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Synthesis, processing, and function of N-glycans in N-glycoproteins

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Abstract

Many membrane-resident and secreted proteins, including growth factors and their receptors are N-glycosylated. The initial N-glycan structure consists of 14 sugar residues (Glc3Man9GlcNAc2) that are first synthesized in the endoplasmic reticulum (ER) as a branched structure on a lipid anchor (dolicholpyrophosphate) and then co-translationally, “en bloc” transferred and linked via N-acetylglucosamine (GlcNAc) to asparagine within a specific N-glycosylation acceptor sequence (Asn-X-Ser/Thr) of the nascent recipient protein. In the ER and then the Golgi apparatus, the N-linked glycan structure is modified by hydrolytic removal of sugar residues (“trimming”) followed by re-glycosylation with additional sugar residues (“processing”) such as galactose, fucose or sialic acid in complex N-glycoproteins. While the sequence of the reactions leading to biosynthesis, “en bloc” transfer and processing of N-glycans is well investigated, it is still not completely understood how N-glycans affect the biological fate and function of N-glycoproteins. Initially, N-glycans have been found to be critical for proper protein folding and quality control by chaperones in the ER, a process now known as the “calnexin/calreticulin cycle” in the unfolded protein response and ER-assisted degradation (ERAD). More recently, N-glycans have been shown to modulate the function of many cell surface proteins involved in migration and adhesion, including those regulating myelination. Currently, the Golgi has emerged as an organelle that is intimately linked to editing the function of N-glycans in N-glycoprotein transport and sorting. For one, it has been shown that mutations in Golgi glycosyltransferases and transport proteins lead to defects in N-glycan processing that cause severe congenital disorders of glycosylation (CDG). On the other hand, it has been found that N-glycans affect transport of glycosylated proteins in the Golgi, including sorting of secreted proteins such as prions and amyloid. Our group has shown that N-glycan-dependent enzyme complex formation may entangle processing of N-glycosylated glycosyltransferases with glycosphingolipid metabolism, which appears to be important for ganglioside class switches during embryonic brain development. This review will discuss the biology of N-glycoprotein synthesis, processing and function with specific reference to the physiology and pathophysiology of the nervous system.

Introduction

Throughout the life cycle of neural cells undergoing a “metamorphosis” from neural stem cells to mature neurons, astrocytes, and oligodendrocytes (or Schwann cells in the peripheral nervous system), the differentiation and function of these cells is critically relying on their response to extra- and intracellular signaling cues. This response depends on the specificity and sensitivity of receptor proteins. It becomes increasingly clear that the sensitivity of receptors is regulated by specific N-glycan residues that affect: 1) secretion, stability, and clearance of the receptor ligands; 2) surface expression, internalization, and recycling or turnover of the receptors; 3) adhesion of neurons and other cells via cell surface receptors and extracellular matrix proteins; and 4) signal induction and transduction by growth factor and neurotransmitter receptors and ion channels. In many of these cases, the N-glycan enhances 1), proper folding of ligand or receptor; 2), solubility or polarity of the ligand or receptor; and 3), binding to extracellular or intracellular factors that induce cell signaling pathways or mediate further processing of the N-glycoprotein. In particular, the latter has gained recent attention since specific N-glycans can regulate protein association in receptor/ligand complexes or sugar-specific binding proteins in the plasma membrane (e.g., galectins) that mediate endo- or exocytosis, transport or sorting, and recycling or turnover of the receptor (1-6).

While these mechanisms are critical for the proportion of receptors expressed on the cell surface or the retrograde transport of signalosomes (protein complexes between ligand and receptor), it can also modulate the exocytotic transport and secretion of proteins with pathological effects such as amyloid or prion protein (7-8). It is not surprising that the particular structure of N-glycans and therefore, the sequence of enzymatic processing steps leading to this structure is focus of intensive research, in particular for the identification of new drug targets. For more than three decades, specific inhibitors of glycosidases involved in N-glycoprotein processing have been tested for their application in antiviral and tumor therapy (9-12). On the other hand, mutations in proteins that mediate N-glycosylation and N-glycan processing can lead to severe diseases, including those of the nervous system (13-16). This is not limited to mutations in trimming or processing glycosidases, but encompasses proteins mediating the transport of N-glycoproteins for their processing in the ER or Golgi, as found in human congenital disorders of glycosylation (CDG) (17-19). To define the function of N-glycans in normal physiology and disease one needs to know their precise structure and the enzymatic steps generating this structure. Currently, the rapid progress in high throughput mass spectrometry analysis has opened a growing field of comprehensive glycomics studies on N-glycans and other proteinogenic glycoconjugates such as O-glycans and proteoglycans (20). Mutation analysis and the genome projects have provided us with the information and tools for any protein involved in N-glycoprotein biosynthesis and processing. And yet, the dynamics of biochemistry on the cellular level requires knowledge beyond the statics of structure and sequence, an insight into the flux of biological reactions.

To understand this flux, one may envision the cell as a gigantic factory with sequential assembly lines for the generation and functional editing of N-glycans. This processing works like a flow chart with individual yes/no decision points for: 1) glycosylation leading to non-

glycosylated or glycosylated proteins; 2) trimming and re-glycosylation leading to “high mannose” and “re-glycosylated” intermediates; and 3) further processing and re-glycosylation leading to “mannose-6-phosphate”, “hybrid”, or “complex” N-glycoproteins as end products. As the result of this editing process, N-glycans act as a specific addresses or tags that regulate the processing or function of their attached proteins by a simple rule: N-glycan-dependent association kinetics between enzymes, receptor proteins, and other factors keeping the N-glycoprotein at a particular compartment or moving it to the next. As a first step of understanding processing of N-glycans we will discuss the initial assembly of the N-glycan and its transfer to the prospective N-glycoprotein.

1. N-glycans are first born on a lipid and then transferred “en bloc” onto the nascent N-glycoprotein in the ER

N-glycans are oligosaccharides by their chemical nature: branched chains of sugar residues attached to each other by α - and β -glycosidic linkages (Fig. 1). However, the N-glycan is not made on the protein, but pre-manufactured on an ER-resident lipid, the polyprenol dolichol pyrophosphate. Further, while after attachment to the protein, the N-glycan points to the lumen of the ER, the assembly of the initial 7 of the 14 sugar residues in the dolichol pyrophosphate (Dol-PP)-linked precursor oligosaccharide is accomplished at the cytosolic side. Hence, the N-glycosylation reaction relies on two critical steps: transport of the partial precursor oligosaccharide (Dol-PP-GlcNAc2Man5) across the ER membrane from the cytosolic to the luminal side and then after further glycosylation reactions, “en bloc” transfer of the mature precursor to the protein. In eukaryotes, the assembly of this precursor oligosaccharide is achieved by a set of ER-resident, transmembrane protein glycosyltransferases of the types a), N-acetylglucosaminyltransferase that attach two GlcNAc residues to Dol-PP in a β 1-N and then β 1-4 linkage, b) mannosyltransferases catalyzing four different glycosidic linkages: β -1,4 (first mannose attached to second GlcNAc), α -1,3 and α -1,6 (mannose at the two branching points of the biantennary oligosaccharide), and α -1,2 (mannose elongation of the middle (B) and two outer (A and C) branches, see Fig. 1) that attach a total of 9 mannose residues to Dol-PP-GlcNAc2 to form a branched Dol-PP-GlcNAc2Man9 structure, and c) glucosyltransferases that attach the terminal three glucose residues in two α 1,3- and one terminal α 1,2-glycosidic linkage onto the outer α 1,3-mannosidic branch (A branch) of the precursor oligosaccharide (Fig. 1). These reactions depend on activated sugars that are provided on the cytosolic and luminal side of the ER in form of UDP-GlcNAc, GDP-mannose (GDP-man), and UDP-glucose (UDP-glc).

Mutations in glycosyltransferases and the flippase transporting the Dol-PP-GlcNAc2Man5 partial precursor from the cytosol into the ER lumen lead to a spectrum of diseases known as congenital disorders of glycosylation type I (CDGs type I) (21-22). The symptoms of CDGs type I often involve the nervous system. For example, mutations of the RFT1 (Requiring Fifty Three 1, CDG-In) yeast homolog, the mammalian flippase, cause sensorineural deafness (13, 15, 21). CDG-In is an extremely rare disease, only 6 patients are known so far. Other CDGs type I resulting from mutations in precursor glycosyltransferases often show defects in multiple tissues that can amount to multiorgan failure as observed with glucosyltransferase II deficiency (CDG-Ih). It should be noted that, while CDGs type I can

be very severe it is not clear which N-glycoprotein dysfunction due to hypoglycosylation accounts for a specific disease phenotype or symptom. The diagnosis of CDG type I is commonly based on testing for hypoglycosylation of transferrin in patient plasma, although this aberrant glycosylation does not account for the entire spectrum of symptoms observed with this disease. As discussed later, the consequences of hypoglycosylation can be severe for a variety of proteins, the proper folding of which relies on the intact N-glycan residue. Therefore, it may not be surprising that CDGs type I can lead to multiple tissue and organ failures.

Following assembly and the flipping reaction of the precursor oligosaccharide, co-translational “en bloc” transfer of the N-glycan from Dol-PP onto the nascent polypeptide is the next critical step in N-glycoprotein biosynthesis. This step is catalyzed by oligosaccharyl transferase (OST), a multimeric enzyme complex composed of 9 subunits in yeast and 4 subunits in higher eukaryotes (19-20, 23-31). The precise function of these subunits is still a subject of ongoing research, however, it is clear that OST will have to manage the association with the ribosome and signal recognition particle receptor for the nascent membrane glycoprotein, the recognition of the acceptor sequence Asn-X-Ser/Thr (X cannot be proline), and the catalytic transfer of the oligosaccharide from Dol-PP onto asparagines (Fig. 1).

As with mutations in glycosyltransferases involved in the precursor oligosaccharide assembly, deficiencies of OST subunits lead to several CDGs of type I. The catalytic subunit of OST, Stt3 in yeast or Stt3A and B in mammals, is a conserved subunit that is already expressed in archaeobacteria. It has been speculated that because of the redundancy conferred by the two mammalian Stt3 homologs, CDGs have not been discovered yet (19, 22, 24, 32). However, mutations in other OST subunits have been associated with various CDGs of type Ix. For example, deficiencies of the ribophorin I subunit RPN2 have been found by screening for hypoglycosylation defects without impairment of precursor oligosaccharide assembly (32). At present, the pathology of these CDGs is unclear, but it is likely to involve nervous system defects. Once OST has transferred the precursor oligosaccharide onto the nascent polypeptide, a process starts that entails an important decision for the newly born N-glycoprotein: *fold or fail*. In particular, the glucose residues on the outer mannosidic (α 1,3- or A) branch are instrumental in assisting the protein folding proofreading and refolding process, which will be discussed in the next section.

2. Trimming, reglycosylation, and remodeling: there are many ways of N-glycoprotein processing in the ER and Golgi

There are two pathways by which the N-linked Glc3Man9GlcNac2 oligosaccharides are processed (“trimmed”): the glucosidase-dependent and independent pathway (Fig. 2). The glucosidase-dependent pathway occurs in the ER and is mediated by a sequential hydrolytic cleavage of the terminal glucose residues by glucosidase I and II (Fig. 2). This sequence is embedded into chaperone-assisted proofreading of protein folding: the calnexin/calreticulin cycle. The glucosidase-independent pathway is catalyzed by endomannosidase which cleaves off a Glc1-3Man1 residue in the Golgi (33-35). The function and regulation of this

reaction is not known. Therefore, we will focus on the glucosidase-dependent pathway and its interaction with the calnexin/calreticulin cycle for protein folding.

To date, the calnexin/calreticulin cycle has been discussed as one of the most important processes underlying the function of N-glycosylation (36-41). It was first described by Ari Helenius in 1994 based on two important observations: a) calnexin and calreticulin are chaperones in the ER that bind to monoglucosylated (GlcMan9GlcNAc2) N-glycoproteins and b) the ER harbors a glucosyltransferase that re-attaches glucose to N-linked Man9GlcNAc2 (38-40, 42-45). Trimming or processing of the terminal glucose residues is thus crucial for the chaperone function of calnexin and calreticulin. The function of a chaperone is to recognize and bind misfolded proteins and then mediate refolding until the proper conformation of the protein is accomplished. If the misfolded protein cannot adopt its proper conformation it will be degraded in the ER by the ERAD (ER-assisted degradation) system, a rather complicated organized sequential process of “unfolded protein response” that first shuttles the misfolded protein into the cytosol, where it is ubiquitinated and then removed by proteosomal degradation (46). Another ERAD-associated system transports the misfolded proteins to the Golgi and then the lysosome for proteolysis. Failure of ERAD can result in severe diseases due to the accumulation and aggregation of misfolded proteins. A prominent example related to neurobiology is Parkinson’s disease that can result from ERAD malfunction involving the E3-ubiquitinase parkin (47). Mutations in glucocerebrosidase (GCase), a lysosomal β -glucosidase deficient in Gaucher disease, can lead to the accumulation of aberrant GCase attached to parkin, which is currently discussed as one of the causes of Parkinson’s disease (47). Since GCase cleaves off glucose from glucosylceramide to generate ceramide, it is an example for the interdependence of N-glycoprotein and glycosphingolipid metabolism, which will be discussed later in this chapter. In addition to the aggregation and accumulation of aberrant proteins, ERAD malfunction may also lead to pre-mature degradation of proteins that are still in the process of refolding, which has been discussed as one of the causes of cystic fibrosis (48). From these examples, it is evident that the N-glycan assisted calnexin/calreticulin cycle for protein folding and thus the function of ERAD critically relies on proper N-glycan processing.

To generate the monoglucosylated N-glycan glucosidase I and II remove the first two glucose residues from the N-linked Glc3Man9GlcNAc2 oligosaccharide (25, 49-51). The resulting GlcMan9GlcNAc2 is bound to calreticulin or calnexin and the protein (partially) refolded, which is followed by removal of the innermost glucose residue by glucosidase II. After this, the N-glycan (Man9GlcNAc2) is transiently reglucosylated to GlcMan9GlcNAc2 by UDP-glucose:glycoprotein glucosyltransferase to prevent further N-glycan trimming of a glycoprotein that has not yet adopted its proper folding state. After glucosidase II has cleaved off the newly added glucose residue, the Man9GlcNAc2 oligosaccharide re-enters the reglucosylation-refolding-trimming cycle until the native conformation of the N-glycoprotein is accomplished. Once the protein is correctly folded, the chaperones do not bind to the protein anymore and the Man9GlcNAc2 oligosaccharide is processed by a series of mannosidases (Fig. 2).

The removal of mannose residues from Man9GlcNAc2 is initiated in the ER and intimately associated with the ERAD response to misfolded proteins (35, 46, 52-54). If correct folding

cannot be achieved the N-glycoprotein is not reglucosylated and ER mannosidase I cleaves off the terminal α 1,2-mannose residue of the middle (B) branch in the N-glycan (Fig. 1) generating a Man8GlcNAc2 oligosaccharide (Fig. 2). How exactly the ERAD machinery distinguishes between properly folded and misfolded Man8GlcNAc2 bearing glycoproteins is not yet fully understood. However, it has been shown that overexpression of ER mannosidase I yields to the acceleration of ERAD-mediated protein degradation. *In vivo*, this function is thought to be mediated by EDEM (ER degradation enhancing α mannosidase like) proteins that accelerate the ERAD response to misfolded N-glycoproteins instead of allowing further exit to the Golgi (54-56). It is expected that mutations in trimming enzymes, in particular glucosidase I, II, and ER mannosidase I would lead to another type of CDGs, called CDG type II since these mutations will affect the N-glycan structure after assembly and transfer to the protein.

Indeed, the first CDG type II known to be caused by trimming glycosidase deficiency is CDG type IIb, which results from mutations in ER glucosidase I (51). CDG type IIb shows multiorgan deficiencies leading to a variety of symptoms such as hepatomegaly, hypoventilation, feeding problems, seizures, and fatal outcome at 74 days after birth. Interestingly, other CDG type II diseases are not directly associated with mutations of trimming enzymes (or are not yet found), but with proteins of the intercisternal Golgi transport machinery such as the COG proteins. Since trimming glycosidases and N-glycan-associated glycosyltransferases are located in distinct Golgi compartments it is expected that mutations in these transport proteins would also lead to disorders of N-glycosylation (17-19). The conserved oligomeric Golgi complex (COG) is a complex of 8 proteins critical for retrograde vesicle transport within the Golgi. It is known to regulate the Golgi distribution of mannosidases and glycosyltransferases important for N- and O-glycoprotein processing (20). Mutations in COG proteins are known to cause CDGs of type II, many of those with presentation of nervous system disorders.

The majority of CDGs caused by COG proteins are associated with abnormal reglycosylation of processed N-glycans due to mislocalization of the respective glycosyltransferases. This process of reshaping the N-linked oligosaccharide by reglycosylation is initiated by 3 distinct Golgi-resident mannosidases and follows (at least) two different routes (Fig. 2). The transport of lysosomal hydrolases requires the attachment of a Mannose-6-phosphate tag (mannose-6-phosphate or M6P-dependent pathway), while other N-glycoproteins of the “hybrid” and “complex” type are further trimmed by removal of additional mannose residues. We will discuss the pathway of further trimming first.

Golgi mannosidase I is distinct from ER mannosidase I in that it cleaves off 3 α 1,2-residues from the Man8GlcNAc2 precursor to yield Man5GlcNAc2 (20, 55, 57-58). This α 1,2 exomannosidase is also different from Man9 mannosidase, which has been cloned and characterized by the author's former group and cleaves 3 mannose residues from Man9GlcNAc2 to yield Man6GlcNAc2, which can then be substrate of Golgi mannosidase I that removes one additional mannose residue (59-62). Regardless of how the Man5GlcNAc2 oligosaccharide is generated, further trimming proceeds after attachment of one GlcNAc residue to the outer mannose (A) branch by UDP-GlcNAc transferase I (Fig. 2). The resulting GlcNAcMan5GlcNAc2 oligosaccharide is then either elongated by the addition of

sugar derivatives such as GalNAc or sialic acid (hybrid N-glycans), or it is subjected to removal of an additional 2 mannose residues by Golgi mannosidase II, which generates N-linked GlcNAcMan3GlnAc2, the “core” glycan structure that is the initial building block for all complex N-glycoproteins (58, 63-67). As with hybrid N-glycans, complex N-linked oligosaccharides are reglycosylated with additional sugar derivatives such as GalNAc or sialic acid, or fucose (Fig. 2). These complex N-glycans come in a large variety of highly branched structures, which can exceed the initial biantennary (2 branching points) structure of high mannose oligosaccharides by far. Biochemically, high mannose N-glycans can be distinguished from hybrid or complex ones by the use of two endoglycosidases that either cleave off the complete N-glycan (glycopeptidase F) or hydrolyze the β -glycosidic linkage between the two GlcNAc residues (chitobiose) of high mannose N-glycoproteins (endoglycosidase H). These enzymes and other glycosidases were discovered early in the history of glycobiology, which in combination with metabolic labeling using radioactive sugars tremendously facilitated the structural analysis of N-glycans (68).

Several glycosylation deficiencies are known to result from mutations of enzymes in the M6P-dependent pathway or aberrant reglycosylation of hybrid or complex N-glycoproteins. In the M6P-dependent pathway for lysosomal enzymes, the Man8GlcNAc glycan is first endowed with 2 GlcNAc phosphate residues that are attached to the subterminal mannose residues of the two outer mannose branches (69-75). Next, the mannose-bound phosphate residues are uncovered by N-acetylglucosaminidase and the terminal mannose residues of the outer (A and C) branches are removed by Golgi mannosidase I (Fig. 2). The resulting P2Man6GlcNAc2 oligosaccharide is now recognized by the M6P receptor in the trans Golgi, which binds to the N-glycoprotein and initiates its transport to the late endosome. While the late endosome matures to lysosomes, the pH value drops and the M6P receptor releases the lysosomal enzyme. The M6P receptor is then recycled to the trans Golgi for further transport of lysosomal enzymes. Deficiencies in the M6P-dependent pathway, in particular caused by mutations of GlcNAc phosphotransferase, the enzyme attaching the GlcNAc phosphate residues to the N-glycan, can lead to severe disorders of glycosylation. Well known examples are I-cell disease or mucopolipidosis type II, and pseudo Hurler polydystrophy or mucopolipidosis type III (69). These diseases are usually not classified as CDG type II but as oligosaccharidosis or mucopolipidosis-type lysosomal storage diseases because failure of transporting enzymes to lysosomes will lead to the accumulation and lysosomal storage of the enzyme substrates, in particular glycosaminoglycans. Mucopolipidosis type II and III lead to severe abnormalities in multiple organs (hepatomegaly, splenomegaly) and delay in cognitive and motor skills development.

As mentioned earlier, the majority of CDGs type II is caused by mutations in glycosyltransferases that generate the complex N-glycan, e.g., CDG IIId, a deficiency of β 1,4 galactosyltransferase I, which leads to psychomotor delay and macrocephaly (15, 21). Also, as discussed earlier, many CDGs type II of complex N-glycoprotein processing are caused by aberrant COG proteins, e.g., CDG-IIe or COG7 deficiency, which leads to hypsialylation of complex N-glycans. In contrast to lysosomal storage diseases related to aberrant M6P-dependent transport of lysosomal enzymes, the molecular cause of the deficiency aka the malfunction of the N-glycoprotein in various CDGs is not clearly defined. The reason is twofold: a) glycosylation defects affect not only one but a variety of complex

N-glycoproteins; and b) for many N-glycoproteins it is not well understood what the physiological function of the N-glycan is, which makes it difficult to understand the malfunction as well. In the next two sections, we will discuss some of these functions, in particular with respect to the significance of N-glycoproteins for brain development and physiology. However, before we move on to these sections, it is necessary to briefly discuss important tools that have helped to elucidate the sequence of N-glycoprotein processing and the function of N-glycoproteins: trimming enzyme-specific inhibitors.

3. The essential toolbox of a glycobiochemist: a brief history of the discovery of N-glycoprotein biosynthesis inhibitors and their impact on our understanding of N-glycan processing

The discovery and development of N-glycosylation and processing inhibitors is intimately linked to the history and progress in N-glycoprotein research – and the professional careers of many leading glycobiochemists. The first inhibitor of N-glycosylation, tunicamycin was found more than 40 years ago in an attempt to screen for antiviral drugs made by bacteria, in particular strains of *Streptomyces* (76-83). This finding is not as surprising at it seems today since the screening procedures at this time were often based on virus hemagglutination and *in vitro* proliferation assays, which were critically affected by the glycoprotein nature of serum proteins and the virus envelope, a well-known fact even decades ago (84). Therefore, one of the tests routinely performed was a competition assay determining if the addition of sugars, in particular N-acetyl aminosugars would reverse the effect of the antiviral antibiotic, which was the case with tunicamycin (78). Later, Alan Elbein discovered that tunicamycin inhibits the transfer of N-acetylglucosamine to dolichol phosphate, the first step in the synthesis of the lipid-linked oligosaccharide that serves as the precursor for all N-glycoproteins (81, 85). It was also found very early that tunicamycin induces the degradation of viral glycoproteins and it was hypothesized that proteolysis was due to the lack of glycosylation, the first inkling of what would later be known as chaperone-assisted N-glycoprotein proofreading or the calreticulin/calnexin cycle in the ERAD response to unfolded proteins (80). Nowadays, tunicamycin is commonly used to induce the “unfolded protein response” or ER stress, unfortunately often without paying attention to its effect on N-glycoprotein biosynthesis. Because of its toxicity tunicamycin has never made its way into virus therapy, although recent studies suggest that it may have antiviral effects against Hepatitis C virus at subtoxic doses (86).

Another antibiotic acting on N-glycoprotein biosynthesis and isolated from *Streptomyces*, the imino sugar deoxynojirimycin (dNM or dNJ) was also discovered more than 40 years ago. It was identified as a member of the validamycin family in a screening assay for antifungal agents that would inhibit the breakdown of trehalose, the storage disaccharide in insects and fungi (10). Deoxynojirimycin is an inhibitor of α -glucosidases and was first designed for treatment of type II diabetes because of its ability to prevent the breakdown of amylase (starch) and mobilization and uptake of glucose in the intestine. Nowadays, acarbose, a natural tetrasaccharide containing a structural isomer of deoxynojirimycin is widely used as an oral medication for diabetes type II. Deoxynojirimycin itself has rather shaped the history of research on N-glycoprotein processing than being used in diabetes therapy.

At the time when acarbose and dNM were discovered, it was well known that neuraminidase-sensitive, glycoprotein-bound sialic acid is important in virus agglutination. Gilbert Ashwell and Anatol Morell reported for the first time a galactose-specific receptor for asialoglycoproteins (87-88). Yet, besides knowing that many glycoprotein-linked oligosaccharides contained terminal sialic acid, units of galactose-4- α -N-acetylgalactosamide, and other di-, tri-, and tetrasaccharides, the actual (branched) structure of the N-linked glycan remained unclear until 1978, when Ellen Li and Stuart Kornfeld proposed a structure with branched mannose chains linked to asparagine via N-acetylglucosamine (89). The 1970's and early 1980's became a well-spring of new discoveries in glycobiology. William Lennarz worked out the biosynthesis pathway of the dolichol-linked precursor oligosaccharide and Amando Parodi reported the "en bloc" transfer of this precursor onto the nascent glycoprotein (90-92).

Deoxinojimycin and other inhibitors such as castanospermine played essential roles in this discovery since they inhibited not only broad spectrum α -glucosidases, but also glucosidase I and II, which are the other components of the N-glycan-driven proofreading machinery. Similar to tunicamycin, dNM and castanospermine prevented correct folding and secretory exit of viral N-glycoproteins and induced ER-resident protein degradation instead. However, inhibitors of Golgi mannosidases such as swainsonine or deoxymannojimycin, did not. Based on this observation and the concurrent characterization of GlcMan9GlcNAc2-binding ER chaperones, Ari Helenius then proposed the calreticulin/calnexin cycle, which is probably the most prominent example for the general function of N-glycans (38). Besides their value in understanding the unfolded protein response to N-glycoproteins, glucosidase I and II inhibitors were also instrumental in the discovery of the M6P-dependent pathway by Kurt von Figura in 1984 (70).

I came into contact with dNM through my graduate student mentor Dr. Gunter Legler, an excellent biochemist of fine and modest character, who majorly contributed to our understanding of the catalytic mechanism of α - and β -glucosidases. He and his mentee, who then became my first post-doctoral mentor, Dr. Ernst Bause, were among the first to use dNM and its derivatives to characterize and purify glycosidases, including those involved in trimming of N-glycoproteins (49, 59, 93-94). At this time, the pre-human genome era, one could not just "blast search" for cDNA sequences, but actually had to purify a protein to homogeneity and then identify the amino acid sequence to synthesize oligonucleotide primers that could be used for RT-PCR to generate a probe for screening of a lambda gt11 library, and eventually, isolate the protein-specific cDNA; a long-forgotten and extremely tedious technique. Using alkylated dNM derivatives for affinity chromatography of trimming enzymes proved to be extremely helpful in this endeavor. Despite of this progress, it took us several years and thousands of plasmid minipreps to generate specific probes that then led to the first cloned cDNAs for glucosidase I and Man9 mannosidase (50, 60-61).

Currently, alkylated dNM derivatives such as N-butyl dNM are tested as antibiotics for treatment of several viral diseases, including HIV (11, 95). Grabowski's and Legler's groups have reported that alkylated dNM derivatives inhibit the two enzymes that regulate the metabolic conversion of ceramide and glucosylceramide into each other, lysosomal β -glucosidase (the "Gaucher enzyme") and glucosyltransferase, with longer alkyl chain length

being more specific for β -glucosidase and shorter chain length for glucosyltransferase (96-101). Therefore, N-butyl dNM (miglustat) has been discussed for treatment of several lysosomal storage diseases involving accumulation of sphingolipids, including Gaucher disease (glucosylceramide accumulation), Niemann-Pick disease (sphingomyelin accumulation), and Tay Sachs or Sandhoff disease (GM2 accumulation) (10, 97, 102-109). While the use of trimming enzyme inhibitors has not (yet) led to breakthroughs in virus therapy, dNM and swainsonine have certainly been invaluable in elucidating the sequence of trimming enzymes and the function of N-glycans. One of these more recently discovered functions intertwines N-glycoprotein processing with glycolipid biosynthesis: the role of N-glycosylation in the subcellular localization and enzyme complex formation of glucosyltransferases in ganglioside biosynthesis.

4. Sweet encounters of proteins and lipids: N-glycans affect the subcellular distribution and complex formation of enzymes in glycolipid biosynthesis

Glycosyltransferases not only transfer sugar residues onto protein linked-glycans, but also onto lipids, in particular sphingolipids. Glycosphingolipids are synthesized from ceramide, a sphingolipid consisting of sphingosine attached to various fatty acids, by sequential glycosylation reactions catalyzed by a series of ER- or Golgi-resident glycosyltransferases (110-115). As expected, the substrate specificity of these enzymes is different from that of glycosyltransferases in N-glycoprotein biosynthesis and processing. After attachment of glucose and then galactose to ceramide, which generates lactosylceramide, the most basic ganglioside, GM3, is synthesized by attachment of sialic acid. This reaction catalyzed by GM3 synthase is followed by enzymatic reactions that split ganglioside biosynthesis into three distinct pathways: a-, b-, and c-series gangliosides (116-124). If N-acetyl galactosamine is the next sugar residue added to GM3, a reaction catalyzed by GM2/GD2 synthase, ganglioside biosynthesis will exclusively follow the a-series pathway. However, if another sialic acid residue is added first, a reaction catalyzed by GD3 synthase, then ganglioside biosynthesis follows the b- or c-series pathway. Note that GD3 synthase can only act on GM3 (thereby generating GD3), while GM2/GD2 synthase can use GM3 (thereby generating GM2) or GD3 (thereby generating GD2) as the substrate. Therefore, the relative location of GD3 and GM2/GD2 synthase determines which pathway of ganglioside biosynthesis is taken. If GM2/GD2 synthase acts first, only a-series gangliosides are made, whereas a sequential reaction of first GD3 synthase and then GM2/GD2 synthase channels ganglioside biosynthesis towards the b-series pathway. Likewise, c-series gangliosides are made if GT3 synthase acts on GD3 before GM2/GD2 synthase does.

Regulation of biosynthetic pathways by the relative location of enzymes in a reaction sequence is not just of academic curiosity, but may actually determine the composition of gangliosides in an organism, tissue, or cell. Robert K. Yu, whose laboratory I joined after my post-doctoral work on N-glycoproteins, has found 30 years ago that the ganglioside pathways undergo a rapid switch from a- to b-series during embryonic brain development, just at the time point when neuroprogenitors start to divide asymmetrically and many intermediate neurons are born (116, 118, 122, 125). He continued to study this fascinating regulatory phenomenon and its biological function till today and will certainly do so in future. His passion gave me the opportunity to pursue my own ideas on the regulation of

ganglioside biosynthesis by the interdependence of glycosylation reactions in glycolipid and glycoprotein biology.

To understand the meaning of this interdependence one has to know that most glycosyltransferases are type II transmembrane proteins with 3-4 N-glycosylation sites. Hence, bearing in mind what we have discussed before – N-glycans are critical for protein folding and transport – N-glycoprotein processing may regulate the subcellular localization, and therefore relative location, of glycosyltransferases in ganglioside biosynthesis. I quickly realized that I was not the only one who pursued this idea. Hugo Maccioni's and our group published in 1998 that inhibition of trimming glucosidases I and II with dNM and castanospermine, but not inhibition of Golgi mannosidase I and II with deoxymannojirimycin and swainsonine prevented transport of GD3 synthase from the ER to the Golgi (126-127). There was some discrepancy between both studies with respect to the effect of glucosidase inhibition on enzyme activity. While in Maccioni's study, dNM and castanospermine still preserved the activity of GD3 synthase, castanospermine increased proteolytic turnover of this enzyme in our study. Since it was known from the Helenius model that N-glycans are required to achieve chaperone-assisted protein folding via the calreticulin/calnexin cycle, both groups concluded that N-glycosylation was necessary to attain and maintain catalytic activity, while glucose trimming was required for the ER-to-Golgi exit of the enzyme(126-127).

In the following years, Maccioni's group made great strides toward understanding the regulation of glycosyltransferase transport and enzyme complex formation, and its significance for glycolipid biosynthesis (112-113, 128-135). However, it still remained unclear how this would switch a-to b-series gangliosides and whether N-glycans are actually critical for this pathway switch. I was working in Bob Yu's group on this problem and discovered that GD3 synthase forms a disulfide bridge-mediated homodimer that turns into a heterodimer with GM2/GD2 synthase (136). Interestingly, binding to GM3 as well as inhibition of trimming by glucosidase I and II retained the GD3 synthase homodimer in the ER, suggesting that the enzyme-substrate complex may participate in protein folding or transport; GM3 as a "lipid co-chaperone" so to speak (Fig. 3). The observation of enzyme (-substrate) complexes in ganglioside metabolism was in line with Saul Roseman's original idea that the biosynthesis of specific gangliosides is best achieved by forming multi-enzyme complexes of glycosyltransferases ("cooperative sequential specificity" model) (137). Moreover, by forming the complex between GD3 synthase and GM2/GD2 synthase, we calculated that ganglioside biosynthesis would be efficiently channeled into the b-series pathway, an enzymatic switch that provided an explanation for the rapid change in the composition of gangliosides during embryonic brain development (125, 136).

In addition to the GD3 synthase homodimer and GD3 synthase-GM2/GD2 synthase heterodimer complex identified by Bob Yu's group, Hugo Maccioni's group discovered that enzyme complexes were also formed between LacCer-, GM3-, and GD3-synthase, and GM2/GD2- and GM1/GD1a synthase (112, 129-130, 132, 134-135). Common to two of these glycosyltransferase complexes described so far is that the stability and/or subcellular localization of at least two of their subunits, GD3 synthase and GM1/GD1a synthase, are critically depending on N-glycosylation and trimming by glucosidase I and II. Moreover,

inhibition of this trimming (by castanospermine) dramatically changes the ganglioside composition by preventing synthesis of higher sialylated, complex b-series gangliosides such as GT1b, the most prominent ganglioside after the “a-to-b series switch” in mouse (and human) embryonic brain at a time point of intense neural progenitor (and intermediate neuron) proliferation and migration (136). It should be noted, that Bob Yu’s group not only was the first to discover this ganglioside pathway switch, but also demonstrated that the simplest b-series ganglioside, GD3, is a robust cell surface marker for mouse (and human) neural progenitor cells (115, 138-139). Yet, it remains to be determined, which functional role the pathway switch plays for brain development and how it is integrated with the regulation for N-glycan processing of glycosyltransferases in ganglioside metabolism.

Conclusions and epilogue: the tale of the tail that wags the dog

At the end of this chapter on N-glycosylation and N-glycoprotein processing, one may miss a discussion of the important functions that cell surface N-glycans play in cell-to-cell recognition and adhesion, in particular in the brain. For example, galectins, cell surface lectins that bind to N-acetylgalactosamine in N-linked glycans, have been found to regulate growth factor receptor endocytosis/recycling, which may contribute to glioma metastasis (140). Another non-discussed example for lectin-like cell surface binding is the interaction of myelin-associated glycoprotein (MAG) with specific gangliosides, which has been suggested to be critical for myelination of axons (141). Loss of b-series complex gangliosides as well as abnormal N-glycoprotein processing of MAG leads to severe nervous system symptoms such as Wallerian degeneration and demyelination (142-143). The tail (protein or lipid-linked glycan) wags the dog (neuron or glia), so to speak.

One may also criticize that other types of protein glycosylation such as O-glycosylation or protein-associated glycans in general, such as proteoglycans are not mentioned (see (20) for a comprehensive review on these). The reason for this is twofold: for one, these glycoconjugates are reviewed in other chapters of this book [and cross-references were made whenever appropriate]. However, more importantly, I wanted to focus on biological processes that are dynamically regulated by the morphing and reshaping of protein-linked N-glycans. As we have seen, the glycosylation and trimming machinery is intimately connected with proofreading and editing of N-glycoproteins. In this regard, it should be noted that the stability, subcellular distribution, and complex formation of glycosyltransferases in ganglioside biosynthesis is the first example of enzymes in a metabolic pathway that may actually be regulated by N-glycoprotein processing. Moreover, as we have discussed, the function of N-glycoprotein processing is by far not completely understood and may involve substrates such as the ganglioside GM3 as “lipid co-chaperones”. And finally, the interdependence between protein-linked N-glycan processing and glycolipid metabolism, a theme that brought me as a researcher trained in glycoprotein biology to pursue studies on glycolipids, holds promise to make future discoveries in uncharted territories with impact on systems biology; a fascinating area of research that tears down the boundaries between over-specialized disciplines in biology and goes back to a more classical, Humboldtian view on life as emerging from the self-organized biology of interacting metabolic systems, such as glycoprotein processing and glycolipid biosynthesis. Therefore, this chapter is not only meant to give an account of what is known about N-

glycosylation (certainly not in an exhaustive manner), but also to ignite interest in young scientists to pursue this area of research in their careers.

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Figure 1. Co-translational, “en bloc” transfer of the Glc3Man9GlcNAc2 precursor oligosaccharide from dolichol pyrophosphate to asparagine, catalyzed by oligosaccharyl transferase in the ER lumen

Figure 2. N-glycoprotein processing: sequential removal (trimming) of glucose and mannose residues from the N-linked glycan in the ER and Golgi, followed by re-glycosylation
N-glycan processing generates signals for chaperone-assisted refolding, mannose-6-phosphate receptor-mediated transport of lysosomal enzymes, and other functions in protein trafficking, enzyme complex formation, and cell adhesion.

Figure 3. Regulation of ganglioside biosynthesis pathways by N-glycan-dependent glycosyltransferase distribution and complex formation

Inhibition of trimming by glucosidase I and II increases proteolytic turnover of GD3 synthase and prevents enzyme complex formation with GM2/GD2 synthase in the Golgi, suggesting that N-glycoprotein processing of glycosyltransferases is critical for ganglioside metabolism. The GD3 synthase-GM2/GD2 synthase complex is hypothesized to promote b-series complex ganglioside biosynthesis. Moreover, our group has proposed that binding of GD3 synthase to GM3 may facilitate enzyme complex formation (“lipid co-chaperone” hypothesis).