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MINI-REVIEW

Recent progress in the molecular biology of the cloned *N*-acetylglucosaminyltransferases

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Several genes which code for the *N*-acetylglucosaminyltransferases have been cloned and characterized. Physiological and pathophysiological roles of the genes still remain to be elucidated but accumulated evidence suggests that the *N*-acetylglucosaminyltransferase genes are implicated in differentiation, morphogenesis and cancer metastasis.

Keywords: *N*-acetylglucosaminyltransferases, molecular biology

Introduction

The sugar structures of complex carbohydrates are mainly determined by 'glyco-genes' which code for glycosyltransferases and glycosidases. The balance between the expression levels of glycosyltransferase and glycosidase, namely biosynthetic enzyme and biodegradation enzyme respectively, may play an important role in the formation of sugar structures.

Regulation of sugar chain biosynthesis is under the control of: expression of glycosyltransferases, substrate specificity of the enzymes, and localization of the enzymes in tissues and organelles. Until recently information on the mechanisms by which glycosyltransferase genes are regulated has been very limited. However, there is growing evidence that the *N*-acetylglucosaminyltransferases may play a key role in branching and elongation processes. Elongation and branching of the sugar structures of N-glycans are important issues that need to be addressed in order to understand the role of sugar chains in terms of differentiation, development and cancer.

There are at least six *N*-acetylglucosaminyltransferases (GnT) which are involved in the biosynthesis of N-glycans. In vertebrate systems they are designated as GnT I-VI as shown in Fig. 1A. Of the six GnTs, the genes for GnT-I, II, III and V have now been cloned. In addition, two other *GnT* genes, which are involved in I antigen biosynthesis and O-glycan biosynthesis designated as IGnT and core 2 GnT respectively, have also been cloned. Each *GnT* gene so far cloned shares a domain structure similar to other cloned glycosyltransferases, and as proposed by Paulson and Colley [1], is a typical type II transmembrane protein with a cytoplasmic domain, a transmembrane anchor domain, a stem region and a catalytic domain. Even though a consensus sequence designated as a sialylmotif [2] was found in other groups of glycosyltransferases, such as the sialyltransferase gene family, no sequence homology was observed in most of the *N*-acetylglucosaminyltransferases with the exception that the core 2 GnT and IGnT share some homology. In this mini-review, we shall attempt to summarize the most recent data available on the cloned *N*-acetylglucosaminyltransferases.

N-acetylglucosaminyltransferase I (GnT-I)

GnT-I catalyses the transfer of GlcNAc from UDP-GlcNAc to the oligomannosyl core structure, Man-5GlcNAc2-Asn, to form the structure shown in Fig. 1B. This essential reaction is the first step in the biosynthesis of complex and hybrid types of N-glycans. The enzyme responsible is widely distributed in most tissues of the body, and has been purified from rabbit liver, for which information on the peptide sequence was obtained [3]. Using mixed oligonucleotide-primed polymerase chain reaction amplification, the cDNA was obtained and when translated *in vivo* [4], yielded a 52 kDa protein with GnT-I activity. Another group also independently cloned

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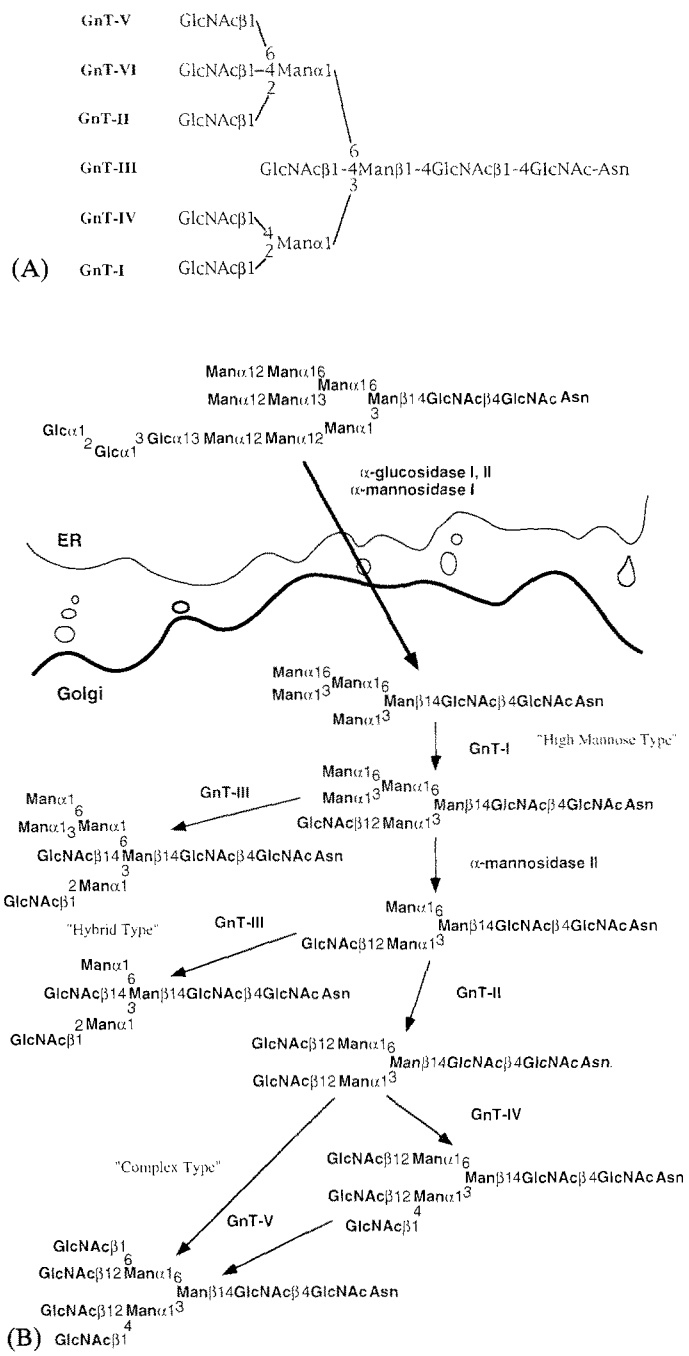

Figure 1. (A) The branching GlcNAc-transferases. (B) The pathway of complex *N*-glycan synthesis.

human GnT-I cDNA by transfecting high molecular weight human genomic DNA into a mutant Chinese hamster ovary cell line (Lec 1) deficient in GnT-I. Transfectant colonies expressing GnT-I activity were identified by screening for colonies that bound sheep erythrocytes conjugated with leukophytohaemmagglutinin (L-PHA), which recognizes the product of GnT-I [5]. The number of amino acids found in human and rabbit GnT-I predicted by cDNA were 447 and 445 respectively, but no N-glycosylation sites were found. GnT-I is localized in the medial-Golgi, and the transmembrane domain is essential for retaining it in the Golgi [6, 7, 8]. The entire coding region of the *GnT-I* gene is located on one exon. Introns, approximately 2 kb upstream from ATG, contain a TATA box, a CAAT box, and the octamer sequence [9]; however, a detailed analysis of the promoter regions has not yet been reported. Southern blot analyses indicate that the gene consists of a single copy, and is located on chromosome 5q35. Schachter's group [10] and Stanley's group [11] independently reported that knocking out the *Mgat-1* gene in mice was fatal to the embryo which died at 9.5–10.5 days. The mice were developmentally retarded, with neural tube formation, vascularization, and determination of left-right body plan asymmetry impaired. GnT-I is requisite for morphogenesis during post-implantation development indicating that other *N*-acetylglucosaminyltransferases which act on N-glycan biosynthesis after GnT-I, may also play an important role in the development and differentiation of neural tissues.

N-acetylglucosaminyltransferase II (GnT-II)

As shown in Fig. 1, GnT-II catalyses the essential step in the biosynthetic pathway leading from high-mannose to complex N-glycans. GnT-II was first purified from rat liver [12], and its cDNA subsequently cloned [13]. Human *GnT-II* also has been cloned, using rat GnT-II cDNA as a probe, showing that the gene is located on chromosome 14q21 [14]. The entire coding regions of human and rat *GnT-II* are on a single exon. There is a 92% homology between the amino acid sequences of the catalytic domain of human and rat GnT-II. Two N-glycosylation sites were found in both the human and rat amino acid sequences. The number of amino acids, predicted from nucleotides, was 447 for the human enzyme and 442 for the rat enzyme. GnT-II is also a typical type II transmembrane protein.

Patients with congenital dyserythropoietic anaemia type II (hereditary erythroblastic multinuclearity with positive acidified serum lysis test: HEMPAS) have an impairment in the formation of sugar chains which is seen in erythrocyte Band 3. Fukuda and colleagues suggested that the abnormality involved a deficiency of α-mannosidase II or GnT-II [15]. However, Schachter's group very recently identified congenital carbohydrate-deficient glycoprotein syndrome (CDGS) type II as an autosomal recessive disease, and showed that in fibroblasts and mononuclear cells taken from such patients, 98% of GnT-II activity was lacking. They also showed that the sugar structures of Band 3 proteins in CDGS type II patients were different from those of a patient with typical HEMPAS [15a]. The pathogenesis of GnT-II deficiency and HEMPAS still requires detailed studies.

N-acetylglucosaminyltransferase III (GnT-III) and *N*-acetylglucosaminyltransferase V (GnT-V)

GnT-III catalyses the addition of bisecting GlcNAc in β1-4 linkage to the β-mannoside of the trimannosyl core of N-glycans, and was originally identified in the hen oviduct membrane [16]. It is well known that the bisecting GlcNAc structure affects the conformation of sugar chains and once GnT-III acts on the biantennary sugar chains, other glycosyltransferases such as GnT-II, GnT-IV, GnT-V and β1-4GalT are no longer able to act on the biantennary sugar chains [17]. GnT-III is therefore a key enzyme in the biosynthesis of N-glycans. The bisecting GlcNAc has been found in glycoproteins in various tissues. In normal rat tissues, GnT-III is abundant in brain and kidney [18]. Whereas virtually no message is found in the normal adult rat liver, the activity of GnT-III is very high in experimental rat hepatomas [19], ascites hepatomas [20, 21], and hepatoblastoma cells [22]. Nishikawa *et al.* [23] purified the enzyme from normal rat kidney using several chromatographic techniques including substrate affinity chromatography. Oligonucleotide primers were designed using amino acid sequences and a cDNA was subsequently obtained. Rat GnT-III cDNA encodes 535 amino acids. Amino acid residues 173–184 were found to be an EGF-like motif, which is a structure similar to that found in human β4 integrin. Three putative N-glycosylation sites were found. Sequence homologies between human and rat GnT-III were found to be 91% at the amino acid level and 86% at the nucleotide level. Human *GnT-III* gene is located on chromosome 22q13.1. Southern blot analysis has also indicated that the gene is composed of a single copy [24]. The localization of GnT-III in the Golgi apparatus was found to be medial as judged by ultramicroscopic analysis using a polyclonal antibody raised against GnT-III (Ihara *et al.* unpublished data).

The mRNA of GnT-III is approximately 4.0 kb, and is highly expressed in the course of mutant LEC rats which develop primary hepatomas [25]. Yoshimura *et al.* reported that GnT-III is highly expressed in leukaemia cells of patients, especially during the blast crisis of chronic myelogenous leukaemia [26]. This observation is interesting when compared with a previous study that reported that GnT-III activities are suppressed during the differentiation of leukaemic cells, HL-60(27). This may imply that there is some special correlation between hematopoietic differentiation and GnT-III expression.

As shown in Fig. 1, GnT-V catalyses the attachment of β1-6GlcNAc to convert branching N-glycans. The enzyme has been obtained in a highly purified form from human serum-free conditioned medium in which human small lung cancer cells were grown [28]. The separative chromatography steps included an affinity column using the substrate as a ligand. Using designed amino acid sequence oligonucleotide primers, a human GnT-V cDNA clone was obtained from a human fetal library [29].

Another group also independently purified GnT-V from normal rat kidney using an inhibitor affinity column, followed by determination of amino acid sequences [30], and finally a rat GnT-V was cloned [31]. The sequence homology between the human and rat enzymes was 97% at the amino acid level. One valine residue was added in human GnT-V. Human and rat GnT-V cDNAs encode 741 and 740 amino acids respectively, and GnT-V is also a type II transmembrane protein. Six putative N-glycosylation sites were found in both human and rat GnT-V.

The human *GnT-V* gene was mapped on chromosome 2q21 as judged by *in situ* hybridization. The gene was divided into 17 exons, spanning 155 kb. In addition, analysis of the 5′ untranslated region of mRNAs from various cells showed multiple sequences depending on the cell types. In the 5′ upstream region of exon 1 consensus sequences matching those for transcription factor sites such as a TATA box, AP-1, AP-2, and for other transcription factor sites such as LF-A1 and HNF1-HP1, were shown. In addition, liver-restricted transcription factor sites were also found in intron 1. The gene employs a multiple promoter system for transcription, and thereby gene expression may be regulated in a tissue-specific fashion [32]. Furthermore two or more transcripts of *GnT-V* were observed in various cell lines and tissues. For example, 7.5 and 9.5 kb mRNAs were found in rodent cells, and 4.5 and 9.5 kb mRNAs in human cells [33]. In murine melanoma cells GnT-V mRNA expression is regulated by TGF-β due to the increased stability of the mRNAs [34]. In the case of a neuroblastoma cell line, however, the level of GnT-V mRNA increases during neuronal cell differentiation following treatment with dibutyryl cAMP, and is well correlated with the increase of GnT-V activity. However, GnT-V activity shows no changes during Schwannian cell-like differentiation, after treatment with bromodeoxyuridine, in spite of specific enhancement of a 9 kb transcript of *GnT-V* [35]. This kind of discrepancy between the transcriptional and activity level of GnT-V was also observed in rat brain [33], and implied that a posttranslational modification of GnT-V is required for enzyme activation. These findings suggest that the regulation of GnT-V expression is complex and varies with different tissues.

Increased expression of elongated N-glycans on the cell surface due to increased β1-6 branching, has been seen in several malignant cell types including baby hamster kidney cells transformed by polyoma [36] and Rous sarcoma viruses [37], or rat2 fibroblast cells in which oncogenes were activated [38]. Branch elongation is initiated by GnT-V, and the enzyme activity is increased

in NIH 3T3 cells [39] after transformation by *N ras* proto-oncogene. Moreover, GnT-V activity correlates well with the metastatic potentials of *ras*-transformed rat2 fibroblasts, SP1 mammary carcinoma cells, MDAY-D2 lymphoma cell line [40, 41], and rat prostate tumour variant R3327-MatLyLu [42]. As described above, GnT-III and GnT-V use the biantennary structure of N-glycans as a substrate, and once a bisecting GlcNAc residue is added to the core mannose by GnT-III, GnT-V is not able to form any further tri-structure.

We have purified GnT-V from a cultured human lung cancer cell supernatant and confirmed that a bisected biantennary sugar chain does not serve as a substrate, whereas biantennary and triantennary sugar chains are utilized by GnT-V [29]. One approach to analysing the role of GnT-V and its product β1-6 branches in metastasis, is to interfere with GnT-V activity in malignant cells with a high tendency to metastasize. Yoshimura *et al.* [43] established a highly metastatic subclone of B16 melanoma cells, and introduced the *GnT-III* gene into the cells. In these transfectants, the glycoproteins showed a reduced affinity to leukoagglutinating phytohaemmagglutinin (L-PHA) [44], whereas their binding to erythroagglutinating phytohaemmagglutinin (E-PHA) [44, 45] was increased. This indicated that the level of β1-6 structure was reduced, due to a competition for substrate between intrinsic GnT-V and ectopically expressed GnT-III. The transfectants displayed decreased invasiveness into matrigel, and inhibited cell attachment to collagen and laminin. Lung metastasis, after intravenous injection of the transfectants into syngeneic and nude mice, was significantly suppressed.

Core 2β1-6 *N*-acetylglucosaminyltransferase (core 2 GnT) and I antigen *N*-acetylglucosaminyltransferase (IGnT)

Both cDNAs have been cloned by expression cloning methods. CHO cells, which express the large T antigen of polyoma virus, have no core 2 GnT and so the reaction proceeds to the left as shown in Fig. 2A with the synthesis of leukosialin, containing tetrasaccharide-glycans. When cDNA, prepared from HL-60 cells in which core 2 GnT is highly expressed, is transfected into CHO cells, the core 2 GnT and the reaction proceed to the right as shown in Fig. 2A, and the cells synthesize leukosialin, containing heptasaccharide O-glycans. A monoclonal antibody, T305, has been prepared which only recognizes heptaoligosaccharides. The cells which express core 2 GnT were therefore able to be concentrated using a panning method with a specific antibody. The plasmids containing core 2 GnT were recovered by Hirt methods [46]. Procedures such as repeated transfection, panning and Hirt methods on three separate occasions made successful cloning of the core 2GnT(47) possible.

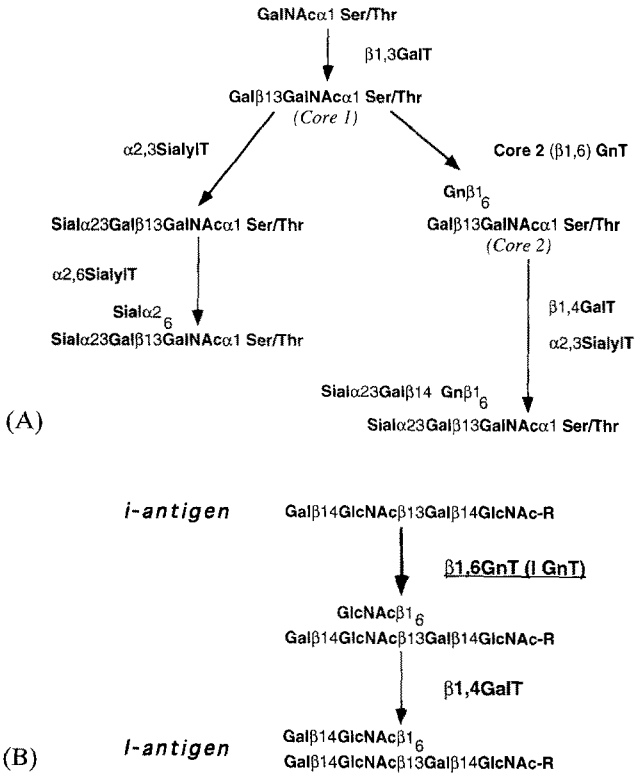

Figure 2. (A) Synthesis of O-glycan tetra- and hexasaccharides. (B) Synthesis of i- and I-antigens.

A similar method was used for cloning the I antigen GnT using CHO cells that have no I antigen. The biosynthetic pathway of I/i antigens is shown in Fig. 2B. Human teratocarcinoma PA-1 cells express a large amount of I antigen. Using a specific antibody to I antigen the cDNA for I antigen GnT was cloned [48].

Core *2 GnT* encodes 428 amino acids, and two putative N-glycosylation sites exist. Northern blot analysis indicates that the major mRNA is 2.1 kb and two other messages with 3.3 and 5.4 kb were also found. Homologies between core 2 and I antigen GnTs were 60% for 120 amino acids, 61% for 33 amino acids, and 59% for 22 amino acids, in three separate regions.

Chromosomal mapping analysis indicates that both genes are located on the same chromosome, 9q21-q22.1, and appear to have the same ancestor genes and consist of a gene family.

Core 2 GnT activity increases significantly in leukaemic cells taken from patients with the disease [49], and is clearly associated with the metastatic potentials of *ras*-transformed rat2 fibroblasts, SP1 mammary carcinoma cells, and the MDAY-D2 lymphoma cell line [41], as is GnT-V activity. Polylactosamine structure is also found in O-glycans [50], and in human B lymphocytes [51] the level of CD43 (leukosialin) is regulated by Core 2 GnT. Moreover, the increase of β1-6 branching GnT (i.e. Core 2 GnT and GnT-V) activities and polylactosa-

mine is closely associated with the differentiation of F9 teratocarcinoma cells into endoderm [52]. In patients with Wiskott-Aldrich syndrome [51, 53] or AIDS [54], a marked activation of core 2 GnT occurs with increased levels of leukosialin sugar chains, suggesting a participation of core 2 GnT in lymphocyte differentiation. Taken together, these observations suggest that expression of polylactosamine may be regulated by the β1-6 branching GnT, during embryogenesis, differentiation and carcinogenesis.

Conclusions

There are over 100 glycosyltransferases in mammalian systems. At present, however, as few as 30 glycosyltransferases have been cloned and characterized. The mechanisms by which glycosyltransferase genes are regulated remain unknown.

Several *GnT* genes have been cloned and characterized (Fig. 3). The domain structure is very similar in terms of type II transmembrane proteins. Core *2 GnT* and *IGnT* have a similar structure; however, sequence homologies are virtually absent among GnT-I, II, III and V enzymes. Recently a novel UDP-GlcNAc: GlcNAcβRβ1-4GnT cDNA was cloned from the pond snail *Lymnaea stagnalis* using bovine β1-4GalT cDNA as a probe. This also has no similarity with cloned GnTs. The overall homology between this novel GnT and β1-4GalT is about 30%. The highest sequence identity was found in the putative catalytic domain, and reaches 50% [55]. This discovery suggests that a more complicated situation exists in glyco-gene families because structural resemblance is found among enzymes that utilize different nucleotide-sugar donors.

Regarding substrate specificity, we still have no information about the active sites of the enzymes. Lack of sequence homology between each GnT enzyme, as described in this review, again supports the view that the active sites are very complex, and more work including experiments using X-ray crystallography and site-directed mutagenesis are necessary.

In some tumour cells, large amounts of GnT-III and GnT-V mRNAs are found. GnT-V has been reported to be highly associated with metastatic potentials. Our approach using transfection of the *GnT-III* gene into melanoma cells resulted in a substrate competition between GnT-III and GnT-V. This competition changed the structure of the sugar chains of cell surface glycoproteins, and resulted in a decrease in cell adhesion and invasion thereby decreasing metastatic potentials in the melanoma cells. The physiological significance of these genes, however, still remains to be studied.

Analysis of the promoter regions of *GnT* genes, and their roles in gene expression also remain unresolved. In this context, it is also necessary to understand the biological reason why GnTs are expressed.

Transgenic mice strategies, such as overexpressed gene or knocked out gene techniques, will provide us with further information as has been observed in the case of GnT-I. Implications that the GnT enzymes are key players in the morphogenesis of neural cells or in metastatic process in cancer cells are highly likely; however, we must await future developments to obtain definitive answers.

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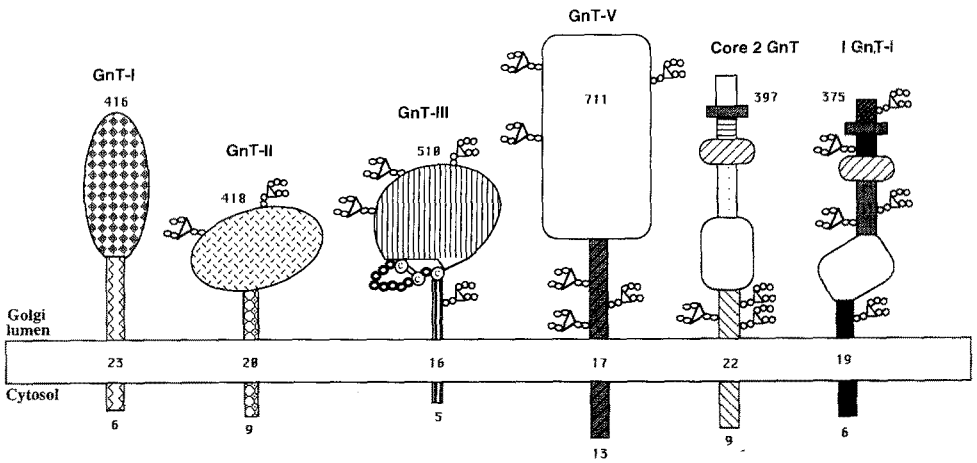

Figure 3. Models of cloned GlcNAc-transferases.

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