Glycoconjugate Journal 17, 465–483, 2000

The joys of HexNAc. The synthesis and function of *N*- and *O*-glycan branches

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This review covers discoveries made over the past 30–35 years that were important to our understanding of the synthetic pathway required for initiation of the antennae or branches on complex *N*-glycans and *O*-glycans. The review deals primarily with the author's contributions but the relevant work of other laboratories is also discussed. The focus of the review is almost entirely on the glycosyltransferases involved in the process. The following topics are discussed. (1) The localization of the synthesis of complex *N*-glycan antennae to the Golgi apparatus. (2) The ''evolutionary boundary'' at the stage in *N*-glycan processing where there is a change from oligomannose to complex *N*-glycans; this switch correlates with the appearance of multicellular organisms. (3) The discovery of the three enzymes which play a key role in this switch, *N*-acetylglucosaminyltransferases I and II and mannosidase II. (4) The ''yellow brick road'' which leads from oligomannose to highly branched complex *N*-glycans with emphasis on the enzymes involved in the process and the factors which control the routes of synthesis. (5) A short discussion of the characteristics of the enzymes involved and of the genes that encode them. (6) The role of complex *N*-glycans in mammalian and *Caenorhabditis elegans* development. (7) The crystal structure of *N*-acetylglucosaminyltransferase I. (8) The discovery of the enzymes which synthesize *O*-glycan cores 1, 2, 3 and 4 and their elongation.

Keywords: *N*-acetylglucosaminyltransferases; glycosylation; glycoproteins; Golgi complex; evolution; development

Introduction

In 1991, I wrote a review entitled "The yellow brick road to branched complex *N*-glycans" [1]. The analogy was intended to illustrate the many choices that are available in the synthetic pathway towards complex *N*-glycans. The same analogy applies to the often serendipitous routes that scientists follow during their careers. This review will attempt to give an overview of some of the routes taken by research on *N*- and *O*-glycan branching over the past 35 years. I hope the reader will forgive me for drawing heavily on my own research and experiences, since this is, in part, what I was requested to do by the Chief Editor of Glycoconjugate Journal; however, I will also try to describe, although somewhat briefly, the contributions of other laboratories.

I obtained my PhD at the University of Toronto's Department of Biochemistry in 1964 under the guidance of Gordon Dixon, a brilliant scientist who, together with George Connell and Oliver Smithies, was at that time working on the structure and genetics of human serum haptoglobin [2,3]. Although my project with Dixon had to do with the structure of chymotrypsin [4], I was asked to carry out sugar analyses on haptoglobin. I was intrigued partly by the fact that the thiobarbituric acid assay for sialic acid gave a beautiful colour but mainly by the realization that very little was known at that time on the biosynthesis of protein-bound glycans. I therefore decided to work on glycosyltransferases with Saul Roseman at Johns Hopkins University in Baltimore MD.

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The role of the Golgi apparatus in glycosylation

I was indeed fortunate to have men of the high calibre of Dixon and Roseman as my mentors at such an early stage of my career. My two years with Saul (1966–68) were an absolute delight. Both the Orioles baseball team and Saul's laboratory were at the height of their successes at that time. Saul and I had the most turbulent and most wonderful arguments! He has mellowed lately but if you try, you can still get him to rise to the bait. My work in Baltimore laid the groundwork for my entire future career. From Saul and his students at the time (Ed McGuire, Steve Roth, Subhash and Manju Basu, Bernie Kaufman and others) and from students who had previously worked with Saul (Bill Jourdian, Ed Kean, Don Carlson), I learned the fundamentals of glycosyltransferases, particularly

how to make the radioactive nucleotide-sugars essential for our work [5]. For example, together with the late Ed Heath, we developed a method for making radioactive GDP-beta-L-fucose [6].

There was an interesting spin-off from the GDP-fucose project. One of the enzymes required was L-fucose kinase which was used to make beta-L-fucopyranosyl-1-phosphate. My assay for this enzyme was based on the conversion of neutral fucose to a negatively charged product. In the belief that I had isolated a crude fucose kinase preparation, I subjected several hundred dollars worth of radioactive L-fucose to my enzyme and found, shortly after, that I had made L-fuconate. Needless to say Saul was not pleased but I think I was eventually forgiven since the pig liver L-fucose dehydrogenase that I had inadvertently discovered led to a rapid enzymatic assay for L-fucose [7,8] and to a mammalian pathway for oxidative degradation of L-fucose to carbon dioxide [9–12].

However, I think that the most important work that came out of my stay in the Roseman laboratory was the first demonstration that the specific activities of several glycosyltransferases are increased by similar amounts in a Golgi apparatus-enriched rat liver membrane fraction [13]. Our investigation was inspired by a large body of data from many laboratories, described in detail in a rather long review that Lennart Rodén and I published in 1973 [14]. The reader is referred to this review for references. The incorporation of radioactive monosaccharide precursors into glycoproteins, using both whole animals and various tissue preparations, had established the endoplasmic reticulum as a major site of carbohydrate incorporation. There was evidence indicating (i) that the Asn-GlcNAc bond is formed primarily on nascent polypeptide during its assembly on membrane-bound ribosomes and passage into the lumen of the rough endoplasmic reticulum; (ii) that lipid carriers may be involved in this reaction; and (iii) that further sequential monosaccharide addition occurs as the polypeptide moves through the lumen from rough to smooth-surfaced endoplasmic reticulum.

The laboratory of C.P. Leblond in Montreal, in a series of elegant papers, had demonstrated by radioautography after incorporation of radioactive monosaccharide precurors and by histochemistry using the Periodic Acid-Schiff (PAS) stain that the Golgi apparatus is the major site for glycosylation of proteins destined for export or the cell surface; I recall Leblond giving a Gordon Conference lecture dressed completely in the pink colour of the PAS stain—suit, shirt, tie—everything! Roseman's group had developed assays for the sialyltransferase (Sialyl-T), galactosyltransferase (Gal-T) and *N*-acetylglucosaminyltransferase (GlcNAc-T) that synthesize the sialyl-Gal-GlcNAc trisaccharide at the non-reducing termini of many complex *N*-glycans. The group of D.J. Morré (and others) had developed methods for preparing membrane fractions from plants and rat liver enriched in Golgi apparatus [15–18]. These ideas and methods led us to show that Sialyl-T, Gal-T and GlcNAc-T can be enriched 10- to 63-fold in a Golgi apparatus preparation [13,19]; enrichments of 100-fold or more were subsequently achieved by others using more efficient methods of Golgi fraction preparation. The laboratory of Becca Fleischer carried out similar experiments at about the same time [20]. It is now established that the endoplasmic reticulum and Golgi apparatus form an endomembrane assembly line along which membrane-bound glycosyltransferases and glycosidases process protein-bound *N*-glycans as the protein moves through the lumen towards its final destination (secretion, cell surface or lysosome) [21,22].

N-glycan processing and the discovery of *N*-acetylglucosaminyltransferases I and II and alpha-3/6-mannosidase II

The Fourth International Symposium on Glycoconjugates held in Woods Hole, Mass, on Sept. 26–Oct. 1, 1977, was a truly extraordinary event in the history of glycobiology. Three major discoveries were presented at this meeting. (i) Hans Vliegenthart's group used 360 MHz high resolution proton nuclear magnetic resonance spectroscopy (NMR) on well-characterized oligosaccharides supplied by Jean Montreuil's group to provide a detailed methodology for the determination of oligosaccharide fine structure [23–28]. (ii) Robert Hill's group showed us for the first time how to use affinity chromatography to purify glycosyltransferases [29–31]. (iii) The groups of Stuart Kornfeld, Phil Robbins and Donald Summers, in three independent studies using viral envelope glycoproteins as probes, provided evidence that complex *N*-glycans are derived from a single high molecular weight $Glc_3Man_9GlcNAc_2$-pyrophosphate-dolichol (lipid-linked oligosaccharide, LLO) precursor by a unique *N*-glycan processing pathway [32–36]. All three of the above discoveries were "quantum leaps" which were highly relevant to my future research.

As a sad point of interest, the Woods Hole meeting was also the occasion of the death of Ward Pigman, a pioneer in mucin research. There was a rather unusual abstract submitted to this meeting by Montreuil's group from Lille, France, showing the words and music of a French song entitled "How to Enjoy Glycoconjugates". We were all supposed to sing this song at the end of the sessions but the celebration was cancelled on news of Pigman's death.

After my return to Toronto in late 1968, I began what proved to be important collaborations with three scientists, Jeremy Carver, Lou Siminovitch and Pamela Stanley. During a casual conversation with Siminovitch, a well-known Toronto geneticist, I learned that there was a post-doctoral fellow in his laboratory by the name of Pamela Stanley who had isolated some lectin-resistant somatic cell mutants using Chinese hamster ovary (CHO) cells. They suspected the mutations had something to do with abnormal glycosylation. Lou asked: "Are you interested?" Luckily for me, I replied: "Sure, why not?". This epic (for me) conversation took place, as I recall, in the all-male swimming pool of the University of Toronto;

I hasten to assure any angry reader that the pool has since been made available to both men and women.

The *N*-acetylglucosaminyltransferase (GlcNAc-T) described by Roseman's group in 1966 [37] was a crude preparation from goat colostrum which incorporated GlcNAc from UDP-GlcNAc into glycoproteins such as alpha-1-acid glycoprotein after treatment with sialidase, beta-galactosidase and beta-*N*-acetylglucosaminidase ("first generation" acceptor substrates) to expose the terminal mannose residues in the *N*-glycan core, Man-alpha-1,6[Man-alpha-1,3]Man-beta-1,4-GlcNAc-beta-1,4-GlcNAc-Asn-X. At that time, it was not known whether one or more separate GlcNAc-Ts were required to initiate the antennae of complex *N*-glycans (Figure 1). Among Jeremy Carver's interests was the binding of carbohydrates to lectins. We collaborated with Jeremy on the purification by high voltage electrophoresis in borate buffer of various glycans and their characterization by NMR [38–40]. The Roseman GlcNAc-T assay was subsequently modified by replacing the glycoprotein acceptor with well-characterized low molecular weight asymmetric oligosaccharides isolated from a human multiple myeloma IgG (Tem) [40]. This use of such "second generation" acceptor substrates isolated from naturally occuring glycoproteins resulted in more specific enzyme assays and allowed accurate product identification by NMR.

Stanley and Siminovitch had isolated several clones of CHO cells resistant to the cytotoxicity of the phytohemagglutinin from *Phaseolus vulgaris* (L-PHA); these clones showed decreased lectin binding to the cell surface. On applying our new GlcNAc-T assays to extracts of these cells, we found a 96% decrease in transferase activity relative to wild type cells with Man-alpha-1,6[Man-alpha-1,3]Man-beta-R as exogenous acceptor but no decrease with Man-alpha-1,6[GlcNAc-beta-1,2-Man-alpha-1,3]Man-beta-R [41,42]. No differences between lectin-resistant and wild-type cells were noted for several other glycosyltransferases. Thus the GlcNAc-T acting on the Man-alpha-1,3 arm of the *N*-glycan core, which we called UDP-GlcNAc:alpha-3-D-mannoside beta-1,2-*N*-acetylglucosaminyltransferase I (GlcNAc-T I, EC 2.4.1.101), was absent in the lectin-resistant cells (Lec1 CHO mutant cells). This results in defective glycosylation of lectin-binding glycoproteins on the cell surface [43]. These experiments proved the existence of a second GlcNAc-T which we called UDP-GlcNAc:alpha-6-D-mannoside beta-1,2-*N*-acetylglucosaminyltransferase II (GlcNAc-T II, EC 2.4.1.143). The data showed that GlcNAc-T II requires the prior action of GlcNAc-T I (Figure 2).

The groups of Stuart Kornfeld [35,44,45] and Colin Hughes [46] isolated similar somatic cell mutants lacking GlcNAc-T I from, respectively, CHO cells (CHO clone 15B) and BHK cells (ricin-resistant baby hamster kidney cell clone Ric^{R14}). The CHO 15B mutant line was shown to have decreased GlcNAc-T activity towards various glycopeptide and glycoprotein acceptors containing terminal nonreducing alpha-linked mannose residues [45], to lack complex *N*-glycans and to accumulate [Man-alpha-1,6(Man-alpha-1,3)Man-alpha-1,6][Man-alpha-1,3]Man-beta-R where R is 1,4-GlcNAc-beta-1,4-GlcNAc-Asn-X($Man_5GlcNAc_2Asn$-X) [47]. The structures of two other glycopeptides found in smaller quantities in clone 15B but not detected in the parent cells were Man-alpha-1,6[Man-alpha-1,3]Man-alpha-1,6-Man-beta-R and Man-alpha-1,3-Man-alpha-1,6[Man-alpha-1,3]Man-beta-R [47], presumably derived by removal of one Man residue from $Man_5GlcNAc_2Asn$-X. The conclusion was drawn that the physiological substrate for GlcNAc-T I is $Man_5GlcNAc_2Asn$-X rather than $Man_3GlcNAc_2Asn$-X and that, therefore, there must be a GlcNAc-T I-dependent alpha-3/6-mannosidase acting in the lumen of the Golgi apparatus to remove two Man residues from the product of GlcNAc-T I [48] (Figure 2). We later proved by direct assay the existence of such a mannosidase (now called alpha-3/6-mannosidase II) in rat liver and showed that it has an absolute requirement for the prior action of GlcNAc-T I [49]. In the test-tube, however, we have shown that GlcNAc-T I also acts quite efficiently on Man-alpha-1,6[Man-alpha-1,3]Man-beta-R′ and even on Man-alpha-1,3-Man-beta-R′ where R′ can be an aglycone; the ability of GlcNAc-T I to act on $Man_3GlcNAc_2Asn$-X provides an alternate mannosidase II-independent processing pathway under certain abnormal conditions such as glucose deprivation or in cells unable to synthesize

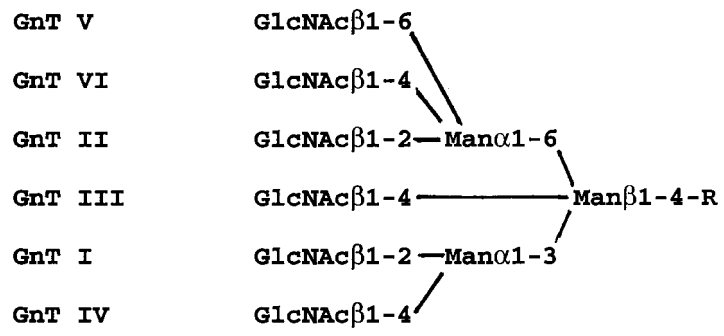

Figure 1. *N*-acetylglucosaminyltransferases I to VI (GnT I–VI) incorporate GlcNAc residues into the Man-alpha-1,6[Man-alpha-1,3]Man-beta-R *N*-glycan core, where R is 1,4-GlcNAc-beta-1,4-GlcNAc-Asn-X.

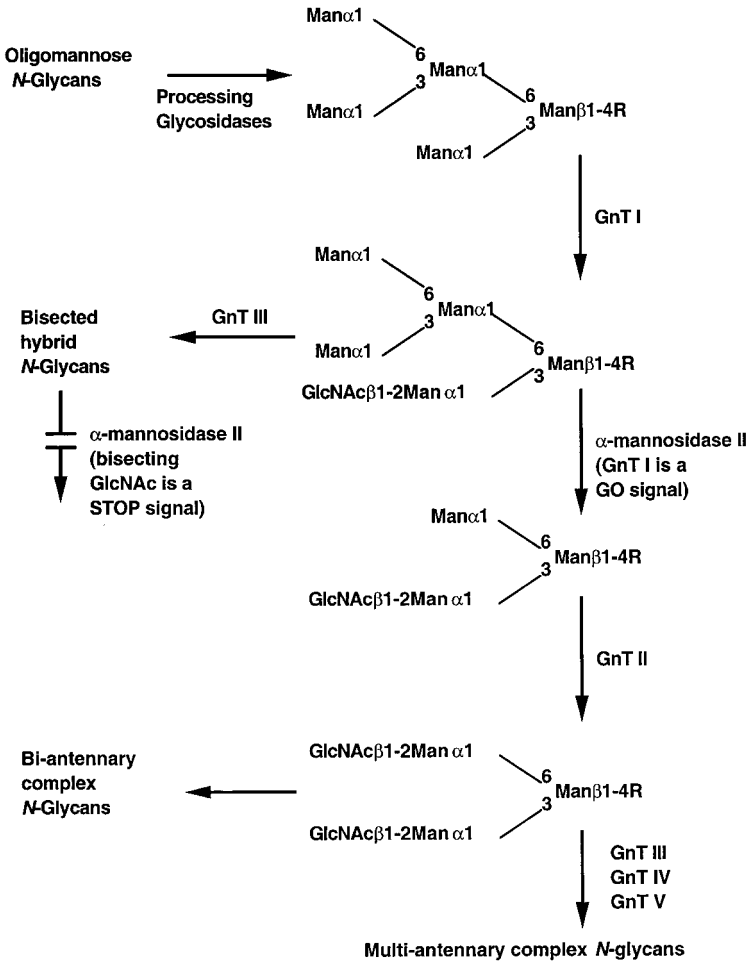

Figure 2. Conversion of oligomannose *N*-glycans to hybrid and complex *N*-glycans. *N*-acetylglucosaminyltransferase I (GnT I) adds a GlcNAc in beta-1,2 linkage to the Man-alpha-1,3-arm of $Man_5GlcNAc_2Asn$-X. Alpha-3,6-mannosidase II removes two Man residues and *N*-acetylglucosaminyltransferase II (GnT II) adds a GlcNAc in beta-1,2 linkage to the Man-alpha-1,6-arm of $GlcNAcMan_3GlcNAc_2Asn$-X to initiate the process of complex *N*-glycan synthesis. *N*-acetylglucosaminyltransferase III (GnT III) competes with alpha-3,6-mannosidase II and, if successful, sends the pathway towards bisected hybrid *N*-glycans.

dolichol-phosphate-mannose [21,50]. Mannosidase II was purified and characterized in Oscar Touster's laboratory [51–53] and was eventually cloned by Kelley Moremen [54–56].

The "Yellow Brick Road" to branched complex *N*-glycans

Whereas all eukaryotes share a common *N*-glycan biosynthetic and processing pathway up to the oligomannose type *N*-glycans, there is an evolutionary boundary between unicellular and multicellular eukaryotes in that only the latter can make complex *N*-glycans. As will be discussed below, there is now good evidence to suggest that multicellular organisms need cell surface complex *N*-glycans for normal embryogenesis and development probably because cell surface glycans serve as a language of communication between various cell surface ligands and receptors.

The synthesis of complex *N*-glycans can be divided into three distinct stages. The first stage occurs primarily in the cytoplasm and rough endoplasmic reticulum and involves the synthesis of $Glc_3Man_9GlcNAc_2$-pyrophosphate-dolichol. The second stage begins with the transfer of $Glc_3Man_9GlcNAc_2$ from $Glc_3Man_9GlcNAc_2$-pyrophosphate-dolichol to an Asn residue of the nascent glycoprotein followed by processing to $Man_5GlcNAc_2$-Asn-X. The third stage occurs primarily in the Golgi apparatus and starts when GlcNAc-T I transfers a GlcNAc residue in beta-1,2 linkage to the Man-alpha-1,3 arm of $Man_5GlcNAc_2$-Asn-X followed by the removal of two mannose residues by mannosidase II to form the substrate for GlcNAc-T II (Figure 2). GlcNAc-T II transfers GlcNAc in beta-1,2 linkage to the Man-alpha-1,6 arm of the product of mannosidase II (Figure 2). The first and second stages are evolutionary predecessors of the third stage and it is likely that the enzymes required for initiation of the third stage (GlcNAc-T I, mannosidase II and GlcNAc-T II) appeared shortly before multicellular eukaryotes.

Once GlcNAc-T I and mannosidase II have acted, the stage is set not only for the initiation of a second antenna by

GlcNAc-T II but also for the actions of various other enzymes, in particular GlcNAc-T III to VI (Figure 1) and the core fucosyltransferases. GlcNAc-T I is an essential prerequisite for the actions of GlcNAc-T II, III and IV, mannosidase II and core alpha-1,6-fucosyltransferase [1,57–61]. GlcNAc-T V and VI require the prior action of GlcNAc-T II and therefore, indirectly, GlcNAc-T I is required for the subsequent actions of these enzymes. GlcNAc-T I action is also a prerequisite for the activities of GlcNAc-T II and beta-1-2-xylosyltransferase in plants [62] and in snails [63] and of the core alpha-1,3-fucosyltransferase which adds fucose in alpha-1,3 linkage to the Asn-linked GlcNAc in plants and insects [60,64].

The synthetic pathway towards complex *N*-glycans is not linear but has many branches, i.e., more than one enzyme may act at various intermediates. This is reminiscent of the problems facing Dorothy and her friends on their way to the Emerald City along the Yellow Brick Road in the land of Oz. For example, if GlcNAc-T III acts on the product of GlcNAc-T I before mannosidase II to form the bisected hybrid structure (Figure 2), the pathway is committed to hybrid structures because mannosidase II cannot act on bisected oligosaccharides. The reverse order of action leads to complex *N*-glycans. The relative abundance of GlcNAc-T III and mannosidase II in a particular tissue controls the pathway towards hybrid or complex *N*-glycans, i.e., the route taken at such divergent branch points is dictated primarily by the relative activities of enzymes which compete for a common substrate. The insertion of a bisecting GlcNAc by GlcNAc-T III prevents the actions of GlcNAc-T II, IV and V, mannosidase II and core alpha-1,6-fucosyltransferase and is an example of a glycosyl residue acting as a STOP signal whereas the action of GlcNAc-T I is a GO signal (Figure 2). Competition and STOP and GO signals serve as "substrate-level" controls of the biosynthetic pathways as opposed to "gene-level" control factors at the transcriptional or translational levels [57]. These ideas were developed by reading the work of Winifred Watkins [65] on the synthesis of the human ABO blood group carbohydrate antigens and by many stimulating talks with André Verbert, Bernard Fournet, Jean Montreuil and my other Lille friends during the EMBO courses on Glycoconjugates held in Lille, France, every two years. It is sad that both Fournet and Verbert died suddenly in the prime of their lives and careers.

Characterization of the GlcNAc-T proteins and genes

GlcNAc-T I and II have been partially purified from various sources [66–68]; rabbit GlcNAc-T I [69] and rat GlcNAc-T II [70] were eventually purified to homogeneity. GlcNAc-T III, IV and VI (Figure 1) were first described by our group [71–75]. GlcNAc-T V was first described by Cummings, Trowbridge and Kornfeld [76] using a lectin-resistant mouse lymphoma cell line ($PHA^R2.1$) that is deficient in GlcNAc-T V; this line is unable to synthesize tetraantennary and certain triantennary complex *N*-glycan species. The total content of repeating N-acetyllactosamine units is greatly decreased in this line indicating that the repeating N-acetyllactosamine sequence is confined primarily to the GlcNAc-beta-1,6-Man-alpha-1,6 arm of complex *N*-glycans [77]. GlcNAc-T III [78], IV [79] and V [80,81] have been purified from various sources.

We have carried out extensive kinetic and substrate specificity analyses on GlcNAc-T I and II. This work would have been impossible without the help of Hans Paulsen, one of the world's great synthetic carbohydrate chemists, and some of his students (especially Folkert Reck who visited our laboratory as a post-doctoral fellow) [69,82–93]. I first met Paulsen, who works in Hamburg, Germany, at the 11th International Carbohydrate Symposium, Vancouver, Canada, in 1982, and we started a highly productive collaboration which consisted primarily of receiving from Hans a large number of low molecular weight synthetic oligosaccharide acceptor substrates and their analogues. These "third generation" synthetic substrates had the same advantages provided by the "second generation" acceptor substrates, i.e., specific assays and accurate product identification. However, in addition, they allowed rapid assays with a minimum of the annoying "endogenous" background caused by the non-specific hydrolysis of radioactive nucleotide-sugars and by incorporation of label into unknown endogenous acceptors. This was achieved by applying the C18 Sep-Pak cartridge assay of Palcic *et al.* [94].

We showed that catalysis by both GlcNAc-T I [69] and II [95] occurs by an ordered sequential Bi-Bi mechanism in which UDP-GlcNAc-Mn^{2+} binds first and UDP leaves last. The minimum substrate requirement for GlcNAc-T I is Man-alpha-1,3-Man-beta-R [69,84]. Essential groups on the beta-linked Man residue are an unsubstituted equatorial hydroxyl at C-4 and an unsubstituted axial hydroxyl at C-2; modifications at C-6 of this residue cause variations in K_m but no major alterations in enzyme activity. Man-alpha-1,6(2-deoxy-Man-alpha-1,3)Man-beta-R is neither a substrate nor a competitive inhibitor indicating that a hydroxyl group at the C-2 position of the Man-alpha-1,3 residue is essential for binding of the substrate to the enzyme. Removal of the hydroxyl groups at C-3, 4 or 6 of the Man-alpha-1,3 residue leads either to a poor or inactive substrate [93]. Man-alpha-1,6(6-*O*-methyl-Man-alpha-1,3)Man-beta-octyl is a competitive inhibitor ($K_i = 0.76$ mM).

The mimimal substrate for GlcNAc-T II is Man-alpha-1,6(GlcNAc-beta-1,2-Man-alpha-1,3)Man-beta-R. The 2-deoxy-Man-alpha-1,6(GlcNAc-beta-1,2-Man-alpha-1,3) Man-beta-R analogue is a competitive inhibitor ($K_i = 0.13$ mM) but the other hydroxyl groups of the Man-alpha-1,6 residue are not essential for activity [88]. Substitution of the C-4 hydroxyl of the beta-linked Man residue but not its removal leads to an inactive substrate. GlcNAc-beta-1,2-Man-alpha-1,3-Man-beta-octyl is a good inhibitor of the enzyme ($K_i = 0.9$ mM) indicating that this trisaccharide moiety is required for substrate binding to the enzyme. It is highly likely that the GlcNAc-beta-1,2-Man-alpha-1,3-Man-beta- moiety will prove to be an essential binding site for all

the enzymes dependent on GlcNAc-T I action (GlcNAc-T II, III and IV, mannosidase II, core alpha-1,6- and alpha-1,3-fucosyltransferases, beta-1,2-xylosyltransferase).

In early 1989, I spent about a week in the laboratory of Richard Simpson in Melbourne, Australia. We wanted to clone some genes but the problem we had was to obtain amino acid sequences from the very tiny amounts of GlcNAc-T I and II protein that we had been able to isolate. Richard was a master at this game and, indeed, produced bits of sequence for us, both from rabbit GlcNAc-T I [69], and with the help of Brad Bendiak who had also visited his laboratory, from rat GlcNAc-T II [70]. In the summer of 1989, I spent 5 months in Utrecht, The Netherlands, working on the snail *Lymnaea stagnalis* in the laboratory of Hans Vliegenthart and Hans Kamerling. I was assigned a bright and eager young graduate student, Hans Mulder, and together with Marijke de Jong-Brink (at the Vrije Universiteit in Amsterdam) we collected snail parts and went about the business of describing several interesting snail glycosyltransferases [63,96–98]. While in Holland, I decided I had to learn some molecular biology so that I could understand what my students were doing. Therefore, while Mohan Sarkar and Eric Hull were learning this new art from Rob Dunn (a Toronto molecular biologist) in my Toronto laboratory, I struggled to do the same under the guidance of a very patient Dutch molecular biologist named J.W.M. Höppener. With further help from Kelley Moremen who taught us the intricacies of "mixed oligonucleotide-primed amplification of cDNA" [54], Mohan was successful in cloning rabbit GlcNAc-T I cDNA [99,100] and I managed to clone human GlcNAc-T I genomic DNA [101,102]. Pamela Stanley, in independent work and with a different approach, cloned the human GlcNAc-T I gene [103]. Giacomo D'Agostaro, our collaborator in Rome, Italy, used Simpson's rat GlcNAc-T II amino acid sequences to clone rat GlcNAc-T II cDNA [104,105] and Jenny Tan in Toronto used Giacomo's rat cDNA sequence to clone human GlcNAc-T II genomic DNA [106,107]. The genes encoding GlcNAc-T I to VI have now been cloned from several species (Table 1).

The open reading frames of both mammalian GlcNAc-T I [102] and II [107] are on a single exon. The 5′-flanking regions of the genes encoding both enzymes have multiple transcription initiation sites, lack a TATA box and are GC-rich

Table 1. *N*-acetylglucosaminyltransferases (GlcNAc-T) involved in complex *N*-glycan branching.

Enzyme	*Species*	*EC No.*	*Accession No.*	*References*
GlcNAc-T I	Rabbit	2.4.1.101	M57301	[100]
	Human		M61829	[102]
			M55621	[103]
	Mouse		X77487-8	[171]
			M73491	[121]
			L07037	[172]
	Rat		D16302	[173]
	Chicken		None	[174]
	Frog		None	[175]
	Chinese hamster		U65791-2	[176]
	Baby hamster		AF087456-7	[177]
	Caenorhabditis elegans		AF082010-12	[149]
	Drosophila melanogaster		AF251495	[152]
GlcNAc-T II	Human	2.4.1.143	U15128, L36537	[107]
	Rat		U21662	[105]
	Frog		None	[175]
	Pig		Y09537	[178]
	Caenorhabditis elegans		AF251126	[151]
	Drosophila melanogaster		None	Unpublished data
GlcNAc-T III	Human	2.4.1.144	D13789	[179]
	Rat		D10852	[78]
	Mouse		L39373	[180]
			U66844	[129]
GlcNAc-T IV	Chicken	2.4.1.145	Not cloned	[74]
	Bovine		AB000628	[79,181]
	Human		AB000616	[182]
GlcNAc-T V	Human	2.4.1.155	D17716	[183]
	Rat		L14284	[184]
	Chinese hamster		U62587-8	[185]
GlcNAc-T VI	Chicken		AB040608	[73,186,187]
	Fish		Not cloned	[188]

[108,109]; these properties are typical for a housekeeping gene. However, GlcNAc-T II may under some circumstances be under the control of Ets transcription factors [110]. However, unlike the GlcNAc-TV promoter [111], the GlcNAc-T II promoter is not activated by *src*.

The role of complex *N*-glycans in mammalian development

We have suggested above that the third stage of complex *N*-glycan synthesis (initiated by GlcNAc-T I) appeared in evolution just prior to the development of multicellular organisms probably because complex *N*-glycans are required for the cell-cell interaction process and normal development of multicellular animals. Studies on human Congenital Disorders of Glycosylation (CDG) and null mutations in mice and *Caenorhabditis elegans* provide support for this concept.

CDG are a group of autosomal recessive diseases with multisystemic abnormalities including a severe disturbance of nervous system development [112–114]. CDG Group I includes all defects in *N*-linked protein glycosylation due to deficiencies in the assembly of $Glc_3Man_9GlcNAc_2$-pyrophosphate-dolichol and/or its transfer to asparagine residues on the nascent polypeptides (Table 2). CDG Group II contains defects in the processing of *N*-glycans; there are only two types at this time, CDG-IIa and CDG-IIb (Table 2). CDG-IIa presents with failure to thrive, psychomotor retardation, facial dysmorphism, hypotonia, kyphoscoliosis, cardiac murmur associated with a ventral septal defect, osteopenia and frequent infection. CDG-IIa was the first CDG in which a specific mutation was determined, a fruitful collaboration between our laboratory and Jaak Jaeken (Leuven, Belgium) who first described CDG in 1980 [115]. Jaeken suspected a GlcNAc-T II defect in this disease from mass spectroscopic structural analyses of serum transferrin *N*-glycans carried out by Genevieve Spik and her group in Lille, France [116–118]. Jaeken was kind enough to send me a large number of blood cell samples from two unrelated CDG-IIa patients, the blood relatives of one of the patients and Belgian controls not related to the CDG patients. Much to our delight, our first assays showed a dramatic absence of GlcNAc-T II activity in both patient samples. We found two distinct point mutations in the human GlcNAc-T II gene (*MGAT2*) in the two CDG-IIa children [116,119,120].

Yet another stroke of very good luck, once again in Vancouver, led to my meeting Jamey Marth in 1990. The Federal Government of Canada had initiated a new Centres of Excellence program to encourage collaborations between Canadian universities which were far apart from one another due to the large size and low population density of the country. I met Jamey while attending a meeting of my network, The Protein Engineering Network of Centres of Excellence (PENCE). I tried to sound excited about the great potential of glycobiology research; as I recall, I suggested we try to convert a mouse to a yeast by knocking out GlcNAc-T I but Jamey claims he has no memory of this conversation. At any rate, we started a collaboration which has continued to this day and which resulted in mice with null mutations in the genes encoding GlcNAc-T I, II and III. Jamey continued his interest in "knock-out glycobiology" after he moved from Vancouver to La Jolla, CA, and has established collaborations with other glycobiologists [121–137].

Table 2. Congenital Disorders of Glycosylation (CDG).

Group/type	*Defect and defective gene (OMIM, Locus Link I.D. numbers in brackets)*[a]		*Acronym*
Group I	**Defects in *N*-linked protein glycosylation due to deficiencies in the assembly of the dolichylpyrophosphate linked oligosaccharide and/or its transfer to asparagine residues on the nascent polypeptides.**		**CDG-I**
Type Ia	Phosphomannomutase 2	*PMM2* (212065, 5373)	CDG-Ia
Type Ib	Phosphomannose isomerase	*MPI* (602579, 4351)	CDG-Ib
Type Ic	Dolichyl-PP-Glc: Man9GlcNAc2-PP-dolichyl alpha-1,3-glucosyltransferase	*ALG6* (603147, 29929)	CDG-Ic
Type Id	Dolichyl-PP-Man: Man5GlcNAc2-PP-dolichyl alpha-1,3-mannosyl-transferase	*ALG3*, *NOT56L* (601110, 10195)	CDG-Id
Type Ie	Dolichol-P-Man synthase 1	*DPM1* (603503, 8813)	CDG-Ie
Group II	**Defects in the processing of *N*-glycans or addition of other glycans to proteins**		**CDG-II**
Type IIa	UDP-GlcNAc: alpha-6-D-mannoside beta-1,2-*N*-acetylglucosaminyl-transferase II (GnT II)	*MGAT2* (212066, 4247)	CDG-IIa
Type IIb	Alpha-1,2-glucosidase I	*GCS1* (601336, 7841)	CDG-IIb
Type x	Genetic basis unknown. OMIM 603585		**CDG-x**

[a]OMIM = Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/).

Marth found that a model of human CDG-IIa can be produced in the mouse. Over 60% of mouse embryos with a homozygous null mutation in the gene encoding GlcNAc-T II (*Mgat2*) [137] survive to term but 99% of newborns die during the first week of post-natal development. Survivors are runted and exhibit facial dysmorphia, kyphoscoliosis, muscular atrophy, tremors, osteopenia and coagulopathy. Patho-histologic findings indicate reduced ossification at the growth plates, increased prevalence of skeletal cartilage, and a calcified bone density that is reduced by over 30%. Female homozygous mutant mice are fertile but males are infertile with a block in spermatogenesis. The production of T and B lymphocytes is reduced, hypocalcinemia is observed, and a ventral septal defect of the heart was noted in one survivor. The majority of *Mgat2* deficient mice die with gastrointestinal blockage. These findings indicate that the mouse and human GlcNAc-T II deficiencies are similar in pathological and biochemical features. We therefore postulate that the majority of humans with CDG-IIa may die in gestation or shortly after birth due to complications associated with gastrointestinal abnormalities, infection, low serum calcium and aberrant heart development. The few surviving *Mgat2* deficient mice probably represent a close model of the human CDG-IIa disease and can be used to study the mechanism of its pathogenesis and for assessing possible future therapeutic approaches.

CHO and BHK somatic mutant cell lines lacking a functional GlcNAc-T I gene (described above) show essentially normal growth in tissue culture. However, mouse embryos with a null mutation in this gene do not survive beyond 10.5 days post-fertilization and show severe multisystemic developmental abnormalities particularly of the brain [123,138]. This phenomenon presumably implies that the affected cell surface carbohydrates are essential for cell-cell interactions during mouse development but not for cell growth under tissue culture conditions. There was no obvious cause of death [124]. Analysis of *Mgat1*-/-blastocysts, positively identified by polymerase chain reaction of genomic DNA, revealed the presence of wild type *Mgat1* RNA [139]. It was concluded that the effects of the *Mgat1* null mutation are not operative until sometime between implantation and E5.5, due to the continued presence of maternally derived *Mgat1* mRNA in pre-implantation embryos.

The GlcNAc-T III null mice produced in Marth's laboratory showed no obvious phenotype [129]. However, GlcNAc-T III has been shown to be elevated in various types of rat hepatoma [140–143], human leukemia [144,145] and other cancers [146]. It was therefore of great interest that the progression of chemically-induced hepatic neoplasms is severely retarded in mice lacking the bisecting *N*-acetylglucosamine on *N*-glycans [147]. The ability of perturbations in complex *N*-glycan structure to affect tumour progression and metastasis is dramatically shown in a recent report [148]. GlcNAc-T V activity is known to increase in malignancies and to correlate with disease progression. Granovsky *et al.* [148] showed that mammary tumor growth and metastases induced by the polyomavirus middle T oncogene were considerably less in *Mgat5*-/-mice than in transgenic littermates expressing both the oncogene and GlcNAc-T V.

The role of complex *N*-glycans in *Caenorhabditis elegans* development

The data from human congenital diseases and null mutations in mice discussed above provide strong evidence that complex *N*-glycans are essential for normal mammalian development. Since complex *N*-glycans appeared in evolution long before mammals, it is of interest to investigate the role of these compounds in multicellular organisms that have an older evolutionary history. The microscopic nematode worm *Caenorhabitis elegans* is a particularly attractive model because of the detailed information available on its development, its relatively simple architecture and the availability of the complete genome sequence. Once again, I have been fortunate to find a patient and brilliant collaborator in the person of Andrew Spence, a Toronto expert in *C. elegans*. Andrew gave my graduate student Shihao Chen not only space in his laboratory but also unlimited access to his vast store of knowledge on the mysteries of *C. elegans* genetics.

We have cloned cDNAs for three predicted *C. elegans* genes homologous to mammalian GlcNAc-T I (designated *gly-12*, *gly-13*, and *gly-14*) [149]. All three cDNAs encode proteins (467, 449, and 437 amino acids, respectively) with the domain structure typical of previously cloned Golgi-type glycosyltransferases. Expression in both insect cells and transgenic worms showed that *gly-12* and *gly-14*, but not *gly-13*, encode active GlcNAc-T I when assayed with a synthetic low molecular weight substrate, Man-alpha-1,6(Man-alpha-1,3)Man-beta-1-octyl (Man_3-octyl). All three genes are expressed throughout worm development (embryo, larval stages L1–L4, and adult worms). The *gly-12* and *gly-13* promoters are expressed from embryogenesis to adulthood in many tissues. The *gly-14* promoter is expressed only in gut cells from L1 to adult developmental stages. Transgenic worms that overexpress any one of the three genes show no obvious phenotypic defects.

We have used ultra-violet light irradiation in the presence of trimethylpsoralen (TMP) as a mutagenic agent to obtain *C. elegans* null mutants for the *gly-12*, *gly-13*, and *gly-14* genes [150]. The *gly-12* null mutant has a 1.6 kb deletion in the *gly-12* gene extending from intron 6 to exon 12 (which contains the STOP codon). This mutation is a molecular null because the deleted fragment encodes most of the GlcNAc-T I C-terminal catalytic domain. Homozygous mutant animals have no obvious phenotypic defects. They show a normal morphology, move normally and have a normal life span, brood size, generation time and defecation cycle; they are also sensitive to lavamisole, show Dauer formation and SDS resistance and male mutants have a normal tail structure and can mate. The *gly-14* mutation is a 1.7 kb deletion in a region containing highly conserved residues indicating that this mutation is also a molecular null. The homozygous *gly-14* mutant also displays a wild type

phenotype. A homozygous double mutant of *gly-12* and *gly-14* has been created. Extracts of the double mutant worms show no detectable GlcNAc-T I enzyme activity when measured with Man_3-octyl as substrate but the worms show a wild type phenotype. The *gly-13* mutation deletes almost the entire gene and the homozygous *gly-13* mutation is associated with lethality at an early larval stage. The lethal mutation has been mapped to within about 8 kb on either side of the *gly-l3* gene indicating with high probability that lethality is due to lack of *gly-13* expression. Preliminary data indicate that the homozygous double mutant of *gly-12* and *gly-14* can synthesize complex *N*-glycans (mass spectroscopy performed by Vernon Reinhold) and that the protein encoded by *gly-13* is in fact an active GlcNAc-T I with physiological substrates. Complex *N*-glycans may therefore be as essential for normal *C. elegans* development as they are for mammalian development. The challenge remains to determine at the molecular level precisely what these essential complex *N*-glycans do.

We have recently cloned and expressed *C. elegans* GlcNAc-T II [151] and *Drosophila melanogaster* GlcNAc-T I [152] and II (J. Tan, unpublished). The *C. elegans* GlcNAc-T II open reading frame is derived from 6 exons. Preliminary results suggest the promoter is expressed in the anterior ganglion, ventral nerve cord and tail ganglion of larvae but is confined to the pharynx in adult worms. The *Drosophila* GlcNAc-T I gene has seven exons and six introns and is the first insect GlcNAc-T I gene to be cloned.

Also of interest to the development problem is our recent finding that there is a human protein similar (64% over 109 amino acids) to human GlcNAc-T I [153]. This gene, which we have named *MGAT I.2*, maps to chromosome 1 whereas GlcNAc-T I maps to 5q35. There is a mouse orthologue with 97% identity to human GlcNAc-T I.2 over 361 C-terminal residues. GlcNAc-T I.2, like GlcNAc-T I, is widely expressed. GlcNAc-T 1.2 cDNA has a 1980 bp open reading frame encoding a 660 amino acid protein; human GlcNAc-T I has 445 amino acids. GlcNAc-T I.2 has the type 2 domain structure typical of Golgi-localized glycosyltransferases. The C-terminal region of human GlcNAc-T I.2 (amino acid residues 227–660) was expressed in the baculovirus/Sf9 system and showed low but consistent activity towards Man_3-octyl. Since the GlcNAc-T I null mutation is lethal in the mouse, GlcNAc-T I.2 may have a physiological function which is different from GlcNAc-T I.

The long road to a crystal structure of GlcNAc-T I

Many of us in the glycosyltransferase business have for a long time been eager to see the three-dimensional structure of a typical Golgi-localized type 2 domain glycosyltransferase (a short N-terminal cytoplasmic domain, a non-cleavable transmembrane anchor domain, a short stem region and a large C-terminal catalytic domain). Despite years of effort by several crystallographers, it was not until 1999 that the first non-bacterial structure, bovine UDP-Gal:GlcNAc-R beta-1,4-galactosyltransferase 1, was published [154]. We have recently published the crystal structure of an enzymatically active form of rabbit GlcNAc-T I, truncated to remove the cytoplasmic and trans-membrane domains and as much of the stem region as was possible without losing enzyme activity [155,156].

I am not sure why others had so much difficulty with their enzymes but I can say that we have been working away at the GlcNAc-T I structure for 5 or more years. The reasons for the delay are not esoteric. X-ray crystallographers ideally want a pure protein at a concentration of about 10 mg protein/ml and they want at least 0.5–1.0 ml of this solution, preferably more, so that they can do many crystallization trials. This certainly seems like a modest request at first glance but many things can go wrong—proteolysis or glycosylation can cause heterogeneity, solubility problems may prevent the achievement of a high enough protein concentration, the yields of recombinant protein may be too low, purification may be difficult, and so on. And then the crystallographers take over and try to make suitable crystals—an even more mystifying process to me. We were successful in expressing recombinant GlcNAc-T I in the Sf9 insect cell/baculovirus system back in 1992 [157,158]. We made sure to attach a nice Ni-binding epitope tag for purification purposes. Nevertheless, although our protein had no *N*-glycan consensus sequences, we were plagued with heterogeneity in our final preparations. Removal of 106 N-terminal amino acids helped to reduce heterogeneity, probably by reducing proteolysis [159] and eventually we succeeded in working out a reproducible purification procedure. This was due to the persistence of Mohan Sarkar in the early stages and of the crystallography team (Jim Rini, Ulug Ünligil, Sihong Zhou and Sivashankary Yuwaraj) later in the project. The yield of pure protein tended to vary but was probably never more than 1–2 mg/litre of culture medium. Crystals were obtained fairly quickly in our case but they were very small, thin and fragile—in the end, however, these same "poor" crystals gave a very good resolution (1.5 to 1.8 Angstrom).

It is not my intention to describe in any detail either the methodology or the results of this project since the paper has been published. However, I will discuss briefly some of the conclusions and potential benefits that seem important to me. Since we had worked out the ordered sequential Bi-Bi catalytic mechanism of the enzyme [69], it was fascinating to see how the structure accounted for it. The UDP-GlcNAc-Mn^{2+} complex must bind first to stabilize a floppy 13-amino acid loop thereby creating the acceptor binding site; the UDP product cannot leave until the loop is disrupted and glycan product is released. One of the roles played by the so-called "DxD" motif (which is EDD for GlcNAc-T I) is to help in the binding of Mn^{2+}, as envisaged by most of us. Although we did not obtain a structure in which the acceptor substrate was seen, the acceptor binding site could be modeled and the Asp residue which serves as the general base catalyst was identified. From the point of view of evolution, it was surprising to find that the three-dimensional structure of

GlcNAc-T I shares common folds with at least three other crystal structures and a common domain structure was suggested at the core of all these enzymes. Using database sequences for modeling, it was further concluded that many other glycosyltransferases share this common domain although there is minimal similarity in the various primary sequences.

There are good reasons why both applied and basic researchers try to obtain protein crystal structures. These reasons apply to GlcNAc-T I. Since the enzyme sits at a very important junction in the *N*-glycan synthesis pathway, inhibitors of the enzyme are potential therapeutic agents against metastasis, inflammation and other diseases. The ability to perturb the *N*-glycan synthetic pathway should also be of great value in understanding the function of complex *N*-glycans in normal development and in disease.

The path to Ser/Thr-linked *O*-glycans is even more tortuous than the path to complex *N*-glycans

My first exposure to the field of *O*-glycan synthesis was in Roseman's laboratory. Saul had for some years been interested in the synthesis of the Ser/Thr-linked *O*-glycans on salivary mucins. I joined Ed McGuire in work on the UDP-Gal:GalNAc-Ser/Thr-X beta-1,3-galactosyltransferase (beta-3-GalT), the enzyme now known as *O*-glycan core 1 beta-1,3-galactosyltransferase (Figure 3) [160]. We were puzzled as to why ovine submaxillary mucin (OSM) has primarily the sialyl-alpha-2,6-GalNAc structure whereas porcine submaxillary mucin (PSM) has longer *O*-glycans containing the Gal-beta-1,3-GalNAc (*O*-glycan core 1) structure. We found that beta-3-GalT cannot act on sialyl-alpha-2,6-GalNAc-mucin. We suggested that the difference between OSM and PSM is dependent on the ratio of beta-3-GalT to alpha-2,6-sialyltransferase levels in the tissue and that this ratio is high in porcine submaxillary glands and low in ovine submaxillary glands. This is an early example of "competition" between glycosyltransferases for a common substrate and of a "STOP" signal (the sialyl residue), as discussed above.

One of my graduate students, David Williams, was given the "sticky" task of working on the mucin problem. He is a good artist and was fond of beginning his talks with a slide of himself crawling slowly through a sticky goo. David, as far as I can determine, is the first to describe and characterize what is now called *O*-glycan core 2 UDP-GlcNAc:Gal-beta-1,3-GalNAc-X [GlcNAc to GalNAc] beta-1,6-*N*-acetylglucosaminyltransferase (core 2 beta-1,6-GlcNAc-T) [161] (Figure 3). X can be porcine or ovine submaxillary mucin treated to expose the core 1 structure or a low molecular weight aglycone. This was, in fact, the first *N*-acetylglucosaminyltransferase shown to act on mucin substrates. This was also the time that I began a collaboration with Khushi Matta who played the same essential "synthetic carbohydrate chemistry" role for the *O*-glycans that Hans Paulsen later played for *N*-glycans. David used mucin substrates he had prepared himself and Khushi's synthetic compounds to characterize the substrate specificity of canine submaxillary gland core 2 beta-1,6-GlcNAc-T [162]. The enzyme product was identified by methylation analysis and high resolution proton nuclear magnetic resonance spectroscopy.

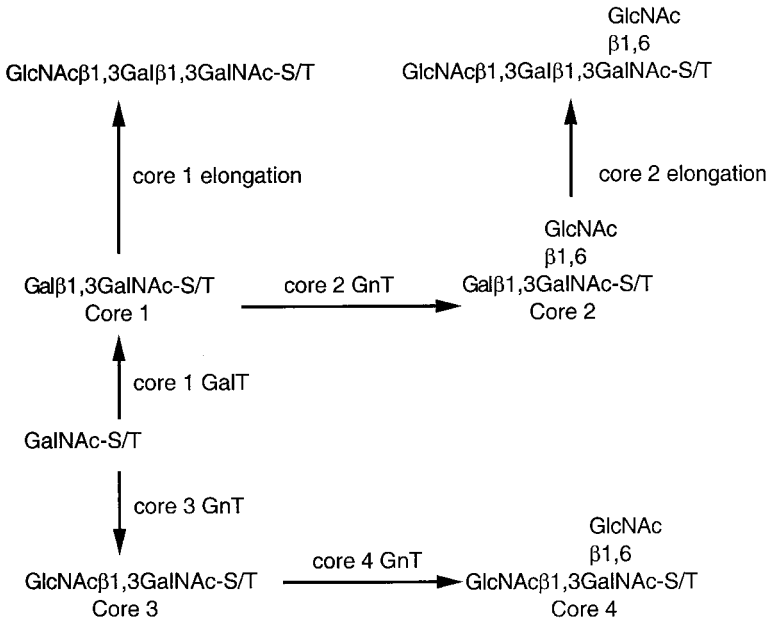

Figure 3. Assembly of the four major Ser(Thr)-GalNAc *O*-glycan cores. The "3-before-6" rule applies, i.e., the core 1 beta-1,3-galactosyltransferase (core 1 GalT) must act before the core 2 beta-1,6-*N*-acetylglucosaminyltransferase (core 2 GnT) and the core 3 beta-1,3-*N*-acetylglucosaminyltransferase (core 3 GnT) must act before the core 4 beta-1,6-*N*-acetylglucosaminyltransferase (core 4 GnT).

Inka Brockhausen was the next graduate student to tackle the mucin project. She was the mother of two young children at the time but that did not seem to have much effect on her productivity—her energy was then, and still is to this day, a remarkable force of nature. In five papers published between 1983 and 1986, Inka continued where David Williams had left off and together they established the pathways for the synthesis of *O*-glycan cores 2, 3 and 4 and for some of the elongation reactions. This field has been reviewed in detail [59,163–165] and only a short discussion will be presented. It will hopefully be clear from this discussion why the license plate on Inka's car reads "GLCNAC".

The first paper in the series [166] described pig gastric UDP-GlcNAc:Gal-beta-1,3(GlcNAc-beta-1,6)GalNAc-alpha-R [GlcNAc to Gal] beta-1,3-*N*-acetylglucosaminyltransferase where R is *O*-nitrophenyl or benzyl. This enzyme elongates *O*-glycan core 2 but not core 1. We developed HPLC techniques [167] to separate the low molecular weight acceptor substrates we obtained from Matta from the enzyme products that we were generating (phenyl, benzyl, and *O*-nitrophenyl glycosides). This method was used to assay core 2 beta-1,6-GlcNAc-T and the core 2 extension beta-1,3-GlcNAc-T described above. This paper [167] and a later paper [168] also describe two novel enzymes in colonic mucosa, UDP-GlcNAc:GalNAc-alpha-R beta-1,3-GlcNAc-T (core 3 beta-1,3-GlcNAc-T) and UDP-GlcNAc:GlcNAc-beta-1,3-GalNAc-R [GlcNAc to GalNAc] beta-1,6-GlcNAc-T (core 4 beta-1,6-GlcNAc-T) (Figure 3). Pig gastric mucosa was shown to contain core 2 beta-1,6-GlcNAc-T as well as one or more elongation beta-1,3-*N*-acetylglucosaminyltransferases which can add GlcNAc in beta-1,3 linkage to the terminal Gal residue of either core 1 or core 2 [169].

In a collaboration with Dirk van den Eijnden's group [170], we showed the presence in pig gastric mucosa of a UDP-GlcNAc:R^1-beta-1,3-Gal(NAc)-R^2 [GlcNAc to Gal(NAc)] beta-1,6-GlcNAc-T capable of producing the I antigenic determinant [GlcNAc-beta-1,3(GlcNAc-beta-1,6)Gal-], of converting Gal-beta-1,3-Gal-beta-1,4-Glc to Gal-beta-1,3(GlcNAc-beta-1,6)Gal-beta-1,4-Glc and of synthesizing both core 2 and core 4. Thus the enzyme can attach a GlcNAc in beta-1,6 linkage either to Gal or GalNAc provided these residues are substituted at the C-3 position with either Gal or GlcNAc in beta-1,3 linkage.

I ceased active research in the *O*-glycan field about 10 years ago and concentrated all my attention on the *N*-glycans. Inka Brockhausen has continued in this area, first in Toronto, and more recently at Queen's University in Kingston, Ont. Many other scientists have also entered the field. Several *O*-glycan glycosyltransferases have now been cloned by Minoru Fukuda, Henrik Clausen, Eric Berger, Larry Tabak, Fred Hagen and others. The *O*-glycans have been shown to play major roles in selectin binding to its ligands, the metastatic behaviour of cancer cells and various other phenomena. Further discussion of this growing area is beyond the scope of this retrospective review.

What lies ahead?

Of course no one knows what lies ahead and I have always felt that it is highly presumptious to play this game. However, we have been asked to gaze into the crystal ball for this Millennium issue and to speculate. If experience is any guide, it is almost certain that the most important advances will come from totally unpredicted directions. It seems ridiculous to have to emphasize again and again that curiosity-directed basic research is the surest path to innovation and creativity. Having made this "motherhood" statement, one must nevertheless choose some specific areas to go fishing. It should be clear from the above review of my own research that I think one such area is developmental biology. I do not want to repeat what I have already said but I will emphasize the clear evidence that multicellular organisms cannot develop normally without cell surface glycans. This is even more evident when one looks at recent developments in proteoglycans (a topic which I did not discuss above). There are undoubtedly other areas which will prove fruitful for glycobiologists but I will not list the "usual suspects"; some have been mentioned in my review and I am sure that most readers can come up with their own lists. It is the choice of which few of these many exciting directions to follow that is the difficult part.

I wish to end this review by thanking most heartily and sincerely all the people who have worked with me and offered advice over the years, my students, fellows, research associates and collaborators, those whose work was mentioned above and those who worked on other projects that I did not discuss [189–204]. All of you have played major roles in my career and many have become close friends during the process. Dorothy had her friends to help her find the Emerald City and I have certainly had mine, especially my wife Judith who put up with me all these years.

Acknowledgements

The author thanks the Medical Research Council of Canada, National Institutes of Health (Bethesda MD), The Terry Fox Special Initiatives Award, Canadian Cystic Fibrosis Research Foundation, Protein Engineering Centres of Excellence (PENCE), The Mizutani Foundation for Glycoscience and the Helen Grossman Research Fund for funding support.

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