinetic parameters, such as rat β1,4-*N*-acetylglucosaminyltransferase-III (Nagai et al. 1997), (4) a decrease in the enzyme solubility/secretion, such as human β1,3-*N*-acetylglucosaminyltransferase-II (Kato et al. 2005), (5) enzyme aggregation, such as rat α2,6-sialyltransferase-I (Chen and Colley 2000). Generally, it may be assumed that enzymes that fail to exit the ER are degraded, so they cannot reveal any activity, as was shown for plant β1,2-xylotransferase from *Arabidopsis thaliana* (Pagny et al. 2003). It should be noted, however, that usually none of these mechanisms contributes to the decrease in enzyme activity independently because many of them intertwine, *e.g.* incorrect localization may be caused by misfolding. In glycoproteins carrying multiple N-glycans, individual chains may vary in importance. In the case of human Gb3/CD77 synthase, its two N-glycans seem to have opposing effects: the N-glycan at position N_{121} seems to be dispensable (with some data, intriguingly, suggesting that its absence may enhance activity and secretion), while the N-glycan at position N_{203} seems to be prerequisite for the enzyme activity, subcellular trafficking and localization. Additionally, N-glycans may impact protein folding and oligomerization, so eliminating N-glycans may change GT activity by altering its ability to oligomerize. Oligomerization may influence subcellular localization in the ER and/or Golgi, and so, indirectly, the activity of a GT (Nilsson et al. 1994; Harrus et al. 2018). Previously, we showed that the Q enzyme of human Gb3/CD77 synthase more readily forms homodimers than the E mutein (Kaczmarek et al. 2013). It is possible that eliminating N-glycans affects the enzyme's capability to oligomerize, resulting in changes in activity.

Since Galα1→4Galβ structures on glycosphingolipids and glycoproteins are recognized by Shiga toxins, we examined the presence of glycoprotein products of Gb3/CD77 synthase glycovariants upon their expression in CHO-Lec2 as well as the sensitivity of CHO-Lec2 cells expressing these glycovariants to Shiga toxins. Strikingly, we found that all kinds of the glycovariants were able to synthesize enough receptors to mediate cytotoxic activity of Shiga toxins, even the double-mutant enzymes, which otherwise revealed only residual activity. Notably, HPTLC orcinol staining showed that all CHO-Lec2 clones produced similar amounts of Gb3. This is not entirely unexpected, because HPTLC assays can readily detect and overestimate even small amounts of GSLs. Nevertheless, the amounts of Gb3 produced by double-mutant glycovariants are sufficient to trigger the cytotoxic effects.

In summary, our study shows that human Gb3/CD77 synthase carries two N-glycans, which dramatically differ in importance. The N-glycan at position N_{121} appears to play little to no role in the activity, with some data suggesting that it muzzles the enzyme and limits its secretion. In contrast, the N-glycan at position N_{203} seems to be necessary for activity and correct localization in the Golgi. The glycanless (i.e. double-mutant) variants were stuck in the ER and could not be efficiently secreted.

The dual role of N-glycosylation in the activity of human Gb3/CD77 synthase is intriguing. Attachment of glycans facilitates and is often necessary for the folding of nascent proteins, so a loss of function is an expected corollary of disrupted glycosylation. In contrast, inconsequence or favorability of underglycosylation is counterintuitive. In the case of Gb3/CD77 synthase, the unexpected role of N_{121}-linked glycan may have several interesting implications. It may represent a novel regulatory mechanism of preventing potentially detrimental effects of hyperactivity. Hyperactive enzymes may cause serious disorders. One example is the human serine protease coagulation factor IX (FIX), whose hyperfunctional variants are ∼5–8-fold more active than the normal FIX and cause hereditary thrombophilias (Simioni et al. 2009; Wu et al. 2021). Alternatively, the N_{121}-linked glycan may toggle substrate preferences of Gb3/CD77 synthase between glycosphingolipids and glycoproteins. Notably, avian Gb3/CD77 synthases readily use glycoproteins as acceptors, in contrast to the human enzymes, which use mainly glycosphingolipids (Bereznicka et al. 2019). N-glycosylation of avian Gb3/CD77 synthases has not been studied and perhaps is the missing link in our understanding of this major interspecies difference. In either scenario, mechanistic aspects of the unusual role of N_{121} glycan will require further studies.

Materials and methods

Antibodies

Mouse monoclonal anti-A4GALT antibody (clone 5C7) was produced by immunization of C57Bl/6 J mice with four subcutaneous injections of the purified human Gb3/CD77 synthase with p.Q211E substitution expressed in insect cells (Kaczmarek et al. 2016) emulsified in incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO). Hybridoma secreting antibodies were produced by fusing immune splenocytes with Sp 2.0-Ag14 mouse myeloma using 50% PEG 1500 solution (Sigma-Aldrich, St. Louis, MO) and subcloned by limiting dilution according to standard methodology (Miazek et al. 1997).

Site-directed mutagenesis

The *A4GALT* gene (GenBank nucleotide sequence databases with accession number NG_007495.2, NCBI, https://www.ncbi.nlm.nih.gov/) encoding full-length Gb3/CD77 synthase and its mutein (with p.Q211E substitution) were used for site-directed mutagenesis, as described previously (Kaczmarek et al. 2016b). Briefly, two N-glycosylation sequons N_{121}-A-S and N_{203}-L-T were disrupted by introducing a codon for alanine in place of serine or threonine, respectively. In the first PCR, two fragments of *A4GALT* gene were created, each containing the overlapping site with an introduced mutation. In the second reaction, the PCR products were duplexed to generate new template DNA. During the overlap extension phase, each fused product was amplified using primers complementary to the pCAG vector (kindly provided by Dr. Peter W. Andrews, University of Sheffield, Sheffield, UK) (PCAG For and PCAG Rev). The resulting full-length gene fragments were digested with XhoI and NotI (Fermentas, Vilnius, Lithuania), cloned into appropriately digested pCAG vector and sequenced (Genomed, Warsaw, Poland) using primers PkSeqFor and PkSeqRev (listed in Table SIII). The plasmids were purified using maxi prep kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instruction. The PCR was performed in a MJ Mini gradient PCR thermal cycler (BioRad, Hercules, CA, USA). About, 20 µl of reaction mixture contained approximately 200 ng of the template DNA, 0.2 mM forward and reverse primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, HF polymerase buffer (1:5 dilution), 1 unit Phusion High-Fidelity DNA Polymerase (Fermentas, Vilnius, Lithuania). The DNA fragments were purified with a gel extraction kit (Gel-Out, A&A Biotechnology, Gdynia, Poland). The sequences of primers are shown in Table SIII, and the conditions of PCR are shown in Table SIV.

Cell culture and transfection

CHO-Lec2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown and maintained in a humidified incubator with 5% CO_2 at 37°C used DMEM/F12 medium (Thermo Fischer Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Gibco, Inc., Waltham, MA, USA) and Pen-Strep (Gibco, Inc., Waltham, MA, USA). Culture medium was changed every second or third day, and after reaching 85–90% confluence, the cells were subcultured by treatment with trypsin (0.25% trypsin, 137 mM NaCl, 4.3 mM $NaHCO_3$, 5.4 mM KCl, 5.6 mM glucose, 0.014 mM phenol red, 0.7 mM EDTA), harvested, centrifuged at 800 × g for 5 min, resuspended in fresh medium and seeded to new tissue culture plates. One day before transfection, cells were seeded at 2×10^5 cells per well in six-well plates, giving at day of transfection about 60% confluence. The medium was replaced with fresh DMEM/F12 (without FBS), and after 4 h the cells were transfected using 60 µg of polyethyleimine (PEI, Polysciences, Warrington, PA, USA). Plasmid DNA in an amount of 1.5 µg was diluted in buffer containing 0.15 M NaCl and 20 mM HEPES (pH 7.5) and then mixed with PEI. The transfection mixture was incubated for 20 min at room temperature and then added dropwise to each well. After 48 h medium was replaced with fresh DMEM/F12 with 10% FBS and gradient concentration (5, 10, 20 and 50 µg/ml) of puromycin (Sigma-Aldrich, St. Louis, MO). The medium with antibiotic was changed daily for 10 days and then every 2 days. Selection was carried out until the non-transfected control cells were dead, and then cells were sorted using FACS. The cells were harvested using trypsin, washed and suspended in PBS containing 0.5% FBS and 5 mM EDTA at a density of 10^6 cells/ml. After 1 h incubation at 4°C with anti-P1 (650 and P3NIL100) antibody (1:400 or 1:200, respectively), the cells were washed with PBS containing 0.5% FBS and 1 h incubated with FITC-conjugated goat anti-murine or goat anti-human $F(ab')_2$ antibodies, respectively. Before cell sorting the cells were filtered through a tubes with cell strainer (Falcon® Round-Bottom Tubes with Cell Strainer Cap, Thermo Fisher Scientific, Waltham, MA, USA) to remove cell aggregates. The analysis was carried out using FACS Aria I cell sorter with FACSDiva software (Becton Dickinson).

Western blotting and lectin blotting

The proteins were separated in the presence of sodium dodecyl sulfate (SDS, Roth, Karlsruhe, Germany) using 10% polyacrylamide gel, according to the Laemmli method (Laemmli 1970) and visualized with Coomassie brilliant blue (CBB R-250, Roth, Karlsruhe, Germany) or transferred to nitrocellulose membrane (Sigma-Aldrich, St. Louis, MO). The PageRuler Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA) was used as a protein standard. The proteins fractionated by SDS-PAGE were transferred to the nitrocellulose membrane (Roth) according to the method of Towbin et al. (Towbin et al. 1979) and detected with mouse anti-A4GALT monoclonal antibody (hybridoma supernatant diluted 1:10, clone 5C7) or mouse anti-c-myc (hybridoma supernatant diluted 1:10, clone 9E10) or with biotinylated lectin *Canavalia ensiformis* agglutinin (ConA) (Vector Laboratories, France) in 1 µg/ml in TTBS (0.05% Tween-20/TBS pH 7.5) with 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ (Cavada BS et al. 2018). For lectin blotting, the nitrocellulose membrane, before blocking in 5% bovine serum albumin (BSA), was desialylated by treatment with 0.025 M sulfuric acid for 1 h at 80°C.

PNGase F digestion

Digestion of CHO-Lec2 protein lysates by PNGase F was performed as described (Maszczak-Seneczko et al. 2011) in denaturing conditions. Briefly, 1 µl of 10% SDS and 0.7 µl of 1 M DTT was added to 50 µg of transfected or non-transfected CHO-Lec2 cell lysates and the samples were incubated in 95°C for 5 min. The deglycosylation reaction was carried out with 500 units of PNGase F for 3 h at 37°C in a final volume of 20 µl. The reaction was stopped with SDS sample buffer and the products were analyzed by immunoblotting. PNGase F digestion in native conditions was carried out without denaturing reagents and skipped incubation at 95°C. Then, the products were used for enzyme activity examination with oligosaccharide-polyacrylamide (PAA) conjugates (Kaczmarek et al. 2016a).

Enzyme activity evaluation

In order to evaluate enzyme activity in cell lysates, CHO-Lec2 cells transfected with vectors encoding Gb3/CD77 synthase and its mutein with substituted N-glycosylation sites were harvested and lysed according to (Cheng et al. 2016). The buffer exchange (from Tris–HCl pH 7.4 to 50 mM sodium cacodylate pH 7.3) was carried out using Amicon Pro 10 kDa cut-off membranes. Activity of de-N-glycosylated recombinant soluble fragment (without transmembrane domain) of human mutein (obtained according to (Kaczmarek et al. 2016a) was performed under non-denaturing conditions using 500 units of PNGase F for 18 h at 37°C in 500 mM ammonium bicarbonate buffer, pH 7.8. The enzymatic activity was evaluated by ELISA with oligosaccharide-polyacrylamide (PAA) conjugates (Kaczmarek et al. 2016a). UDP-Gal (Sigma-Aldrich, St. Louis, MO) was used as a donor and three different conjugates were used as acceptors: Galβ1→4Glc-PAA (Lac-PAA, the precursor of Gb3), Galβ1→4GlcNAcβ1→3Galβ1→4Glc-PAA (nLc4-PAA, the precursor of P1), GalNAcβ1→3Galα1→4Galβ1→4Glc-PAA (Gb4-PAA, the precursor of NOR1) (Bovin 1998; Tuzikov et al. 2021). ELISA microtiter plates (Nunc, MaxiSorp, Roeskilde, Denmark) were coated overnight in 4°C with conjugates (2 µg/well) in phosphorane buffer (50 mM, pH 7.4). Enzyme samples (100 µg/well) in cacodylate buffer containing Mn^{2+} and the donor substrate (50 mM sodium cacodylate pH 7.4, 14 mM $MnCl_2$, 200 µM UDP-Gal, pH 6.3) were loaded in triplicates. The reactions were run overnight (16–18 h) in 37°C. The plates were then washed twice with distilled water and thrice with PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% Tween 20) and blocked with 5% BSA in PBST. Next, dilutions of antibodies recognizing the reaction products (1:50, 1:100 and 1:100 for anti-P1 P3NIL100, anti-P1 650 and anti-NOR nor118, respectively) were added and incubated for 90 min in room temperature, followed by sequential 1-hour incubation with biotinylated anti-mouse or anti-human IgM antibody (each diluted 1:1000) and ExtrAvidin-alkaline phosphatase conjugate (diluted 1:10000). Wash steps were carried out using PBST/1% BSA. Finally, color reactions were developed with *p*-nitrophenyl phosphate (1 mg/ml in Tris–HCl with 1 mM $MgCl_2$) (Sigma-Aldrich, St. Louis, MO). Plates were read using 2300 EnSpire Multilabel Reader (PerkinElmer, Waltham, MA) at 405 nm at several time points within 1 hour. Data were analyzed using Microsoft Office Excel (Microsoft Corp, Redmond, WA). Negative controls were set up by adding incomplete reaction mixtures (lacking UDP-Gal or enzyme) to coated wells; by adding complete reaction mixtures to uncoated wells; or by omitting primary or secondary antibodies.

Flow cytometry

The cells were incubated with 100 µl appropriately diluted primary antibodies (anti-P1 P3NIL100 1:200, anti-P1 650 1:400, anti-NOR nor118 1:20) for 60 min on ice. Then the cells were washed (all washes and dilutions were done with PBS) and incubated with 100 µl (diluted 1:50) FITC-labeled anti-mouse IgM antibody for 40 min on

ice in the dark. The cells were washed and approximately 5×10^5 cells were suspended in 750 µl of cold PBS and analyzed by flow cytometry using FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). The number of events analyzed was 10,000/gated cell population. The analysis of the results was carried out using Flowing software (Perttu Terho, University of Turku, Turku, Finland) (Sahraneshin Samani et al. 2014). For quantification of cell surface antigens, we used Quantum™ FITC-5 MESF (Bio-Rad, Hercules, CA, USA), which carry defined quantities of FITC, and thus allow plotting calibration curves (mean fluorescence intensity versus Molecules of Equivalent Soluble Fluorochrome units). The cells were then analyzed by flow cytometry and the antibody binding capacity (ABC, the number of antibody molecules bound per cell) was calculated by interpolation from the calibration curve as described in the manufacturer's protocol and based on the fluorophore-to-protein molar ratios of the FITC-antibody conjugates. Negative control results (secondary antibody only) were subtracted from the sample results to obtain specific antibody binding capacities.

Extraction and purification of glycosphingolipids from CHO-Lec2

The isolation and fractionation of glycosphingolipids and the orcinol staining were performed as described previously (Duk et al. 2001). Cellular lipids were extracted with chloroform/methanol method from 10^7 transfected or non-transfected CHO-Lec2 cells. The neutral glycosphingolipids were separated from the phospholipids and gangliosides, purified in peracetylated form, then de-O-acetylated and desalted. Glycosphingolipid samples were solubilized in chloroform/methanol (2:1, v/v), applied to HPTLC plates (Kieselgel 60, Merck, Darmstadt, Germany), and developed with chloroform/methanol/water (55:45:9, v/v/v). The dried plates were immersed in 0.05% polyisobutylmethacrylate (Sigma-Aldrich, St. Louis, MO) in hexane for 1 min, dried, sprayed with TBS (0.05 M Tris buffer, 0.15 M NaCl (pH 7.4) and blocked in 5% HSA. For antibody assays, the plates were successively overlaid with (1) primary antibody diluted in TBS/1% BSA (TBS-BSA) for 1–1.5 h (anti-P1 650 1:100, anti-P1 P3NIL100 1:50, anti-NOR nor118 1:50); (2) biotinylated goat anti-mouse Ig antibody (Dako, Glostrup, Denmark), diluted 1:5000 with TBS-BSA; (3) ExtrAvidin-alkaline phosphatase conjugate (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:1000 with TBS/BSA/0.2% Tween 20 for 1 h; and (4) the substrate solution (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, Sigma-Aldrich, St. Louis, MO). Other details were as described previously (Duk et al. 2001; Duk et al. 2007). Each HPTLC experiment was repeated three times (without significant differences between consecutive repetitions), and GSLs samples were solubilized in the same volumes of chloroform/methanol (2:1, v/v).

HPTLC orcinol staining

Orcinol staining was performed using standard procedures as previously described (Kuśnierz-Alejska et al. 1999; Duk et al. 2001). Briefly, dried HPTLC plates were sprayed with a solution of orcinol (0.2% w/v) in 3 M aqueous sulfuric acid and incubated in an oven at 110°C for 10 min.

Quantitative analysis of transcripts level

Total RNA from transfected or non-transfected CHO-Lec2 cells was prepared using Universal RNA Purification Kit (Eurx, Gdansk, Poland) and the complementary DNAs (cDNAs) were synthesized using SuperScript III First-Strand Synthesis kit (Life Technologies, Carlsbad, CA, USA) with oligo(dT) primers. Quantitative polymerase chain reaction (qPCR) was performed on 30 ng of cDNA using the 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instruction. The *A4GALT* transcripts were detected with Custom TaqMan Gene Expression Assay. The ORF sequences were chosen in assay design to enable detection of transcripts originating from plasmids. A predesigned TaqMan assay targeting exon 2–3 boundary (Hs00213726_m1; Life Technologies) was also used to ensure equal amount of the endogenous *A4GALT* transcript in transfected and non-transfected cells. The transcript quantities were normalized to hamster *GAPDH* endogenous control (sequence in Table SVB). All samples were run in triplicates. Data were analyzed using Sequence Detection software Version 1.3.1 (Life Technologies, Carlsbad, CA, USA). Target nucleotide sequences are shown in Table SV, while qPCR conditions are in Table SVI.

MALDI-TOF Mass Spectrometry of CHO-Lec2 GSLs

MALDI-TOF mass spectrometry was carried out on a MALDI TOF/TOF ultrafleXtreme™ instrument (BrukerDaltonics, Bremen, Germany). Samples were dissolved in chloroform/methanol (2:1, v/v). Norharmane (9H-Pyrido[3,4-b]indole, Sigma-Aldrich, St. Louis, MO) was used as a matrix (10 mg/ml, chloroform/methanol, 2:1, v/v). Spectra were scanned in the range of *m/z* 700–1600 in the reflectron-positive mode. External calibration was applied using the Peptide Calibration Standard II (BrukerDaltonics, Bremen, Germany).

Cytotoxicity assay

2×10^4 CHO-Lec2 cells were seeded in 96-well plates (Wuxi NEST Biotechnology Co., Ltd, China) in complete DMEM/F12. After 24 h the medium was replaced by 100 µl/well of serum-free DMEM/F12 containing 0.1, 0.5 and 1 ng/ml of Stx1 or Stx2 holotoxins (all concentrations were run in triplicates). After 20 h of toxin treatment, 20 µl/well of MTS tetrazolium compound (CellTiter 96® AQueous One Solution Assay, Promega, Madison, WI) was added. Plates were incubated in humidified, 5% CO_2 atmosphere for 2.5 h, then absorbance at 490 nm was recorded on ELISA plate reader. Background absorbance registered at zero cells/well was subtracted from the data and the absorbance of wells incubated in the medium without Stx was taken as 100% of cell viability. Each experiment was performed at least three times.

Immunolocalization of Gb3/CD77 synthase

Immunogold. For ultrastructural analysis in transmission electron microscopy (TEM) 5×10^6 transfected or non-transfected CHO-Lec2 cells were fixed in cooled 4% formaldehyde solution (FA), diluted in PBS for 20 min at room temperature (RT) (Thermo Fisher Scientific, Waltham, MA, USA). After fixation, the cells were scraped and the cell suspensions were centrifuged three times at 2100 × g for 8 min followed by rinsing the samples with PBS and distilled water. After adding 1 drop of bovine thrombin (Biomed, Lublin, Poland) to 2 drops of fibrinogen (1 mg/ml; Sigma-Aldrich, St. Louis, MO; Merck KGaA) the cells were entrapped within the fibrin clots. Next, the cell clots were post-fixed for 7 min in 0.25% (w/v) osmium tetroxide OsO_4 diluted in PBS (Serva Electrophoresis, Heidelberg, Germany). Subsequently, the samples were rinsed with PBS three times for 5 min.

The cell clots were dehydrated in the increasing concentration of ethanol, EtOH 50, 70, 96, 99.8% (Stanlab, Lublin, Poland) for 10 min at RT. Afterwards, the samples were incubated for 3 h

at RT in the mixture of EtOH and LR White resin (Polysciences, Inc., Warrington, PA, USA) in the following proportions: 2:1, 1:1 and 1:2, respectively. Finally, the samples were embedded in pure resin. Polymerization of the resin blocks was carried out at 55°C for 48 h.

LR White blocks were cut into semithin, 600-nm-thick sections with the Histo Diamond Knife (Diatome, Nidau, Switzerland). The semithin sections were stained with a dye solution (Serva, Alchem, Torun, Poland) and closed with a Euparal mounting agent (Carl Roth, Mannheim, Germany), followed by examination on a light microscope in order to remove excessive resin and select a group of no fewer than 30 cells for TEM documentation. Finally, ultrathin, 70-nm-thick sections were cut with the Ultra 45° Diamond Knife (Diatome), arranged with a loop, and collected onto the dull side of nickel grids (200 mesh, Ted Pella, Redding, CA, USA). Resin-embedded materials were prepared using an ultramicrotome Power Tome XL (RMC, Tucson, USA).

All incubation steps were performed on top of drops of appropriate reagents at RT. First, the ultrathin sections were incubated in 0.02 M glycine (Biotechnology grade, BioShop Canada Inc., Burlington, Canada), dissolved in PBS (one time for 10 min) to quench free aldehyde groups, followed by gentle rinsing with PBS. Then, the cells were permeabilized two times for 5 min with 0.1% Triton X-100 (Reagent grade, BioShop), followed by washing three times for 5 min with PBS. In order to block non-specific antigen-binding sites, the grids were transferred for 1 h to 1% bovine serum albumin PBS solution (Albumin fraction V, Carl Roth) and rinsed with PBS for 5 min. For immunogold reaction, the grids were incubated with mouse anti-A4GALT monoclonal antibody (hybridoma supernatant diluted 1:10, clone 5C7) for 1 h, followed by washing the sections in PBS three times for 5 min. Subsequently, secondary antibody conjugated with colloidal gold nanoAbcam, Cambridge, UK, preadsorbed) in 1% BSA in PBS (dilution 1:10) was applied for 1 h (dark chamber).

Next, the grids were rinsed with PBS and distilled water, six times for 5 min. Additionally, the specimens were post-fixed with 1% glutaraldehyde (Serva Electrophoresis, Heidelberg, Germany) diluted in PBS for 5 min, followed by rinsing with distilled water three times for 5 min. To improve contrast, the ultrathin sections were counterstained with uranyl acetate (10 min) and lead citrate trihydrate (5 min) (Serva), and then rinsed three times in distilled water. The sections were then examined using TEM JEM-1011 (Jeol, Tokyo, Japan) with the accelerating voltage of 80 kV. Digital micrographs were collected using TEM imaging platform iTEM1233 equipped with a Morada Camera (Olympus, Münster, Germany) at magnifications ranging from 4 to 50 K.

Immunofluorescence. Millicell EZ slides were used for culturing cells prior to staining. Cells were fixed on the second day of culture with 4% paraformaldehyde (PFA) in PBS added onto slides for 5 min. Afterwards, cells were washed three times for 5 min with PBS. To inhibit non-specific binding sites the slides were incubated 1 h with blocking solution (1% (w/v) BSA and 0.1% (w/v) saponin in PBS). After blocking, cells were incubated with primary antibodies (Table SI) for 3 h at 37°C. Washing (3 × 5 min) was carried out with the blocking solution. Subsequently, slides were incubated with secondary antibodies (Table SI) for 1 h at 37°C. Counterstain with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO) for 10 min at room temperature was performed for nuclei visualization. Finally, slides were mounted onto glass coverslips using fluorescence mounting medium (Dako). Slides were examined with a LEICA SP8 confocal microscope and pictures were analyzed using ImageJ software (NIH).

Data availability statement

Raw MS data of GSLs analyzed in the paper are available at the GlycoPOST, Announced ID GPST000166 (https://glycopost.glycosmos.org/).

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Abbreviations

GSL: glycosphingolipid; Gb3: globotriaosylceramide; RBC: red blood cell; Stx: Shiga toxin; STEC: Shiga toxin-producing Escherichia coli; Stx1B: Shiga toxin 1 B subunit; Stx2B: Shiga toxin 2 B subunit; ConA: Canavalia ensiformis agglutinin; ER: endoplasmic reticulum; HUS: hemolytic-uremic syndrome; PNGase F: peptide-N-glycosidase F; ABC: antigen binding capacity; nLc4: paragloboside; Gb4: globoside; HPTLC: high performance thin layer chromatography; qPCR: quantitative polymerase chain reaction; MALDI-TOF: matrix-assisted laser desorption/ionization-time of flight

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Supplementary data

Supplementary data are available at Glycobiology online.

Conflict of interest statement

The authors declare that they have no conflict of interest with the contents of this article.

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Supplementary data

Missing the sweet spot: one of the two N-glycans on human Gb3/CD77 synthase is expendable

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Contents

Table SI. Antibodies used in the study.

Antibody	Clonality	Dilution IF / WB / HPTLC / FACS	Host	Manufacturer
Anti-A4GALT	Monoclonal, clone 5C7	1:10 (WB)	Mouse	Hybridoma supernatant
Anti-c-myc	Monoclonal, clone 9E10	1:10 (WB)	Mouse	Hybridoma (purchased from ATCC) supernatant
Anti-6x-His	Monoclonal, clone HIS.H8	1:1000 (WB, HPTLC)	Mouse	Thermo Fischer Scientific
Anti-P1	Monoclonal, clone 650	1:100 (WB, HPTLC, FACS)	Mouse	Ce-Immundiagnostika
Anti-P1	Monoclonal, clone P3NIL100	1:100 (WB, HPTLC, FACS)	Human	Immucor Inc.
Anti-NOR	Monoclonal, clone nor118	1:20, 1:100 (WB, HPTLC, FACS)	Mouse	Hybridoma supernatant
Biotynylated anti-IgG/A/M (H/L)	Polyclonal	1:1000 (WB)	Goat	Bio-Rad Laboratories
Anti-mouse IgM-FITC	Polyclonal	1:100 (FACS)	Goat	Thermo Fisher Scientific
Anti-human IgM-FITC	Polyclonal	1:100 (FACS)	Goat	Thermo Fisher Scientific
Anti-mouse IgG-FITC	Monoclonal	1:100 (FACS)	Goat	Santa Cruz Biotechnology
Anti-calnexin	Polyclonal	1:100 (IF)	Rabbit	Abcam
Anti-syntaxin16	Monoclonal	1:100 (IF)	Rabbit	Abcam
Anti-LAMP1	Polyclonal	1:100 (IF)	Rabbit	Abcam
Anti-rabbit Alexa Fluor 568 (secondary)	Polyclonal	1:1000 (IF)	Goat	Thermo Fisher Scientific
Anti-mouse Alexa Fluor 488 (secondary)	Polyclonal	1:1000 (IF)	Goat	Thermo Fisher Scientific

Table SII. Specificity of anti-P1 (650 and P3NIL100) and anti-NOR (nor118) antibodies used in western blotting and flow cytometry. Cer, ceramide; R, core N-glycan structure; GSL, glycosphingolipid; GP, glycoprotein.

Glycan structure	Anti-P1		Anti-NOR (nor118)
	650	P3NIL100	
Gb3 (Galα1→4Galβ1→4Glc-Cer)	+	-	-
P1 on GSL (Galα1→4Galβ1→4GlcNAcβ1→3Galβ1→4Glc-Cer)	+	+	-
P1 on GP (Galα1→4Galβ1→4GlcNAcβ1→R)	+	+	-
NOR1 (Galα1→4GalNAcβ1→3Galα1→4Galβ1→4Glc-Cer) **NOR2** (Galα1→4GalNAcβ1→3Galα1→4GalNAcβ1→3Galα1→4Galβ1→4Glc-Cer)	-	-	+

Table SIII. Nucleotide sequences of primers used in site-directed mutagenesis and sequencing. Changed nucleotides in codons are shown in red.

Primers	Sequence [5' → 3']
PkSeqFor	TCGCACTCATGTGGAAG
PkSeqRev	AGTACATTTTCATGGCCT
Pkcon_S123A_sens	GC AAC GCC GCA CTG CCC CGG CAC
pCAGanty	ACA AAC GCA CAC CGG CCT TAT TCC
Pkcon_S123A_anty	GTG CCG GGG CAG TGC GGC GTT GC
pCAGsens	CGT GCT GGT TGT TGT GCT GTC TCA
Pkcon_T205A_sens	CTG CGG AAC CTG GCA AAC GTG CTG G
PCAGanty	ACA AAC GCA CAC CGG CCT TAT TCC
Pkcon_T205A_anty	C CAG CAC GTT TGC CAG GTT CCG CAG
pCAGsens	CGT GCT GGT TGT TGT GCT GTC TCA

Table SIV. PCR conditions used in site-directed mutagenesis.

Mutagenesis – first step				**Mutagenesis – second step**		
	Temp. [°C]	**Time [s]**	**Cycle**	**Temp. [°C]**	**Time [s]**	**Cycle**
Initial denaturation	**94**	**180**	**1**	**94**	**180**	**1**
Denaturation	**94**	**15**	**30**	**94**	**15**	**30**
Annealing	**62-72**	**20**	**30**	**70**	**20**	**30**
Extension	**72**	**10**	**30**	**72**	**20**	**30**
Final extension	**72**	**300**	**1**	**72**	**300**	**1**

Table SV. A) Target nucleotide sequences within *A4GALT* open reading frame used for design of Custom TaqMan Gene Expression Assay. **B)** Target nucleotide sequences within hamster *GAPDH* open reading frame (NM_001244854.2) and TaqMan probe nucleotide sequence used for design of Custom TaqMan Gene Expression Assay (endogenous control).

A)

Name of target sequence	Sequence [5' → 3']
A4gtf	CTGCACCCT
A4gtr	TTCTCAAGAAC

B)

Name of target sequence	Sequence [5' → 3']
Gapdhf	TGGAAAGCTTGTCATCAAC
Gapdhr	GAAGACGCCAGTAGATTCC

TaqMan probe	Sequence [5' → 3']
GAPDH	AGGCCATCACCATCTTCCAG

Table SVI. qPCR conditions used for quantitative analysis of *A4GALT* transcripts.

qPCR system	Reaction format	Reaction volume	Thermal cycling conditions			
			Parameter	Initial denaturations	PCR (40 cycles)	
					Denaturation	Annealing/Extension
			Temperature [°C]	95 °C	95 °C	60 °C
7500 Fast	96-well plate	20 µl	Time (mm:ss)	10:00	0:15	1:00

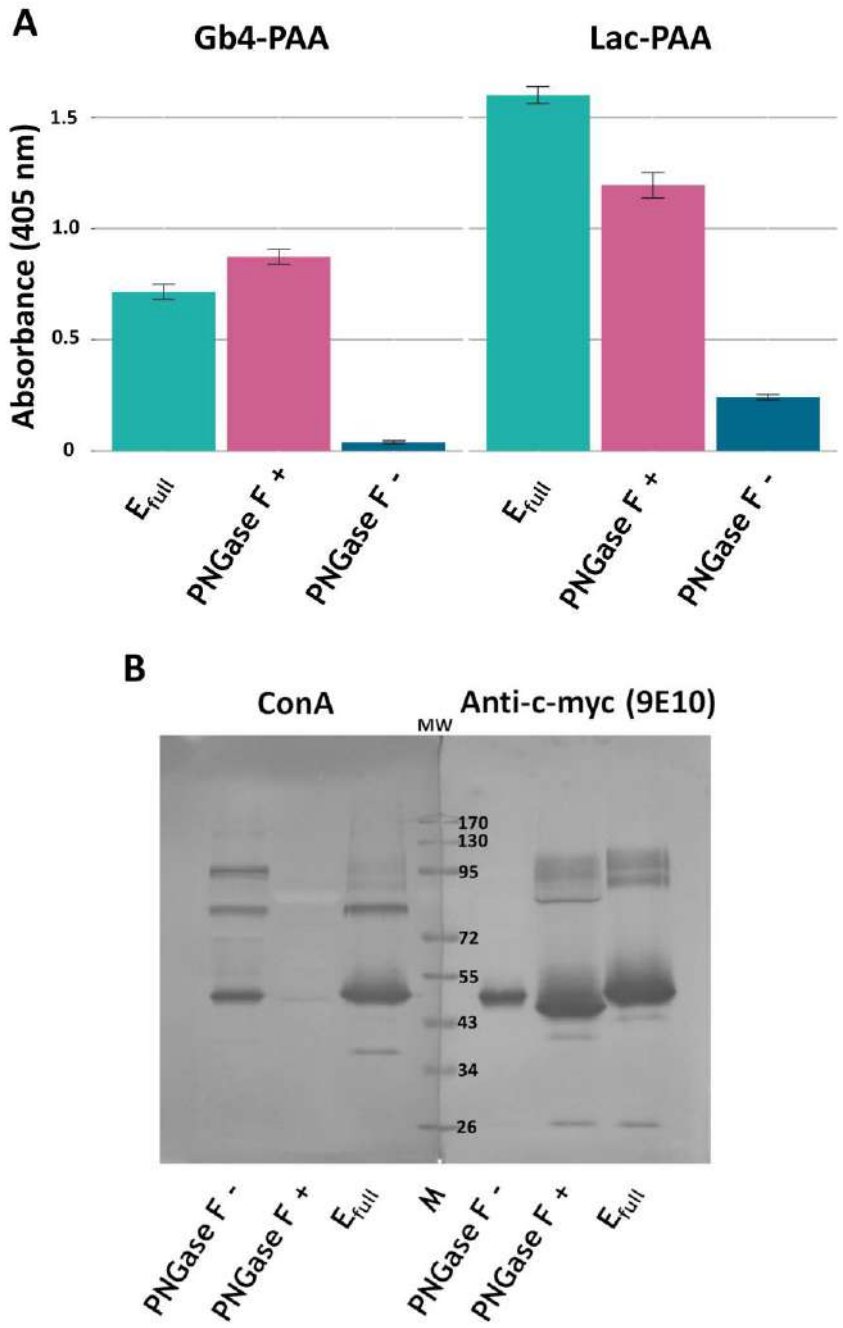

Fig. S1. **Activity of human recombinant mutein obtained in insect cells after de-N-glycosylation.** (**A**) *In vitro* activity of untreated human mutein (E_{full}), enzyme treated with PNGase F (PNGase F +) or incubated in deglycosylation buffer (PNGase F -). Enzymatic activity was evaluated using PAA-conjugates, serving as precursors of Gb3 antigen (Lac-PAA acceptor) and NOR (Gb4-PAA acceptor) [Szymczak K., Kaczmarek R. et al. 2016]. (**B**) Lectin blotting and western blotting of untreated human mutein (E_{full}), enzyme treated with PNGase F (PNGase F +) and treated only with deglycosylation buffer (PNGase F -). The blots were overlaided with ConA lectin, which is specific for core oligosaccharide of N-glycans with α-linked mannose [Cavada B.S., Pinto-Junior V.R. et al. 2018] and with anti-c-myc antibody (clone 9E10), which recognizes c-myc tag at C-terminus of soluble human Gb3/CD77 synthase [Kaczmarek R., Duk M. et al. 2016].

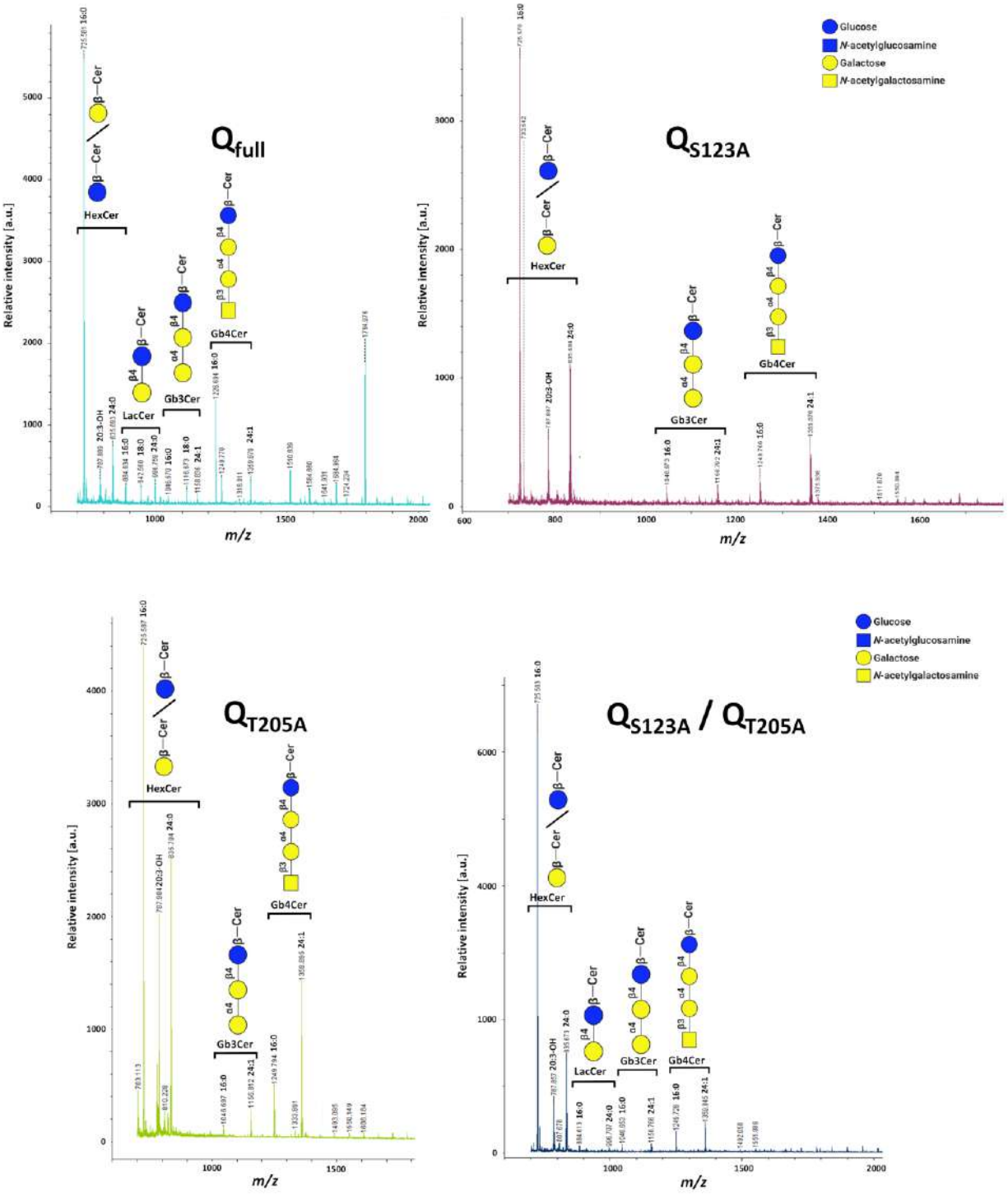

Fig. S2. **Reflectron-positive mode MALDI-TOF mass spectra of glycosphingolipids isolated from CHO-Lec2 cells expressing the Gb3/CD77 synthase glycovariants.** The GSLs samples from CHO-Lec2 cells transfected with vectors encoding fully N-glycosylated Q_{full} enzyme as well as Q_{S123A}, Q_{T205A} and Q_{S123A}/Q_{T205A} glycovariants.

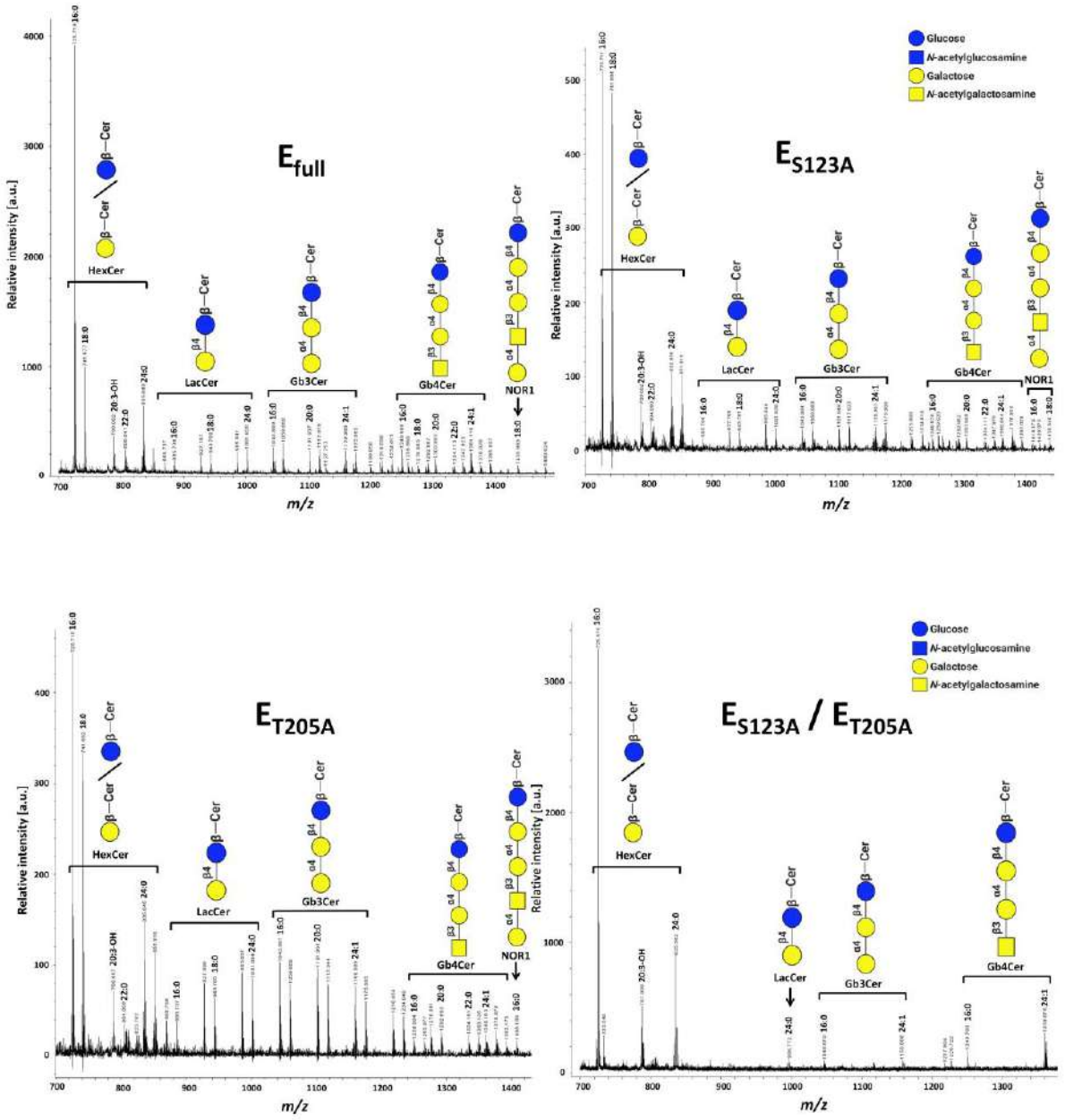

Fig. S3. **Reflectron-positive mode MALDI-TOF mass spectra of glycosphingolipids isolated from CHO-Lec2 cells expressing glycovariants of mutein.** The GSLs samples from CHO-Lec2 cells transfected with vectors encoding fully N-glycosylated E_{full} enzyme as well as E_{S123A}, E_{T205A} and E_{S123A}/E_{T205A} glycovariants.

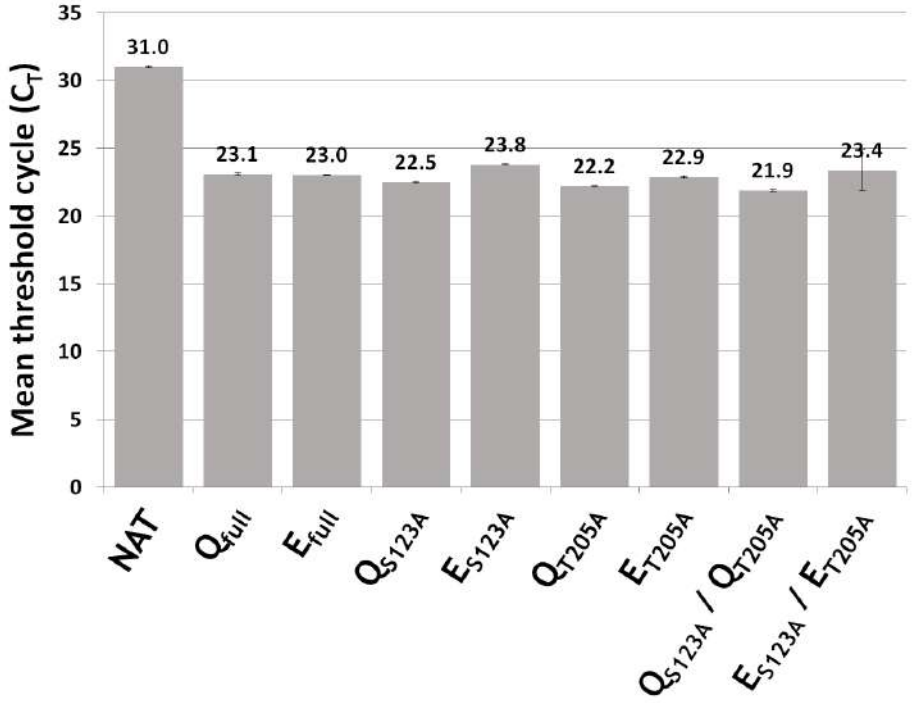

Fig. S4. **Comparisons of mean threshold cycle (C_T) values between Gb3/CD77 synthase and its mutein glycovariants.** Q_{full}, fully N-glycosylated of Gb3/CD77 synthase; E_{full}, fully N-glycosylated mutein Gb3/CD77 synthase; Q_{S123A}, Gb3/CD77 synthase with p.S123A substitution; E_{S123A}, mutein Gb3/CD77 synthase with p.S123A substitution; Q_{T205A}, Gb3/CD77 synthase with p.T205A substitution; E_{T205A}, mutein Gb3/CD77 synthase with p.T205A substitution; Q_{S123A}/Q_{T205A}, Gb3/CD77 synthase with p.S123A/p.T205A substitutions; E_{S123A}/E_{T205A}, mutein Gb3/CD77 synthase with p.S123A/p.T205A substitutions.

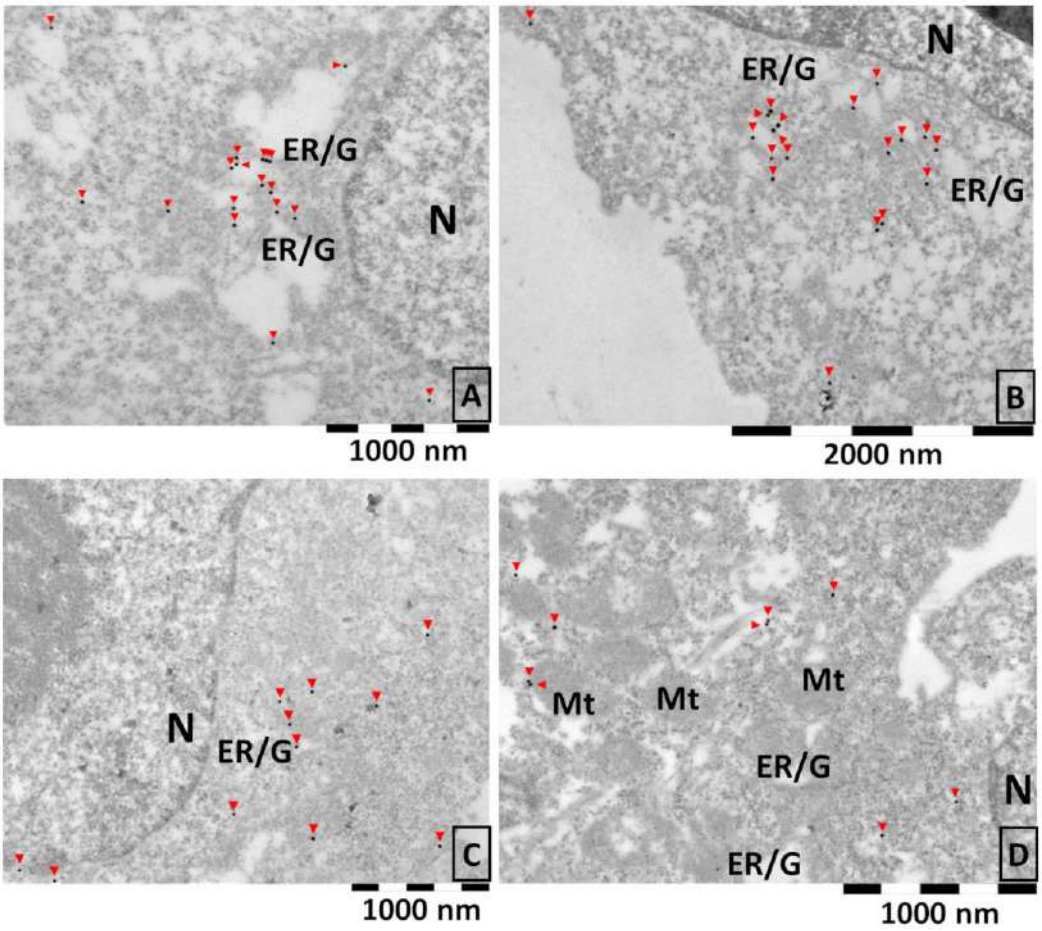

Fig. S5. **Subcellular localization of human Gb3/CD77 synthase glycovariants in CHO-Lec2 cells using immunogold reaction**. (**A**) Fully N-glycosylated Gb3/CD77 synthase. (**B**) Glycovariant Q_{S123A} with p.S123A substitution. (**C**) Glycovariant Q_{T205A} with p.T205A substitution. (**D**) Glycovariant Q_{S123A}/Q_{T205A} with p.S123A/p.T205A substitutions. Red arrows indicated gold nanoparticles which correpond Gb3/CD77 synthase localization. N, nucleus; ER/G, endoplasmic reticulum or Golgi apparatus; Mt, mitochondrion.

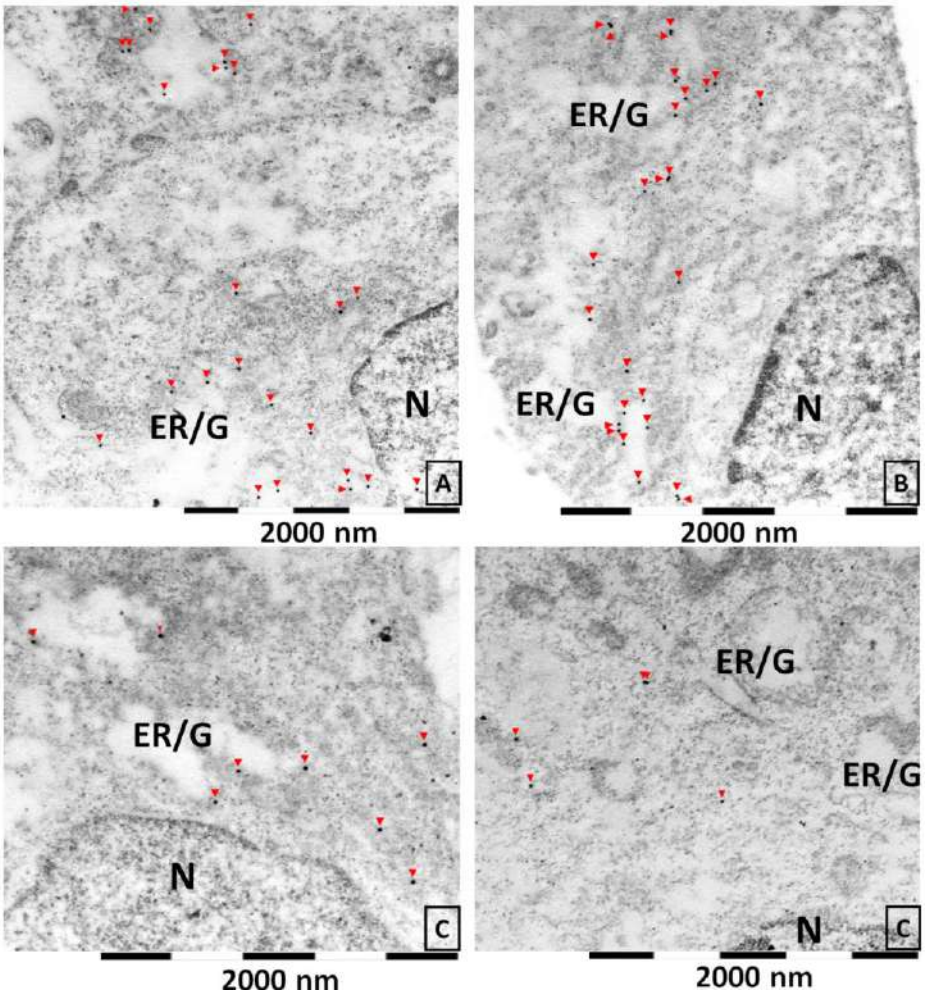

Fig. S6. **Subcellular localization of mutein glycovariants in CHO-Lec2 cells using immunogold reaction**. (**A**) Fully N-glycosylated Gb3/CD77 synthase. (**B**) Glycovariant E_{S123A} with p.S123A substitution. (**C**) Glycovariant E_{T205A} with p.T205A substitution. (**D**) Glycovariant E_{S123A}/E_{T205A} with p.S123A/p.T205A substitutions. Red arrows indicated gold nanoparticles which correpond mutein localization. N, nucleus; ER/G, endoplasmic reticulum or Golgi apparatus.

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One of the two N-glycans on the human Gb3/CD77 synthase is essential for its activity and allosterically regulates its function

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ABSTRACT

N-glycosylation is a posttranslational modification that influences many protein properties, such as bioactivity, folding or solubility. The same principles apply to key enzymes in glycosylation pathways, including glycosyltransferases, that also undergoing N-glycosylation, changes in which may affect their activity. Human Gb3/CD77 synthase (encoded by *A4GALT*) is a Golgi-resident glycosyltransferase, which catalyzes the synthesis of Galα1→4Gal disaccharide on glycosphingolipid- and glycoprotein-derived acceptors, creating Gb3 or P1 antigens and P1 glycotopes (Galα1→4Galβ1→4GlcNAc-R), respectively. The molecules that contain Galα1→4Gal serve as receptors for pathogens and Shiga toxins, which are the major virulence factors of Shiga toxin-producing *Escherichia coli* (STEC). Human Gb3/CD77 synthase contains two N-glycosylation sites at positions N_{121} and N_{203}. Using the recombinant soluble glycovariants of human Gb3/CD77 synthase with mutated N-glycosylation sequons expressed in HEK293E cells, we show that the glycovariants devoid of N-glycan at position N_{203} or simultaneously at N_{121} and N_{203} sites reveal no enzymatic activity. In contrast, the N-glycan at position N_{121} plays a negligible role, whereas the presence of both N-glycans is required for efficient secretion of the enzyme. Moreover, utilizing specific glycosidases, we have found that the fully N-glycosylated enzyme contains one complex and one hybrid/oligomannose N-glycan, while single mutants contain only the complex type. Finally, *in silico* analysis using the AlphaFold enzyme model showed that N-glycan attached to N_{203} sequon is located in a protein motif near the active site and may allosterically influence the activity. All these findings highlight the prerequisite role of N-glycosylation in human Gb3/CD77 synthase activity (N_{203} sequon) and solubility (both N_{121} and N_{203}), with a particularly prominent role of N-glycan at position N_{203} in the regulation of enzyme activity.

1. Introduction

Human Gb3/CD77 synthase (α1,4-galactosyltransferase, EC 2.4.1.228) encoded by the *A4GALT* gene, is a Golgi-resident type II transmembrane glycosyltransferase (GT) with C-terminal globular catalytic domain facing the Golgi lumen and N-terminal cytoplasmic domain. The enzyme belongs to the CAZy glycosyltransferase family 32 (Carbohydrate Active Enzymes database, CAZy, http://www.cazy.org/) and adopts the GT-A fold. Structurally, the enzyme contains a cytoplasmic domain (spanning 1–22 amino acid residues), a transmembrane domain (23–43 amino acid residues), which enables localization in the Golgi membrane, and a luminal domain (44–353 amino acid residues), which contains the catalytic site (according to UniProt Q9NPC4, https://www.uniprot.org/.) [1–4]. Similarly to other GT-A enzymes, human Gb3/CD77 synthase is a retaining glycosyltransferase and contains the DXD motif (D_{192}TD according to UniProt Q9NPC4), which interacts with the divalent metal ion (usually Mn^{2+}) [5,6]. In humans, the *A4GALT* gene occurs as a high-frequency variant that encodes an enzyme with Q at position 211 (p.Q211), as well as a rare gene variant

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0006-291X/

(identified only in two families worldwide), encoding a protein with p.Q211E substitution (rs397514502), hereinafter called E [1]. Human Gb3/CD77 synthase catalyzes the transfer of galactose from UDP-galactose to lactosylceramide (LacCer), producing globotriaosylceramide (Gb3, P^k, Galα1→4Galβ1→4Glc-Cer), and thus initiating the globo series glycosphingolipid (GSL) pathway. In addition, it generates the P1 antigen (nLc5, Galα1→4Galβ1→4GlcNAcβ1→3Galβ1→4GlcCer) from paragloboside (nLc4), which belongs to the neolacto series. The presence of c.631C>G mutation (rs397514502) in *A4GALT* (p.Q211E substitution) steps up the enzyme's promiscuity by broadening its specificity, rendering it able to also synthesize NOR1 and NOR2 antigens, terminating with a Galα1→4GalNAc disaccharide [1,2].

All antigens synthesized by human Gb3/CD77 synthase belong to the human P1PK histo-blood group system (International Society of Blood Transfusion, No. 003) [7]. For many years, there was a general agreement that human Gb3/CD77 synthase is GSL-specific, but recently it was found that the enzyme can also cap complex-type N-glycans, synthesizing Galα1→4Galβ1→4GlcNAc (the P1 glycotope)-terminated oligosaccharide chains on glycoproteins [8,9].

Gb3 is a major neutral GSL of human RBCs, kidney, heart, lung, smooth muscle, and epithelium of the gastrointestinal tract; it participates in the binding and internalization of Shiga toxins (Stxs) and pathogens [7–10]. Shiga are AB_5 toxins produced by *Shigella dysenteriae* serotype 1 and Shiga toxin-producing *Escherichia coli* (STEC). In the USA, STEC cause over 265 000 infections annually. The symptoms range from mild diarrhea to hemorrhagic colitis, which can often progress into hemolytic-uremic syndrome (HUS) characterized by high morbidity and mortality. Both Stx1 and Stx2 were shown to efficiently bind Gb3, but in the recent reports demonstrated that only Stx1 could engage P1-capped glycoproteins and trigger cytotoxicity [8,9].

The role of N-glycosylation in GT activity has been studied extensively, showing that N-glycans may heavily impact the subcellular localization and enzymatic activity of GTs [4]. The human Gb3/CD77 synthase harbors two occupied N-glycosylation sites: N_{121}AS and N_{203}LT. In our recent study, we analyzed the full-length Gb3/CD77 synthase expressed in Chinese Hamster Ovary (CHO-Lec2) cells, and we found that the N-glycan at position N_{203} is required for the activity and subcellular localization. In contrast, the presence of N-glycan at N_{121} seemed to be expendable, with some data suggesting that it may curtail the activity [4]. However, expression of full-length Golgi-residing enzymes as intracellular proteins has limitations such as cumbersome analysis of enzyme activity and N-glycan structures. Therefore, we decided to investigate soluble glycovariants of the human Gb3/CD77 synthase, using modified human embryonic kidney cells (HEK293-EBNA1). Additionally, we performed *in silico* modeling of human Gb3/CD77 synthase with N-glycans attached to sequons at positions N_{121} and N_{203} to study their mechanistic roles in the folding and activity of the enzyme.

2. Material and methods

2.1. Cloning, expression and purification of the human Gb3/CD77 synthase (Q) and its mutein (E) glycovariants in HEK293E cells

The *A4GALT* gene fragment (NG_007495.2) encoding the catalytic domain (130–1059 nucleotides of the ORF, corresponding to 44–353 aa of the enzyme's polypeptide sequence) with c.631C (for the Q enzyme) or c.631C> G substitution (p.Q211E; the E enzyme) were used as templates for site-directed mutagenesis to obtain different glycovariants as described [2,4]. The glycovariants were constructed by introducing short oligonucleotides, with codons encoding A instead of the amino acid at the third position of the canonical sequence NXS at the N-glycosylation site [11]. Six glycovariants of human Gb3/CD77 synthase were created: single glycomutants Q_{S123A}, Q_{T205A}, E_{S123A}, E_{T205A}, and double glycomutants Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A} (Fig. 1). These constructs were cloned in-frame with Strep-Tag II and 6x-His-Tag into EBA181-Bio vector (EBA181-bio Addgene plasmid #47744) [12].

PCR amplification, DNA sequencing and preparation of the plasmid constructs were carried out as previously described [4]. Cloning was performed using NotI and EcoRI restriction sites added to the forward and reverse primer, respectively (Table 1). Sequences encoding Strep-Tag II and 6x-His-Tag were introduced into the forward PCR primer. Sequences of all the primers used in this study are listed in Table 1.

HEK293-EBNA1 (HEK293E, IBA Bioscience) cells were grown in suspension in FreeStyle™ 293 Expression Medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1% of fetal bovine serum (FBS, Gibco, Waltham, MA, USA), 4 mM glutamine and 25 μg/mL G418 at 37 °C with 8% CO_2. The cultures were maintained at a density below 2.0×10^6 cells/mL in Erlenmeyer flasks shaken at 120 rpm (Infors) in a humidified incubator at 37 °C with 8% CO_2.

Cell cultures were diluted to 2×10^6 cells/mL in the FreeStyle™ medium without FBS 24 h before transfection. The transfection mix was prepared in 1 mL of the FreeStyle™ medium with 20 μl of DNA plasmid (1 μg/mL) and 50 μl of linear PEI 25 kDa (1 μg/mL, Polysciences Inc.). The mix was incubated for 15 min at room temperature and added to cell cultures. After 96 h of incubation (37 °C, 8% CO_2, in a humidified incubator) with shaking (120 rpm), the medium was collected and filtered using Stericup® Filter Units with the cut-off 0.22 μm (Merck) and loaded on HisPur™ Ni-NTA Resin (Thermo Fisher Scientific, Waltham, MA, USA) as described previously [2].

2.2. SDS-PAGE and western blotting

The proteins were separated in the presence of SDS (Roth) using

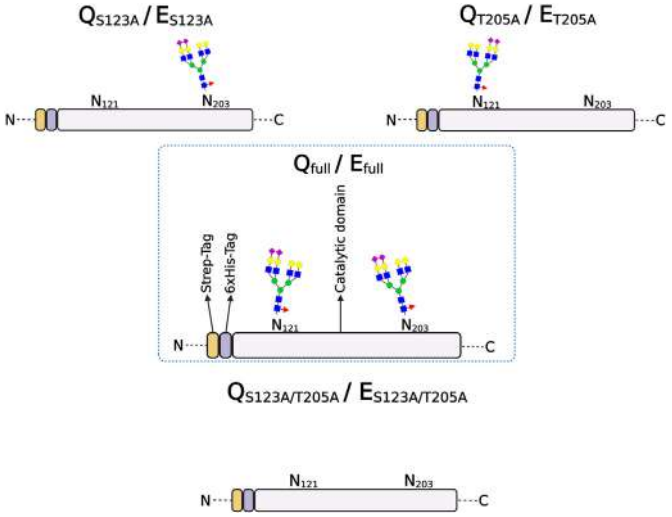

Fig. 1. Schematic representation of truncated (44–353 aa) recombinant human Gb3/CD77 synthase (Q and E) and its glycovariants with locations of N-glycosylation sites (N_{121} and N_{203}). The Q enzyme contains glutamine at position 211 in contrast to E which harbors glutamic acid at the same site. Q_{full}, fully N-glycosylated Gb3/CD77 synthase; E_{full}, fully N-glycosylated mutein Gb3/CD77 synthase; Q_{S123A}, Gb3/CD77 synthase with p.S123A substitution; E_{S123A}, mutein Gb3/CD77 synthase with p.S123A substitution; Q_{T205A}, Gb3/CD77 synthase with p.T205A substitution; E_{T205A}, mutein Gb3/CD77 synthase with p.T205A substitution; $Q_{S123A/QT205A}$, Gb3/CD77 synthase with p.S123A/p.T205A substitutions; $E_{S123A/ET205A}$, mutein Gb3/CD77 synthase with p.S123A/p.T205A substitutions [Figure was created with BioRender.com].

Table 1
Nucleotide sequences of primers used in the study. Changed codons are underlined and shown in red. Restriction sites are underlined; the added sequences encoding 6x-His-Tag and Strep-Tag II are shown in orange and blue, respectively. For A4G_HEK_sens and A4G_HEK_rev primers, the *A4GALT* ORF coding nucleotide sequence is italicized.

Name of primer	Sequence [5' → 3']
A4G_Seq_sens	TCGCACTCATGTGGAAG
A4G_Seq_rev	AGTACATTTTCATGGCCT
A4G_S123A_sens	GCAACGCCGCACTGCCCCGGCAC
pCAG_rev	ACAAACGCACACCGGCCTTATTCC
A4G_S123A_rev	GTGCCGGGGCAGTGCGGCGTTGC
pCAG_sens	CGTGCTGGTTGTTGTGCTGTCTCA
A4G_T205A_sens	CTGCGGAACCTGGCAAACGTGCTGG
pCAG_rev	ACAAACGCACACCGGCCTTATTCC
A4G_T205A_rev	CCAGCACGTTTGCCAGGTTCCGCAG
pCAG_sens	CGTGCTGGTTGTTGTGCTGTCTCA
A4G_HEK_sens	AAAAAGCGGCCGCCCATCACCATCACCATCACTGGAGCCATCCTCAGTTTGAAAAGTCCGCT*GGAGAGCCCAAGGAGAAAG*
A4G_HEK_rev	AAAAAGAATTC*TCACAAGTACATTTTCATGGCCTC*

10% polyacrylamide gel and visualized with Coomassie Brilliant Blue R-250 (Roth, Karlsruhe, Germany) or transferred to the nitrocellulose membrane (Roth, Karlsruhe, Germany) as described [4]. The PageRuler Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA) was used as a protein standard. All samples were run in triplicates with equal amounts of protein in each lane.

The proteins fractionated by SDS-PAGE were transferred to the nitrocellulose membrane (Roth) and detected with mouse anti-A4GALT antibody (hybridoma culture supernatant 5C7, obtained as described in Ref. [4]) or anti-6x-His antibody (clone HIS.H8, Thermo Fisher Scientific, Waltham, MA, USA). Goat anti-mouse IgG (H + L) conjugated with alkaline phosphatase was used as a secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. PNGase F and endo H digestion

Digestion with glycosidases was performed on the glycomutants of Q and E. Briefly, 1 μl of 10% SDS and 0.7 μl 1 M DTT were added to 20 μg of each glycomutant, and the samples were incubated at 95 °C for 5 min. Then they were treated overnight with recombinant peptide-N-glycosidase F (PNGase F, Promega, Madison, WI) in 50 mM sodium phosphate buffer, pH 7.5 at 37 °C or endoglycosidase H (Endo H, Promega, Madison, WI) in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl and 5 mM EDTA at 37 °C according to the manufacturer's instructions.

2.4. Evaluation of the enzymatic activity

The enzymatic activity was evaluated by ELISA with oligosaccharide-polyacrylamide (PAA) conjugates as acceptors: Galβ1→4Glc-PAA (Lac-PAA, precursor to Gb3), Galβ1→4GlcNAcβ1→3Galβ1→4Glc-PAA (nLc4-PAA, precursor to P1), GalNAcβ1→3Galα1→4Galβ1→4Glc-PAA (Gb4-PAA, precursor to NOR1) as described previously [2,4]. PAA-conjugates were gifted to us by Dr. Nicolai V. Bovin (Auckland University of Technology, Auckland, New Zealand). Data were analyzed using Microsoft Office Excel (Microsoft Corp, Redmond, WA).

2.5. Molecular modeling

The structure of human Gb3/CD77 synthase has not been determined to date, so it was modeled using the AlphaFold [13]. To this end, the UniProt Q9NPC4 sequence was used and the obtained model was trimmed to the region of high confidence (78–353 aa). To assess the structural impact of the N-glycans on each sequon, GlycoSHIELD was used to graft glycan conformers derived from extensive molecular dynamics simulations [14]. Representatives of the three classes of N-glycans were used: oligomannose (Man5,GlcNac(2),-Man(5)), hybrid (Man5_Gal5_Fuc1, GlcNac(3),Man(5),Gal(1),Fuc(1)) and large complex (Fuc1_Neu4, GlcNac(6),Man(3),Gal(4),Sialic Acid(4),Fuc(1)). The grafting procedure was performed using the coarse-grained protein and glycan representation and a cutoff radius of 3.5 Å. In each case, uniform glycosylation was applied to both sites and glycan conformers were randomized. Protein surface accessibility reduction was calculated using GlycoSASA applied to 2500 grafted glycan conformations and probe radii of 1.4 and 7 Å, with the remaining parameters set to default. Visualization was performed using VMD [15], and Anisotropic Normal Mode [16] calculations were performed using ProDy implementation [17], with a standard cutoff radius of 15 Å and unit force constant. The motion associated with the lowest non-trivial mode was visualized with VMD and NMWiz plugin.

3. Results and discussion

3.1. Expression and purification of the human Gb3/CD77 synthase (Q) and its mutein (E) glycovariants in HEK293E cells

In order to evaluate the role of N-glycans in the enzyme activity, we used site-directed mutagenesis to obtain the soluble enzymes with changed N-glycosylation patterns. The recombinant glycovariants were expressed in HEK293E cells which enables highly effective episomal replication of expression plasmids [12]. The recombinant glycovariants of both E and Q enzymes produced in HEK293E cells were evaluated by immunoblotting using an anti-A4GALT antibody (hybridoma supernatant, clone 5C7) (Fig. 2A and B) [4]. The fully N-glycosylated enzymes (Q and E) migrated as approximately 52 kDa bands, while the single mutants of Q enzyme (Q_{S123A} or Q_{T205A}) and E (E_{S123A} or E_{T205A}) showed a reduced molecular weight; three bands with approximately 49 kDa, 45 kDa and 41 kDa were visible. The enzymes without any N-glycans (Q_{S123A}/Q_{T205A} and E_{S123A}/E_{T205A}) could not be detected. To answer the question of whether the double mutants accumulate in the cells, we analyzed the lysates of HEK293E cells transfected with plasmids encoding double mutants and fully N-glycosylated control enzymes (Fig. 2C). The bands detected by the anti-A4GALT antibody represented both Q- or E-derived double mutated glycovariants, with MW of about 37 kDa, in contrast to the fully N-glycosylated enzyme with MW of approximately 52 kDa (Fig. 2C). The difference in MW suggests that both N-glycosylation sites were occupied. Both Q and E double mutants accumulated in the cells, likely as a result of misfolding (Fig. 2C). Thus, we propose that human Gb3/CD77 synthase requires N-glycans at both sequons (N_{121} and N_{203}) for effective secretion.

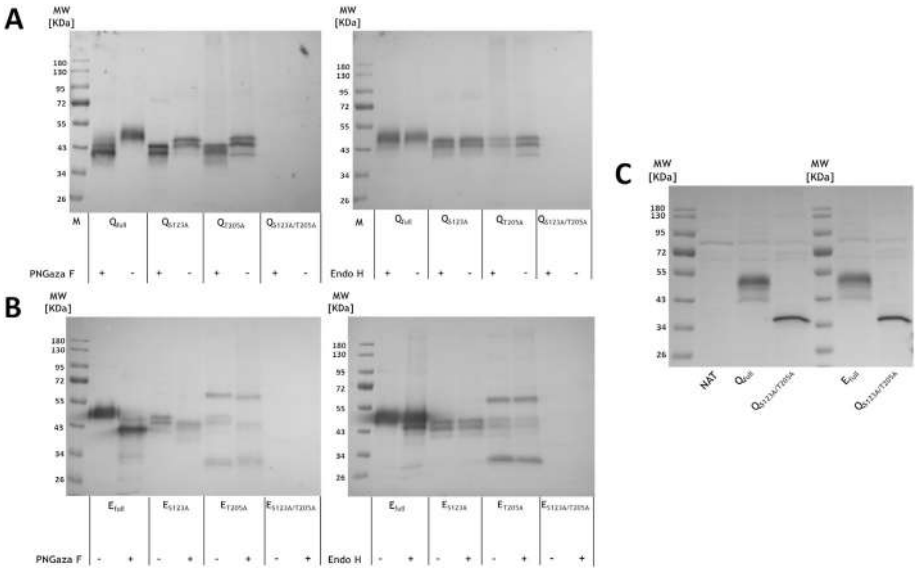

Fig. 2. Western blotting analysis of recombinant human Gb3/CD77 synthase Q (**A**) and E (**B**) glycovariants expressed using HEK293E cells as well as HEK293E cell lysates transfected using plasmids encoding fully N-glycosylated Q and E enzymes and double glycomutants (**C**). Immunoblotting with anti-A4GALT which specifically recognizes human Gb3/CD77 synthase alongside a molecular weight marker. NAT, untransfected HEK293E cell lysate control.

The HEK293E cells synthesize predominantly complex and hybrid N-glycans [18,19], so such N-glycans should be present in recombinant glycoproteins expressed in these cells. In order to analyze the N-glycosylation patterns of recombinant human Gb3/CD77 synthase glycovariants, we used two glycosidases with different specificities: PNGase F and Endo H, which cleave all or hybrid and oligomannose N-glycans, respectively. All investigated single glycomutants were susceptible to PNGase F treatment but resistant to Endo H. In contrast, the fully N-glycosylated Q and E enzymes were sensitive to both glycosidases, with an additional band of about 45 kDa appeared after Endo H treatment. This suggests that one of the two N-glycans attached to the enzyme is a hybrid or oligomannose chain (Fig. 2A and B). Thus, N-glycosylation sites in fully N-glycosylated human Gb3/CD77 synthase (Q and E) are occupied by complex and hybrid/oligomannose N-glycans, while the N-glycans in single glycomutants contain only complex structures. The difference in N-glycan types may be dictated by accessibility of respective sequons for glycosylation machinery in the HEK293E cells. However, a more precise characterization of which N-glycan is attached to which N-glycosylation sequon requires further studies. The emergence of extra faster-migrating bands for the Q and E enzymes after treatment with glycosidases could be caused by the presence of other PTMs (human Gb3/CD77 synthase has two predicted O-glycosylation, one methylation and seven phosphorylation sites according to UniProt Q9NPC4) or partial proteolysis of the enzymes.

3.2. *N-glycan at N_{203} is crucial for human Gb3/CD77 synthase activity*

The glycovariants of Gb3/CD77 synthase were evaluated by the ELISA activity assay, using the following antibodies: human *anti*-P1 (clone P3NIL100) which binds only to P1 antigen, mouse *anti*-P1 (clone 650), which recognizes both Gb3 and P1 antigens, and anti-NOR (clone nor118 [20]), which binds NOR antigens. Enzymatic activity toward Lac-PAA acceptor was detected only in the case of glycovariants with p.S123A substitution (Q_{S123A} and E_{S123A}) (Fig. 3). The glycovariants with p.T205A substitution (Q_{T205A} and E_{T205A}) and double mutants did not show any catalytic activity. The loss of

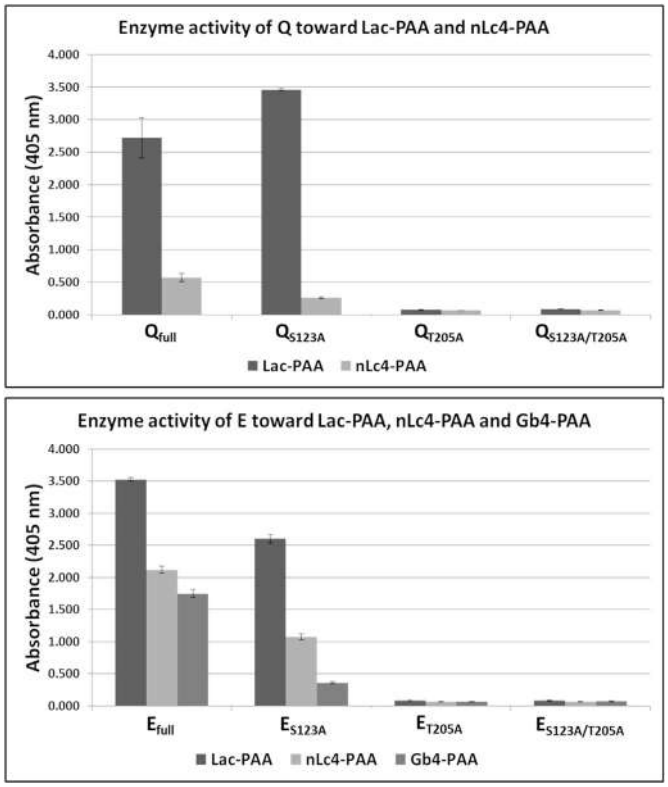

Fig. 3. Evaluation of the enzymatic activity of recombinant human Gb3/CD77 synthase and its mutein glycovariants by ELISA. Activities of the recombinant enzymes were measured against Lac-PAA, nLc4-PAA, and Gb4-PAA conjugates used as acceptors. Q_{full}, fully N-glycosylated Gb3/CD77 synthase; E_{full}, fully N-glycosylated mutein Gb3/CD77 synthase; Q_{S123A}, Gb3/CD77 synthase with p.S123A substitution; E_{S123A}, mutein Gb3/CD77 synthase with p.S123A substitution; Q_{T205A}, Gb3/CD77 synthase with p.T205A substitution; E_{T205A}, mutein Gb3/CD77 synthase with p.T205A substitution; $Q_{S123A/QT205A}$, Gb3/CD77 synthase with p.S123A/p.T205A substitutions; $E_{S123A/ET205A}$, mutein Gb3/CD77 synthase with p.S123A/p.T205A substitutions; Neg, control without the enzyme-containing lysates; -UDP, control without UDP-Gal donor; −Ab, control without primary antibodies.

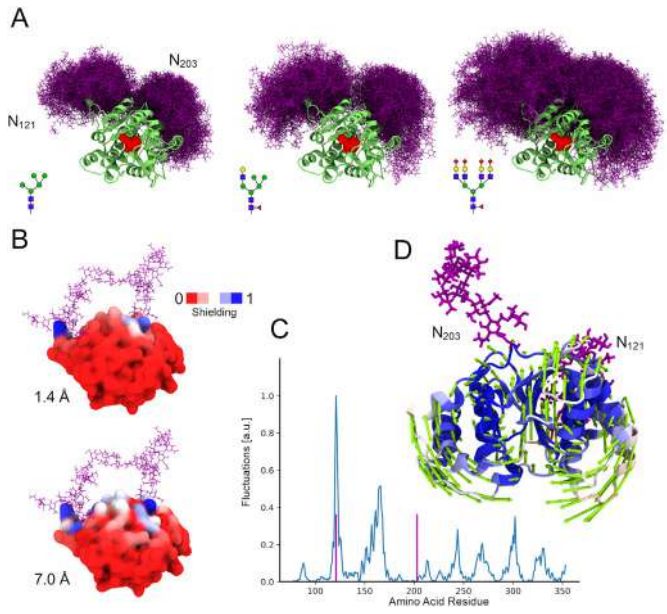

Fig. 4. Molecular modeling of the human Gb3/CD77 synthase. (A) Arrays of 250 conformers (purple) of representatives of three glycan classes (insets) grafted onto the AlphaFold model of the Gb3/CD77 synthase (green). The putative position of the active site is indicated in red. (B) Reduction of the surface accessibility (shielding) calculated for 2500 glycan conformers. (C) Fluctuations of Cα atoms calculated from the slowest mode of the ANM model. Glycan positions are indicated in magenta. (D) Visualization of the slowest mode. Arrows indicate the direction of motion for each Cα atom with the length of the arrow proportional to the motion magnitude. Protein (cartoon representation) is colored according to the magnitude of predicted fluctuations in (C), with red corresponding to the largest and blue smallest fluctuations. Glycans are shown as purple licorice. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

activity could be caused by instability or misfolding and aggregation of the p.T205A glycovariants [21]. These findings pinpoint that N-glycan at position N_{203} is essential for the activity of human Gb3/CD77 synthase.

In our previous work, we evaluated the full-length human Gb3/CD77 synthase expressed in CHO-Lec2 cells and we found that the N-glycan at position N_{203} is required for activity and proper subcellular localization in the Golgi apparatus. On the other hand, elimination of N-glycan from N_{121} had no significant impact on the activity. The double mutants (devoid of N-glycans) accumulated in the ER and showed negligible activity [4]. However, using full-length enzymes in that study limited our ability to evaluate N-glycan structures attached to the enzyme. Therefore, we produced recombinant soluble enzymes in human HEK293E cells, which use extensive and well-defined glycosylation machinery, and efficient secretory pathways, making them a favorite cell line for the production glycoproteins that are difficult to express [22].

3.3. Molecular modeling reveals potential allosteric activity of N203

To better understand the molecular basis of the observed relationship between N-glycosylation and the activity of the human Gb3/CD77 synthase, we sought to determine the structural impact of both glycosites. Glycans are typically much more dynamic than proteins and their conformations span large swathes of protein surface [23]. To test whether such glycan conformations may directly influence the enzyme's active site, we grafted arrays of conformations onto the truncated Gb3/CD77 synthase AlphaFold model using GlycoSHIELD [14] with high mannose, hybrid and complex N-glycans (Fig. 4A). As the resulting glycan umbrellas are located on the far side of the protein in relation to the putative active site (residues 192–194) they do not reach the entry channel, corroborated by the reduction of surface accessibility ("shielding", Fig. 4B) calculated for water-sized probe radius (1.4 Å) and a larger probe (7 Å), comparable with the size of UDP-Gal.

In addition to directly influencing the active site or ligand entry, N-glycans can modulate enzymatic activity by affecting the long-range allosteric dynamics, despite their generally small effect on protein structure [24,25]. To explore this possibility, we analyzed the locations of the N_{121} and N_{203} N-glycosylation sequons. Whereas the former is located on a detached loop, the latter lies on the hinge of the beta-sheet structure forming the core of the active site and could influence the internal dynamics of the protein. To test this hypothesis we applied the Anisotropic Network Model (ANM) which has been successfully used to predict the internal motions of proteins [26]. The fluctuations associated with the slowest mode confirmed the large mobility of the N_{121} site and the relatively rigid neighborhood of the N_{203} site (Fig. 4C). This rigidity together with the position in the hinge region, suggests that N_{203} may allosterically modulate the activity. Further corroborating our findings, the slowest ANM mode revealed breathing-like motion (Fig. 4D), potentially participating in the enzymatic activity. Taken together, these results suggest an intriguing mechanism of allosteric modulation of the human Gb3/CD77 synthase, whereby N-glycans located at N_{203}, but not N_{121}, could modulate the enzyme activity, in agreement with our experimental results.

In summary, the N-glycan at N_{203} is essential for enzymatic activity, in contrast to the N-glycan at N_{121}, whose role is negligible. *In silico* modeling suggests that the position of the N_{203} N-glycan on a rigid hinge of beta-sheet near the active site underlies its direct impact on the enzyme activity. In turn, both N-glycans (N_{121} and N_{203}) are required for effective secretion of the enzyme. The fully N-glycosylated Gb3/CD77 synthase seems to carry two different N-glycan types (complex versus hybrid/oligomannoses), while the single glycomutants have only complex N-glycans. Finally, the N-glycan at position N_{203} may allosterically regulate the enzyme activity.

Failure to solve the spatial structure of human Gb3/CD77 has stood in the way of generating conclusive mechanistic data about the enzyme. Here, we circumvented this limitation by employing the latest innovations in protein modeling. Previously, we showed that one (N_{203}) of the two N-glycans on Gb3/CD77 synthase is essential for its activity, but the exact mechanism of its impact remained unknown. Changes in N-glycosylation may affect enzymatic activity by impairing its ability to fold into a functional protein or altering the kinetic properties of a correctly folded protein through interactions with the active site. Our protein modeling suggests that the N_{203}-linked glycan allosterically modulates the Gb3/CD77 synthase activity, adding to the long list of its unique properties.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.05.085.

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WNIOSKI

1. Oba sekwony N-glikozylacji ludzkiej syntazy Gb3/CD77 o pełnej długości mają przyłączone N-glikany. W przypadku form rozpuszczalnych enzymu, w pełni N-glikozylowana syntaza Gb3/CD77 ma dwa typy struktur N-glikanowych: kompleksowe i wielomannozowe. Warianty pozbawione jednego N-glikanu mają wyłącznie struktury kompleksowe.

2. N-glikan w pozycji N_{121} nie ma wpływu na aktywność enzymatyczną ani lokalizację wewnątrzkomórkową enzymu o pełnej długości, natomiast N-glikan w pozycji N_{203} jest niezbędny do zachowania aktywności i prawidłowej lokalizacji w aparacie Golgiego. W przypadku enzymu rozpuszczalnego, eliminacja N-glikanu w pozycji N_{121} powodowała obniżenie aktywności enzymatycznej, a enzym pozbawiony N-glikanu w pozycji N_{203} był nieaktywny.

3. Wszystkie warianty glikozylacyjne enzymu o pełnej długości produkują glikotop P1 na glikoproteinach.

4. Całkowita eliminacja N-glikanów z ludzkiej syntazy Gb3/CD77 o pełnej długości powoduje utratę aktywności enzymatycznej. Enzym rozpuszczalny nie jest wydzielany do medium, tylko akumuluje się wewnątrz komórki.

5. Wszystkie analizowane komórki CHO-Lec2 transfekowane genami kodującymi warianty glikozylacyjne ludzkiej syntazy Gb3/CD77 o pełnej długości były wrażliwe na toksyny Shiga.

6. Analizy *in silico* wykazały że reszty cukrowe przyłączone do sekwonu w pozycji N_{203} ludzkiej syntazy Gb3/CD77 znajdują się w pobliżu miejsca aktywnego enzymu i mogą allosterycznie regulować aktywność enzymatyczną enzymu.