The role of N-glycosylation in human Gb3/CD77 synthase activity

 $P1/P^k$. Human Gb3/CD77 synthase $(\alpha 1, 4$ -galactosyltransferase, synthase 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 Gala1 \rightarrow 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 Gala1 \rightarrow 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 Gal α 1 \rightarrow 4Gal β 1 \rightarrow 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 (N₁₂₁AA and N₂₀₃LA) 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 $Gal\alpha 1 \rightarrow 4Gal$ 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 N₂₀₃ 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 N₂₀₃ 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 N₁₂₁ 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 N₂₀₃.

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.