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REVIEW ARTICLE

Comparing N-glycan processing in mammalian cell lines to native and engineered lepidopteran insect cell lines

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In the past decades, a large number of studies in mammalian cells have revealed that processing of glycoproteins is compartmentalized into several subcellular organelles that process N-glycans to generate complex-type oligosaccharides with terminal *N*-acetlyneuraminic acid. Recent studies also suggested that processing of N-glycans in insect cells appear to follow a similar initial pathway but diverge at subsequent processing steps. N-glycans from insect cell lines are not usually processed to terminally sialylated complex-type structures but are instead modified to paucimannosidic or oligomannose structures. These differences in processing between insect cells and mammalian cells are due to insufficient expression of multiple processing enzymes including glycosyltransferases responsible for generating complex-type structures and metabolic enzymes involved in generating appropriate sugar nucleotides. Recent genomics studies suggest that insects themselves may include many of these complex transferases and metabolic enzymes at certain developmental stages but expression is lost or limited in most lines derived for cell culture. In addition, insect cells include an *N*-acetylglucosaminidase that removes a terminal *N*-acetylglucosamine from the N-glycan. The innermost *N*-acetylglucosamine residue attached to asparagine residue is also modified with α(1,3)-linked fucose, a potential allergenic epitope, in some insect cells. In spite of these limitations in N-glycosylation, insect cells have been widely used to express various recombinant proteins with the baculovirus expression vector system, taking advantage of their safety, ease of use, and high productivity. Recently, genetic engineering techniques have been applied successfully to insect cells in order to enable them to produce glycoproteins which include complex-type N-glycans. Modifications to insect N-glycan processing include the expression of missing glycosyltransferases and inclusion of the metabolic enzymes responsible for generating the essential donor sugar nucleotide, CMP-*N*-acetylneuraminic acid, required for sialylation. Inhibition of *N*-acetylglucosaminidase has also been applied to alter N-glycan processing in insect cells. This review summarizes current knowledge on N-glycan processing in lepidopteran insect cell lines, and recent progress in glycoengineering lepidopteran insect cells to produce glycoproteins containing complex N-glycans.
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Keywords: **insect cell, baculovirus, glycosylation, neuraminic acid, glycoprotein, recombinant DNA biotechnology, metabolic engineering**

Abbreviations: **CMP-NeuAc, cytidine-5′-monophospho-*N*-acetylneuraminic acid; Fuc, Fucose; Gal, galactose; GalT, galactosyltransferase; GDP-Fuc, guanosine-5′-diphosphofucose; GDP-Man, guanosine-5′-diphosphomannose; GlcNAc, *N*-acetylglucosamine; GlcNAc-1-P, *N*-acetylglucosamine-1-phosphate; GlcNAc-6-P, *N*-acetylglucosamine-6-phosphate; GnT I, *N*-acetylglucosaminyltransferase I; GnT II, *N*-acetylglucosaminyltransferase II; HPLC, high performance liquid chromatography; KDN, 2-keto-3-deoxy-D-glycero-D-galactononic acid; Man, mannose; ManNAc, *N*-acetylmannosamine; ManNAc-6-P, *N*-acetylmannosamine-6-phosphate; NeuAc, *N*-acetylneuraminic acid; NeuAc-9-P, *N*-acetylneuraminic**

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acid-9-phosphate; PA, pyridylamino; SiaT, sialyltransferase; UDP-Gal, uridine-5′-diphosphogalactose; UDP-GalNAc, uridine-5′-diphospho-*N*-acetylgalactosamine; UDP-GlcNAc, uridine-5′-diphospho-*N*-acetylglucosamine.

Introduction

A large number of studies have been devoted to interpreting processing pathway of N-glycans in mammalian cells. Glycosidases involved in the early stages of N-glycan processing and glycosyltransferases in the later stages of N-glycan processing have been extensively studied. In addition, many genes encoding these processing enzymes have been cloned from several mammalian sources. Insect cells are also capable of N-glycosylation. However, recent studies revealed that N-glycans attached to the insect-cell produced glycoproteins are composed primarily of paucimannosidic glycans and oligomannose-type glycans. Such characteristic glycosylation patterns are considerably different from those of mammalian-cell hosts, which typically generate complex-type N-glycans often terminating in *N*-acetylneuraminic acid (NeuAc).

The lepidopteran insect cell - baculovirus expression vector system (BEVS) is widely used for production of various recombinant proteins due to its advantages of safety, ease of use, and high productivity. Lepidopteran insect cells infected with baculovirus usually express glycoproteins bearing the typical paucimannosidic or oligomannosidic, "insect-type" N-glycans. Examples are the insect cells derived from *Spodoptera frugiperda* [1–14], *Mamestra brassica* [2,5,15], *Bombyx mori* [1,5], *Trichoplusia ni* [1,16], *Estigmene acrea* [6,7], and *Lymantria dispar* [17]. Several studies have revealed that processing of the N-glycans in insect cells begins with the addition of the $Glc_3Man_9GlcNAc_2$ oligosaccharide onto nascent polypeptides followed by trimming by glycosidases similar to those of mammalian cells. The differences in insect and mammalian N-glycans processing occur at the later stages of processing. Current understanding about N-glycosylation potential of most lepidopteran insect cell lines can be summarized as follows: (i) an inability to synthesize sialylated complex-type N-glycans [18–20]; (ii) cleavage of a terminal GlcNAc residue from intermediate N-glycans leading to generation of predominantly paucimannosidic structures; (iii) addition of α(1,3)-fucose to the Asn-linked GlcNAc, which is recognized as a potential allergenic epitope. These essential differences in N-glycan structure may result in poor biological activities and allergenic reactions in human [21–25], rendering the insect cell-BEVS less suitable for production of human-like glycoproteins. In this review, we summarize the current knowledge on N-glycan processing in lepidopteran cell lines and compare it to that in mammalian cells, which have been more extensively studied. Processing in insect cell lines will also be examined in the context of current biochemical and genomic analysis of N-glycan processing in insects, which may include more complex processing pathways at various stages or in different tissues. We also review several important recent studies aimed at producing heterologous glycoproteins bearing human-type N-glycans using the insect cell-BEVS. Readers are also referred to other recently published reviews on N-glycosylation in insects and insect cells [19,20,26].

Processing and maturation of N-glycans in mammalian cells

In eukaryotic cells, processing of N-glycans on cellular and secreted glycoproteins occurs in multiple steps in several subcellular compartments. Many enzymes, sugar nucleotides, and sugar nucleotide transporters participate in this complex intracellular process. Enzymes are known to be localized in particular subcellular compartments, *i.e.*, the rough endoplasmic reticulum (rER) and the Golgi apparatus (cis-, medial-, and trans-Golgi), trans-Golgi network, and the transport vesicles between these compartments (for review, see [27–29]).

Trimming of N-glycans in the early steps by processing glycosidases

The early stage of the pathway consists of several trimming steps that generate a key intermediate, $Man_5GlcNAc_2$, from a glucosylated precursor, $Glc_3Man_9GlcNAc_2$ originally transferred to nascent polypeptides in the rER (Figure 1).

The enzyme, α-Glucosidase I, initiates modification of N-glycans in the ER and is present in all eukaryotic cells [30] except trypanosomes [31]. This enzyme, localized to the nuclear envelope and the ER [32], catalyzes the cleavage of the terminal α(1,2)-linked glucose residue from $Glc_3Man_9GlcNAc_2$. A cDNA encoding human α-glucosidase I has been cloned [32]. Subsequent removal of α(1,3)-linked glucose is catalyzed in the ER by α-glucosidase II. These steps of processing are also important for generation of complex, hybrid,and oligomannose–type N-glycans. Furthermore, the processing of the terminal glucose residues involves critical signals for controlling the interaction of proteins with calnexin and calreticulin to mediate the correct folding and processing of the protein (for review, see [33]). An alternative pathway present in some organisms uses an endo-α-mannosidase which forms $Man_8GlcNAc_2$ isomer A from $Glc_{1\text{-}3}Man_9GlcNAc_2$ [34] (for structure, see Figure 1). The endo α-mannosidase is present in the Golgi and it prefers mono-glucosylated oligosaccharides as a substrate [35,36]. Following removal of all three glucose residues from $Glc_3Man_9GlcNAc_2$, ER and Golgi α(1,2)-mannosidases act together to remove four α(1,2)-linked mannose residues from $Man_9GlcNAc_2$ to form $Man_5GlcNAc_2$. Several α(1,2)-mannosidases have been purified and cloned [37–47]. The α(1,2)-mannosidases, essential for processing to complex and hybrid N-glycans in mammalian cells [27–29], generate a series of oligomannose-type N-glycans in

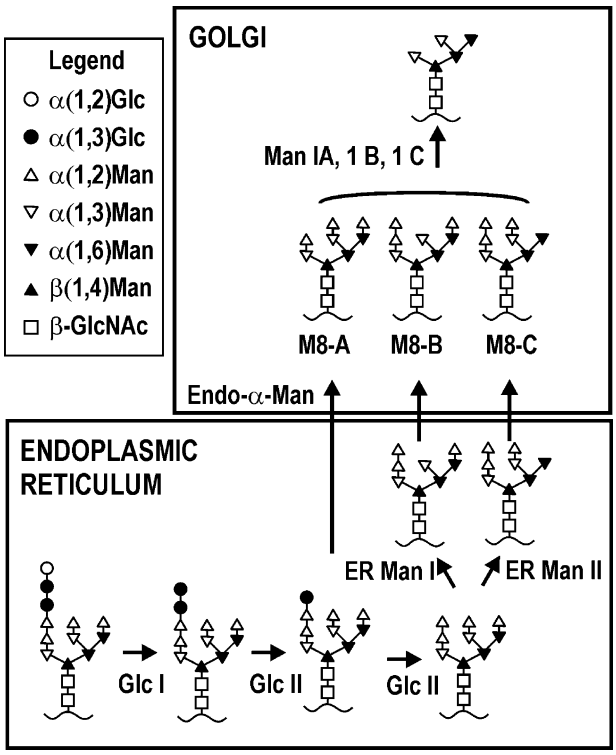

Figure 1. Early stage of N-glycan processing by several processing glycosidases. Glc I, α-glucosidase I; Glc II, α-glucosidase II; ER Man I, ER α-mannosidase I; ER Man II, ER α-mannosidase II; Endo-α-Man, endo-α-mannosidase; Man 1A, α-mannosidase IA; Man 1B, α-mannosidase IB; Man 1C, α-mannosidase IC; M8-A, $Man_8GlcNAc_2$ isomer A; M8-B, $Man_8GlcNAc_2$ isomer B; M8-C, $Man_8GlcNAc_2$ isomer C.

the ER and the Golgi. One of the $\alpha(1,2)$-mannosidases, the ER-resident $\alpha(1,2)$-mannosidase (ER Mannosidase I) specifically removes a single mannose residue from $Man_9GlcNAc_2$ to form $Man_8GlcNAc_2$ isomer B that lacks a single terminal $\alpha(1,2)$-mannose from the middle branch of the N-glycan [48,49] (for structure, see Figure 1). The ER contains another α-mannosidase (ERα-mannosidase II) which generates the $Man_8GlcNAc_2$ isomer C [50,51] (for structure, see Figure 1). In addition to the ER α-mannosidases, three different Golgi resident $\alpha(1,2)$-mannosidases termed Golgi $\alpha(1,2)$-mannosidase IA, IB, and IC play an important role in mammalian cells to form $Man_5GlcNAc_2$ [46,52]. However, the terminal $\alpha(1,2)$-mannose attached to the middle branch of the N-glycan precursor is relatively resistant to these Golgi enzymes. In this way, the specificity of ER $\alpha(1,2)$-mannosidase I, which cleaves this particular mannose residue, is complementary to that of the Golgi mannosidases [46,49,52].

Synthesis of complex-type N-glycans in the later steps by glycosyltransferases

The final elaboration of N-glycans is the most varied stage in eukaryotic organisms and is the major cause for the extreme diversity of mature N-glycans on glycoprotein from different cells and tissue types. In the case of mammalian cells, several steps in the latter half of the N-glycan processing pathway are used to build up complex-type N-glycans. This stage includes initiation of complex-type N-glycan synthesis, chain elongation, branching, and additional modifications catalyzed by several glycosyltransferases which are localized in the Golgi apparatus (Figure 2).

Following the synthesis of $Man_5GlcNAc_2$ intermediate, a single GlcNAc residue is transferred to Man$\alpha(1,3)$-branch of $Man_5GlcNAc_2$ from UDP-GlcNAc by the action of *N*-acetylglucosaminyltransferase I (GnT I) (for review, see [53]). GnT I gene has been cloned from several mammalian species [54–62]. The reaction catalyzed by GnT I is the first step that adds a sugar to the intermediate N-glycans in the eventual biosynthesis of complex-type N-glycan. At this point, two enzymes participate in modification of the $GlcNAcMan_5GlcNAc_2$. In the case of mammalian-cell expressed N-glycans, Asn-linked GlcNAc residue is often modified with an $\alpha(1,6)$-linked fucose. This fucosylation first takes place on the $GlcNAcMan_5GlcNAc_2$ by the action of the core $\alpha(1,6)$-fucosyltransferase (also called FucT 8) [63,64]. This enzyme requires the presence of $\beta(1,2)$-linked GlcNAc on the Man$\alpha(1,3)$-branch of the core $Man_3GlcNAc_2$ glycan. Biantennary oligosaccharides/glycopeptides also serve as substrates for this mammalian core $\alpha(1,6)$-fucosyltransferase [64–66]. A second modification of $GlcNAcMan_5[\pm Fuc\alpha(1,6)]GlcNAc_2$ is catalyzed by α-mannosidase II (for review, see [67]), which depends on prior action of GnT I [68]. The enzyme cleaves off a terminal $\alpha(1,6)$- and a terminal $\alpha(1,3)$-linked Man from the $GlcNAcMan_5[\pm Fuc\alpha(1,6)]GlcNAc_2$, and yields $GlcNAcMan_3[\pm Fuc\alpha(1,6)]GlcNAc_2$. Immunoelectron microscopy has demonstrated that α-mannosidase II is localized in the Golgi complex in rodent cells, but the sub-Golgi localization is variable in different cell types [69]. The enzyme was purified and characterized from rat liver [70,71] and mung bean seedlings [72], and behaved as a ~136-kDa disulfide-linked homodimer [70]. The genes encoding α-Mannosidase II have been cloned from human [73], mouse [74,75], *Drosophila melanogaster* (*D. melanogaster*) [76,77], and other sources. In addition to the above mentioned classical pathway, recent studies proposed alternative pathways which allow removal of $\alpha(1,3)$- and $\alpha(1,6)$-linked terminal Man residues from $Man_{5-6}GlcNAc_2$ (but not from $GlcNAcMan_5GlcNAc_2$). In one study, Bonay and Hughes examined the activity of a purified α-mannosidase from rat liver microsome fraction, using $Man_{4-9}GlcNAc$ as substrates, and they found that the purified enzyme could convert $Man_{4-9}GlcNAc$ to $Man_3GlcNAc$ [78]. This α-mannosidase activity was enhanced (19.5%) and stabilized in the presence of 1 mM $CoCl_2$ [78], and appeared to be mainly localized in the Golgi [79]. Chui *et al.*[80] also found similar enzyme activity which could hydrolyze $Man_5GlcNAc$-PA (PA, pyridylamino) to $Man_{2-4}GlcNAc$-PA, in various types of mouse cells and tissues. This enzyme was named

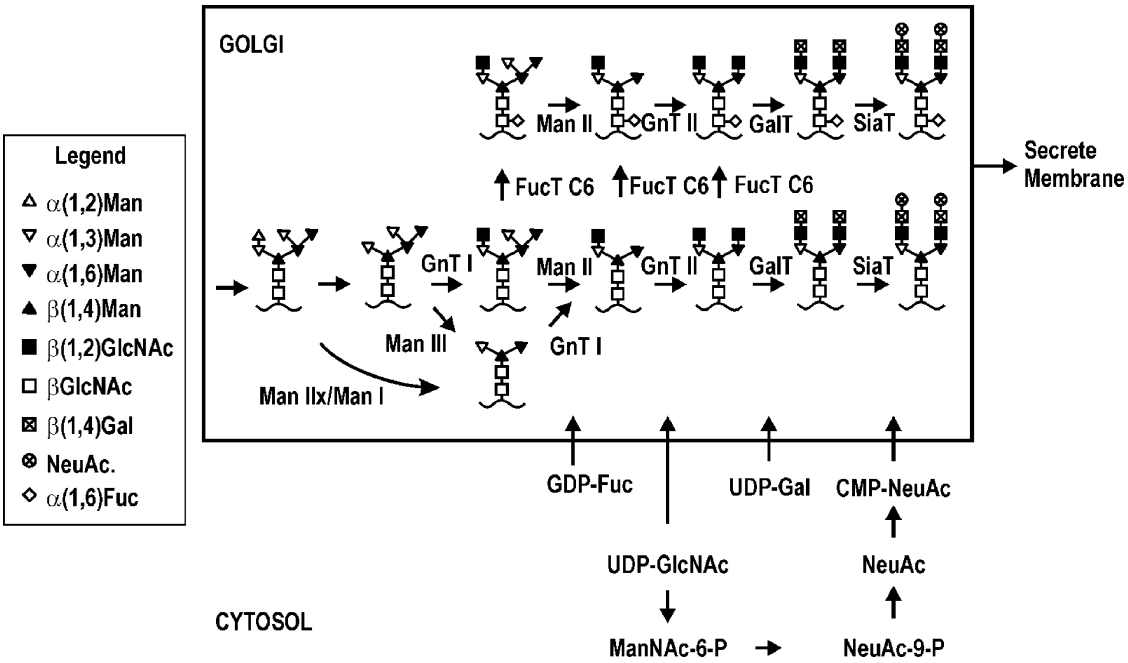

Figure 2. Late stage of N-glycan processing by several glycsosyltransferases. Man I, α-mannosidase I; Man IIx, α-mannosidase IIx; GnT I, *N*-acetylglucosaminyltransferase I;, GnT II, *N*-acetylglucosaminyltransferase II; GalT, galactosyltransferase; FucT C6, core α(1,6)-fucosyltransferase; SiaT, sialyltransferase.

α-mannosidase III as it is presumed to catalyze an alternative processing pathway converting $Man_5GlcNAc_2$ to $Man_3GlcNAc_2$ in cells [80]. Separately, Misago *et al.* [73] cloned a new α-mannosidase II-like enzyme termed α-mannosidase IIx from human. According to the results from *in vivo* experiments, this enzyme appears to be able to remove α(1,3)- and α(1,6)-linked terminal mannose residues from $Man_6GlcNAc_2$ and generate $Man_3GlcNAc_2$ in conjunction with the action of endogenous α-mannosidase I [81]. In both pathways, the final product, $Man_3GlcNAc_2$, can serve as substrate for the mammalian GnT I [82,83]. Another α-mannosidase was purified from rat brain microsomes [84]. Interestingly, this enzyme was able to release mannose residue from both oligomannose-type oligosaccharide ($Man_{4\text{-}9}GlcNAc$) and hybrid-type oligosaccharide.

In mammalian cells, $GlcNAcMan_3GlcNAc_2$ formed by either the classical pathway or the alternative pathway serves as an acceptor for *N*-acetylglucosaminyltransferase II (GnT II), which adds a second GlcNAc to the Manα(1,6)-branch to yield a biantennary complex-type N-glycans terminating with two β(1,2)-linked GlcNAc residues. GnT II gene has been cloned from human [85] and rat [86]. In addition to GnT I and II, three other *N*-acetylglucosaminyltransferases, GnT IV, V, and VI further add additional GlcNAc to the α(1,3)- or α(1,6)-linked Man to fabricate multiantennary N-glycans (not shown in Figure 2).

In mammalian cells, terminal GlcNAc residues attached to the α(1,3)- or α(1,6)-linked Man are modified with β(1,4)-linked Gal by the action of β-1,4-galactosyltransferase (β4GalT). Several different β4GalTs have been found in human and mouse, and β4GalT 1, 2, 3, 4, 5, and 6 can perform such galactosylation of N-glycans (for review, see [87,88]). Recent studies revealed that these βGalTs have different branch specificity with respect to the acceptor [89]. *N*-Glycans attached to glycoproteins produced by human cells are primarily modified with α(2,6)-linked NeuAc, while N-glycans attached to glycoproteins produced by other mammalian cells are often modified with both α(2,3)- and α(2,6)linked NeuAc. From a standpoint of glycoprotein production for pharmaceutical applications, sialylation with either α(2,3)- or α(2,6)- linked NeuAc is likely to be sufficient. Sialyltransferases are classified into four families based on the linkages the enzymes form: β-galactoside α(2,3)-sialyltransferase (ST3Gal), β-galactoside α(2,6)-sialyltransferase (ST6Gal), GalNAc α(2,6)-sialyltransferase (ST6GalNAc), and α2,8-sialyltransferase (ST8Sia) [90,91]. Addition of α(2,6)-linked NeuAc to the terminal β(1,4)-linked Gal attached to GlcNAc in the N-glycan is synthesized by ST6Gal. So far, two different sialyltransferases, ST6Gal I [92] and ST6Gal II [93,94], that can perform this type of sialylation have been cloned from humans. Studies on the substrate specificity and expression levels of these ST6Gal enzymes suggested that ST6Gal I is the major enzyme that sialylates β(1,4)-linked Gal in the Galβ(1,4)GlcNAc sequence attached to glycoproteins [93,94].

Processing of N-glycans in insect cells

Compared to the knowledge about glycoprotein processing in mammalian cells, much less is known about enzymes and

other components involved in processing of N-glycans in insect cells. N-glycans attached to glycoproteins produced by most lepidopteran insect cells are typically composed of paucimannosidic-type and oligomannose-type N-glycans. The occurrence of such N-glycans in the final product mix suggests that lepidopteran insect cells initiate N-glycan processing similar to that in mammalian cells as summarized below.

Potential of processing glycosidases in the early stages: α-glucosidase I, II, and α-mannosidase I

Many glycoproteins produced by lepidopteran insect cells include a variety oligomannose-type N-glycans. In addition, paucimannosidic glycans are often the major population in the glycoproteins produced by lepidopteran insect cells. The existence of these structures suggests that lepidopteran insect cells contain substantial levels of processing glycosidase activity similar to mammalian cells. For example, N-glycans on human IgG and hTF produced by Tn-5B1-4 cells contain oligomannose and paucimannosidic glycans [16,95] indicating that the precursor N-glycan $Glc_3Man_9GlcNAc_2$ is digested by α-glucosidase I, II, and $\alpha(1,2)$-mannosidase. Indeed, inhibition studies on specific glycosidases revealed that lepidopteran insect cells express α-glucosidase I, II, and α-mannosidase for processing [96]. The α-mannosidase I, purified from the Golgi fraction of Sf21 cells [97], could digest $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_9GlcNAc_2$ substrates with a preference for $Man_6GlcNAc_2$ [97]. A gene encoding α-mannosidase I has been cloned from Sf9 cells (SfMan I) [98], and the amino acid sequence of the SfMan I exhibits similarity to all three known human Golgi α-mannosidase IA, IB, and IC (identity 44–50%). The substrate specificity of SfMan I [99] is similar to that of human α-mannosidase IC rather than the others [46], and SfMan I localizes in the Golgi apparatus in Sf9 cells [100]. Structural analysis performed by our group indicated presence of $Man_8GlcNAc_2$ isomer B in N-glycans attached to human IgG [16] and hTf [95] expressed by Tn-5B1-4 cells. The same structure was also found in N-glycans attached to hTf expressed by Ld652Y cells [17]. The presence of these structural isomers suggest that at least some lepidopteran insect cells have an mammalian ER $\alpha(1,2)$-mannosidase-like enzyme that is able to remove the $\alpha(1,2)$-linked mannose residue specifically from the middle branch of $Man_9GlcNAc_2$. It should be noted that the $\alpha(1,2)$-linked mannose attached to the middle branch is the last mannose residue released by SfMan I. To the best of our knowledge, there is no report on purification or cloning of any kind of ER $\alpha(1,2)$-mannosidase from lepidopteran insects. It is not known if lepidopteran insects have more than one Golgi $\alpha(1,2)$-mannosidase as found in mammalian cells. Although its presence is expected, α-glucosidase I and II have not been cloned from lepidopteran insects.

Presence of GnT I and α-mannosidase II

Substantial GnT I activities were observed in several insect cells including Sf9, Sf21, Mb0503, and Bm-N [101,102] and the gene has been cloned from *D. melanogaster* [103]. As in mammalian cells, a single GlcNAc is added to Man$\alpha(1,3)$-branch of $Man_5GlcNAc_2$ by the action of GnT I. It was demonstrated that Sf21, Mb0503, and Bm-N cells have substantial level of α-mannosidase II [104]. An α-mannosidase II , purified from Golgi-like membrane fraction of Sf21 cells [105], removed terminal Man$\alpha(1,3)$ and Man$\alpha(1,6)$ from a $GlcNAcMan_5GlcNAc_2$ structure. Like its counterpart from mammalian cells, α-mannosidase II from insect cells requires GlcNAc on the Man$\alpha(1,3)$-branch for its action [104]. These early studies suggest that lepidopteran insect cells are capable of processing $Man_5GlcNAc_2$ to GlcNAc$\alpha(1,2)$Man$\alpha(1,3)$[Man$\alpha(1,6)$]GlcNAc$\beta(1,4)$GlcNAc through $GlcNAcMan_5GlcNAc_2$. In our studies, GlcNAc-$Man_5GlcNAc_2$ structure was observed at very low levels for glycoproteins produced by Tn-5B1-4 [16,95] and Ld652Y cells [17] despite the fact these cells expressed a large amount of the subsequent downstream products, $Man_{1-3}GlcNAc(\pm Fuc\alpha 3/\alpha 6)GlcNAc$. These results suggest that some lepidopteran insect cells have a high level of α-mannosidase II activity in order to process the $GlcNAcMan_5GlcNAc_2$ intermediate to the next glycosylation step. As mentioned in an earlier section, alternative processing pathways have been proposed in mammalian cells that lead to $Man_3GlcNAc_2$ without participation of GnT I. Recently, a gene encoding a Class II α-mannosidase from Sf9 cells was purified and cloned. In contrast to α-mannosidase II, this α-mannosidase from Sf9 cells could hydrolyze $Man_5GlcNAc_2$ but could not hydrolyze $GlcNAcMan_5GlcNAc_2$ [106], and thus was named α-mannosidase III (SfMan III). It is not clear whether or not SfMan III actually plays an important role *in vivo* because its steady-state expression level was very low in both uninfected and baculovirus infected Sf9 cells [107].

Potential of branch formation: GnT II

In mammalian cells, GlcNAc$\beta(1,2)$Man$\alpha(1,3)$[Man$\alpha(1,6)$] Man$\beta(1,4)$GlcNAc$\beta(1,4)$GlcNAc serves as an acceptor for GnT II in order to generate a biantennary structure. However, Sf9, Sf21, Mb0503, and Bm-N cells have been shown to have 1% or less of the GnT II activity of mammalian cells [101]. The lack of sufficient GnT II activity is one of the major bottlenecks in using lepidopteran insect cells for the production of multiantennary complex-type N-glycans. Interestingly, the gene for GnT II has been cloned from *D. melanogaster* [108,109].

Potential of galactosylation

A terminal GlcNAc residue(s) is usually galactosylated by $\beta(1,4)$-galactosyltransferase (β4GalT) in mammalian cells. In contrast, galactosylated N-glycans are found only rarely in glycoproteins from lepidopteran cells. In fact, negligible levels of GalT activities were detected in Sf9, Tn-5B1-4, and Mb0503 cells [110–112]. Recently, GalT activities in Sf9 and Tn-5B1-4 cells were reexamined using a high sensitive

Eu-fluorescence-based assay method [113]. Tn-5B1-4 cells were observed to contain about 10% of GalT activity detected in Chinese hamster ovary (CHO) cells, while Sf9 cells did not contain any detectable levels of GalT activity [113]. The insufficient level of GalT activity appears to represent yet another bottleneck in the production of mammalian-type N-glycans by lepidopteran insect cells. Recently, a gene encoding β4GalNAc transferase has been cloned from Tn-5B1-4 (*Trichoplusia ni, T. ni*) cells [114]. It has been shown that this enzyme preferentially transferred GalNAc (from UDP-GalNAc) to N-glycan and glycolipid acceptor substrates *in vitro* [114]. Furthermore, co-expression of *T. ni* β4GalNAc in Sf9 cells with GST-SfManI, a GST-tagged soluble domain of the *Spodoptera frugiperda* class I Golgi α-mannosidase, produced GST-SfManI terminally modified with GalNAc *in vivo*. However, such a modification with terminal GalNAc was not detected in GST-SfManI when it was expressed in native Sf9 cells under the same culture condition [114]. These results suggest that the gene is present in some lepidopteran insects, but the expression level of this enzyme in native insect cell lines such as Sf9 cells is not sufficient to produce glycoproteins modified with GalNAc at a significant level.

Potential of sialylation

Sialyltransferase, ST6Gal I (and ST6Gal II), adds NeuAc to the terminal β(1,4)-linked galactose residues on N-glycans in mammalian cell. Sialyltransferase activity has yet to be detected in numerous lepidopteran insect cells such as Sf9 [112,115,116], Sf21 [116], Tn-5B1-4 [115], Mb0503 [115], and Ea4 cells [116] using radiolabeled or fluorescent-labeled CMP-NeuAc as the donor substrate. The limitation in expression of sialyltransferase (SiaT) thus represents another significant hurdle to producing mammalian-type N-glycans.

This lack of sialyltransferase activity in lepidopteran insect cell lines contrasts with previous studies that suggested NeuAc is present at particular insect development stages. Indeed, sialylated glycoconjugates were observed in embryo of *D. melanogaster* [117] in which NeuAc was detected by cytochemistry using LFA (*Limax flavus agglutinin*) and by a combination of gas-liquid chromatography and electron-impact mass spectrometry (GLC-EI-MS) [117]. This group also demonstrated the presence of α(2,8)-linked polyneuraminic acids in *D. melanogaster* embryo using anti-polyneuraminic acid antibody [117]. NeuAc has also been detected in Malpighian tubules of larvae of cicada (*Philaenus spumarius*) [118] by cytochemistry using SNA (*Sambucus nigra* agglutinin), LFA, and anti-polyneuraminic acid antibody. The neuraminic acid was identified to be *N*-acetylneuraminic acid by high-performance liquid chromatography (HPLC) using a DMB (1,2-diamino-4,5-mehylene dioxybenzene) derivative and GLC-EI-MS [118]. In support of these earlier biochemical studies, Koles *et al.* [119] recently demonstrated that the genome of *D. melanogaster* contains a gene that encodes a functional ST6Gal. *In situ* hybridization revealed that DmST6Gal is expressed during embryonic development in a tissue- and stage-specific fashion, with elevated expression in a subset of cells within the central nervous system. These results suggest that cell type- and/or developmental stage-specific sialylation occurs in some dipteran insects.

Indeed, the fact that sialylation is restricted to particular cell types or stages may explain why numerous lepidopteran insect cell lines lack significant sialyltransferase enzyme activity. A limited number of studies [120–122] have also reported sialylation in insect cell lines to suggest that, at least in a few cases, sialyltransferase and other genes may be turned on during particular cell culture conditions or sialylation may occur with a particular glycoprotein as discussed in the earlier review on this issue by Marchal *et al.* [20]. Nonetheless, the absence of sialyltransferase activity in the preponderance of insect cell lines and studies to date suggests that sialyltransferase expression remains a significant bottleneck to the generation of human-like glycoproteins in most insect cell lines.

Degradation of the intermediate oligosaccharides: β-*N*-acetylglucosaminidase

Aβ-*N*-acetylglucosaminidase that can specifically remove terminal β(1,2)-linked GlcNAc on the Manα(1,3)-branch was found in Sf21, Bm-N, and Mb0503 cells [123], and it was suggested that this enzyme was localized in the Golgi-like membrane fraction in Sf21 cells [123]. Similar enzymatic activity was also detected in the cell lysate and cell culture supernatant of insect cells derived from *Spodoptera frugiperda*, *Trichoplusia ni*, *Bombyx mori*, and *Malacosoma disstria* [124]. Structural analysis of N-glycans from human IgG [16] expressed in Tn-5B1-4 cells, hTf [95] expressed in Tn-5B1-4 cells, and hTf expressed in Ld652Y cell [17] suggested the presence of such a β-*N*-acetylglucosaminidase in Tn-5B1-4 and Ld652Y cells. The further removal of additional Man residues by α-mannosidase(s) can lead to generation of the structures with fewer than three Man residues, as has been observed in many structural studies. It is possible that the β-*N*-acetylglucosaminidase prevents not only elongation of the Manα(1,3)-branch, but also prevents branch formation on the Manα(1,6)-arm because GnT II requires β(1,2)-linked GlcNAc attached to Manα(1,3) [125]. The insect membrane associated β-*N*-acetylglucosaminidase appears to be yet another bottleneck in lepidopteran insect cells for production of human-type glycoproteins.

Fucosylation in insect cells

N-Glycans with terminal β(1,2)GlcNAc can be further modified by core α(1,6)- and core α(1,3)-fucosyltransferase (called FucT C6 and FucT C3, respectively) in some lepidopteran insect cells [126]. N-Glycans modified with Fucα(1,6) or both Fucα(1,6)and Fucα(1,3) at the Asn-linked GlcNAc have been identified in membrane glycoproteins from Mb0503, Sf21, and Bm-N cells. Glycoproteins from Mb0503 cells contained highest levels of core α(1,3)-fucosylated N-glycans [5]. Similar

core-fucosylation was reported for Tn-5B1-4-produced recombinant human IgG [16], human serum transferrin [95], neuropsin [127], and CR3 domain of latent TGF-beta binding protein-1 [128]. On the other hand, human interferon ω1 expressed in Sf9 cells showed no α(1,3)-fucosylated N-glycan [3]. A similar study on human serum transferrin produced by Ld652Y cells indicated a lack of α(1,3) fucosylation as well [17].

Activities of FucT C3 and FucT C6 were detected in Mb0503 cells, in which the former was much higher than the latter [126]. FucT C6, but not FucT C3 was easily detected in Sf9 cells [126]. Independently, FucT C6 activity was detected in Sf9, Mb0503, and Bm-N cells [129]. Interestingly, in the same study, FucT C3 activity was detected in Mb0503 cells but not in Sf9 and Bm-N cells. A gene corresponding to the observed FucT C3/C6 has not been cloned from any lepidopteran insects, although a gene encoding FucT C3 has been recently cloned from *D. melanogaster* [130]. Several studies suggested that α(1,3)-linked fucose attached to the innermost GlcNAc is a potential epitope that causes allergenic reactions in human [21–25]. This type of modification of N-glycans in insect cells will also be a limitation to the use of some lepidopteran insect cells for expressing human glycoproteins.

Sugar nucleotides in lepidopteran insect cells

All glycosyltransferases require their respective sugar nucleotides as donor substrates, and the sugar nucleotide concentrations have been evaluated in several lepidopteran insect cells [115,131]. These studies demonstrated that no detectable levels of CMP-NeuAc are present in any of these widely used insect cell lines while significant levels of UDP-hexose, UDP-*N*-acetylhexosamine, GDP-Fuc, and GDP-Man are present.

Insufficient expression of several enzymes in NeuAc and CMP-NeuAc synthesis pathways

Of particular significance is the absence of CMP-NeuAc in lepidopteran insect cells. In mammalian cells, NeuAcs are synthesized from UDP-GlcNAc through multiple enzymatic reactions. The bifunctional enzyme, UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase, is believed to be a key enzyme in the biosynthesis of NeuAc in rat liver [132]. This enzyme converts UDP-GlcNAc to ManNAc-6P, which is further converted to NeuAc by *N*-acetylneuraminate-9-phosphate (NeuAc-9-P) synthase and NeuAc-9-P phosphatase. NeuAc is then converted to CMP-NeuAc by the action of CMP-NeuAc synthase. Effertz *et al.* [133] reported that the UDP-*N*-acetylglucosamine-2-epimerase activity in Sf9 cells was about 30 times lower (in terms of specific activity) than that in the rat liver cytosol fraction. Interestingly, Sf9 cells had 50 times higher *N*-acetylmannosamine kinase activity than the 2-epimerase activity [133]. We found that Sf9 cells contained minimal levels of NeuAcs, and negligible NeuAc-9-P synthase and CMP-NeuAc synthase activities [134,135].

In summary, the inability of lepidopteran insect cell lines to produce mammalian-type N-glycans is mainly attributable to (i) insufficient expression of Gn TII, β4GalT and SiaT, (ii) the inability to produce significant levels of CMP-NeuAc, (iii) the presence of a β-*N*-acetylglucosaminidase which removes β(1,2)-linked GlcNAc from the Manα(1,3)-branch of the core oligosaccharide, and (iv) potentially allergenic core α(1,3)-fucosylation from lepidopteran insect cell lines.

Genetic and metabolic engineering of insect cell glycosylation pathways

While insect cells are used widely for the production of various recombinant proteins, the system is not yet ideal for the production of pharmaceutical-quality glycoproteins. One of the major reasons is due to differences in the glycosylation pattern between insect cell-expressed glycoproteins and those obtained from mammalian hosts. N-linked glycans obtained from insect cells such as *Spodoptera frugiperda* [1,3–12,116,128,136–138], *Trichoplusia ni* [1,16,128,136], *Mamestra brassica* [2,5], *Bombyx mori* [5,14], *Estigmene acrea* [7,139], *Lymantria dispar* [14,17], and *Heliothis virescens* [14] are typically paucimannosidic with one to three Man and one or two GlcNAc residues with or without Fuc attached to the Asn-linked GlcNAc. In contrast, mammalian cells usually produce terminally sialylated complex-type N-glycans. Given the role of NeuAc residues for *in vivo* circulatory half-life, insect-cell derived glycoproteins will be cleared rapidly during animal trials. As a result, mammalian cells are the predominant host for the commercial production pharmaceutical glycoproteins. In order for insect cells to be considered as a suitable system for production of pharmaceutical glycoproteins, the limitations described in the previous section must be overcome. A general strategy for humanizing glycoproteins produced by the insect cell-baculovirus expression system is summarized in Figure 3.

Addition of GlcNAc to the Manα(1,3)-branch

In order to synthesize biantennary (and more highly branched) complex-type N-glycans, a GlcNAc must be added to the Manα(1,3)-branch, because GlcNAcT II requires the presence of this GlcNAc residue for its action. Unfortunately, N-glycans containing a GlcNAc residue on the Manα(1,3)-branch generally represent a minor fraction of the total N-glycans of insect glycoproteins. Wagner *et al.* has reported that the *N*-acteylglucosaminylation of the Manα(1,3)-branch of fowl plague virus hemagglutinin from Sf9 cells could be improved by overexpressing human GnT I [6,7]. Unfortunately, some lepidopteran insect cells contain considerable levels of β-*N*-acetylglucosaminidase activity that removes the terminal GlcNAc from the Manα(1,3)-branch [123], and this β-*N*-acetylglucosaminidase represents a serious obstacle to N-glycan elongation. It is absent or very low in *Estigmene acrea* cells, which produced N-glycans containing terminal GlcNAc residues, but is very active in Sf9 cells, which produced

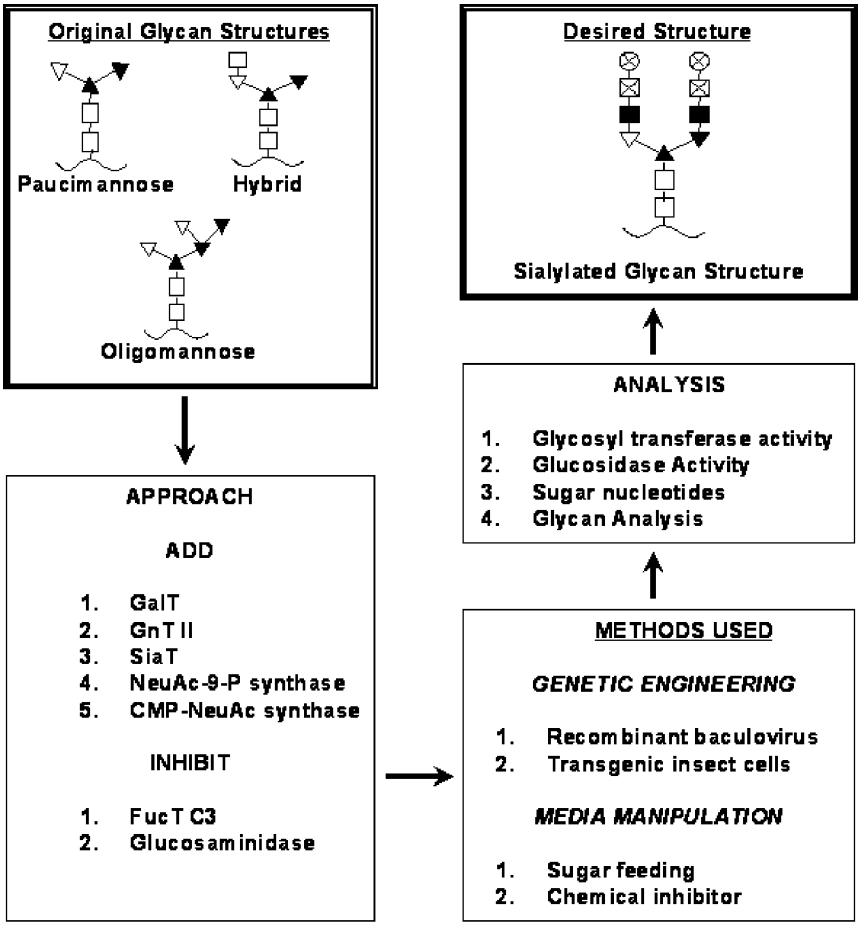

Figure 3. Strategies for humanization of glycoproteins produced by lepidopteran cell-baculovirus expression system. GalT, galactosyltransferase; GnT II, *N*-acetylglucosaminyltransferase II; SiaT, sialyltransferase; NeuAc-9-P synthase, *N*-acetylneuraminate-9-phosphate synthase; CMP-NeuAc synthase, CMP-neuraminate-9-phosphate synthase. Sugar symbols in glycan structures are same as in Figures 1 and 2.

primarily paucimannosidic N-glycan [139]. These studies suggested that inhibition of the β-*N*-acetylglucosaminidase activity might improve the N-glycosylation pattern of some lepidopteran insect cells. In this regard, Watanabe *et al.* [140] examined the effect of using a β-*N*-acetylglucosaminidase inhibitor, 2-acetamide-1,2-dideoxynojirimycin (2-ADN) on the glycosylation pattern of bovine interferon-γ (bIFN-γ) from Tn-5B1-4 cells. They found some sialylated bIFN-γ by inhibiting the β-*N*-acetylglucosaminidase activity. The authors suggested that the inhibitor allowed for the accumulation of substrates possessing a β(1,2)-linked GlcNAc which enabled further elongation by GalT and SiaT to form sialylated N-glycans. However, the appearance of sialylated glycans was demonstrated by lectin staining only, and no detailed structural analysis was performed. This report is in contrast to the previous observations that no sialyltransferase activity was detected in Tn-5B1-4 [115] and insignificant amounts of CMP-NeuAc were present in the same cell line [131]. These results suggest either that the inhibition of β-*N*-acetylglucosaminidase induced these transferases and metabolic enzymes or that the level of sialylation of bIFN-γ observed in the above study might have been very low.

Addition of Gal to Manα(1,3)-branch

The lack of significant GalT activity in insect cells has been reported by multiple investigators [110–113]. To address this limitation, a mammalian β4GalT was expressed in insect cells using a baculovirus vector under the control of an immediate early (*ie1*) promoter and this operation led to the addition of galactose residues onto a viral glycoprotein, gp64 glycoprotein [141] as indicated by *Ricinus communis* agglutinin (RCA) lectin and later by electrophoretic mobility [142]. The authors hypothesized that the mammalian β4GalT reacted with terminal GlcNAc residue before it was eliminated by intracellular β-*N*-acetylglucosaminidase activity. In subsequent studies, Sf9 and Tn-5B1-4 cells stably transformed to contain β4GalT in the host cell genome (Sfβ4GalT from Sf9 [110] and Tn5β4GalT from Tn-5B1-4 [143]) generated galactosylated gp64 [110,143] and

tissue plasminogen activator (t-PA) [110]. The ability of recombinant β4GalT to compete with β-*N*-acetylglucosaminidase was proven in a subsequent study in our laboratories in which the N-glycans of hTf obtained from Tn-5B1-4 cells were examined using two-dimensional HPLC analytical methods [95]. While 13% of the N-glycans were galactosylated in cells co-infected with a recombinant baculovirus encoding human β4GalT, no galactosylation was observed for Tn-5B1-4 in the absence of the recombinant β4GalT expression. Furthermore, the number of N-glycans containing GlcNAc on the Manα(1,3)-branch increased from 6.5% in the absence of GalT to 15.7% following co-expression with the β4GalT. This increase in GlcNAc levels demonstrated that the addition of galactose by the recombinant GalT protected the GlcNAc on Manα(1,3)-branch from cleavage by β-*N*-acetylglucosaminidase.

Interestingly, no complex-type structures containing Gal residues on both Manα(1,3)- and Manα(1,6)-branches were detected even in the Tn-5B1-4 cells co-expressing β4GalT. HPLC analysis revealed that all galactosylated N-glycans included terminal galactose residues exclusively on the Manα(1,3)-branch of the N-glycans. Since Gal residues were not observed on the α(1,6)-Man branch even in the GalT expressing cells, a limitation in the number of GlcNAc acceptors available on the Manα(1,6)-branch was indicated. The presence of N-glycans containing Gal only one the Manα(1,3)-branch was confirmed later for engineered Sfβ4GalT cells as well using mass spectrometric analysis [144].

Elongation of Manα(1,6)-branch with GlcNAc and Gal

Early studies on lepidopteran insect cells indicated the presence of low but detectable GnT II activity responsible for the addition of GlcNAc on the Manα(1,6)-branch [101]. However, hTf generated by Tn-5B1-4 cells contained only 0.7% of N-glycans with GlcNAc on the Manα(1,6)-branch without any terminal Gal residues to indicate a lack of sufficient GnT II activity [95]. To counter this deficiency, a mammalian GnT II was expressed in transgenic insect cell lines (SfSWT-1 [144] or Tn5β4GalT [143]), or using the baculovirus expression vector system [145]. In the latter case, Tn5β4GalT cells were infected with a recombinant baculovirus containing GnT II and hTf, and N-glycans of the produced recombinant hTf were examined by mass spectrometry, multidimensional HPLC, and exoglycosidase digeston [145]. The five major oligosaccharides representing 69% of the total N-glycans are shown in Figure 4. Of special significance was the finding that more than 50% of the N-glycans were fully galactosylated on both termini. Thus, the expression of recombinant GnT II in combination with GalT could yield a glycosylation pattern in which more than half of the structure were biantennary complex-type N-glycans terminating in galactose. However, some of the biantennary structures also included α(1,3)-linked Fuc to the innermost GlcNAc, and a limited number of paucimannosidic structures were also present [145].

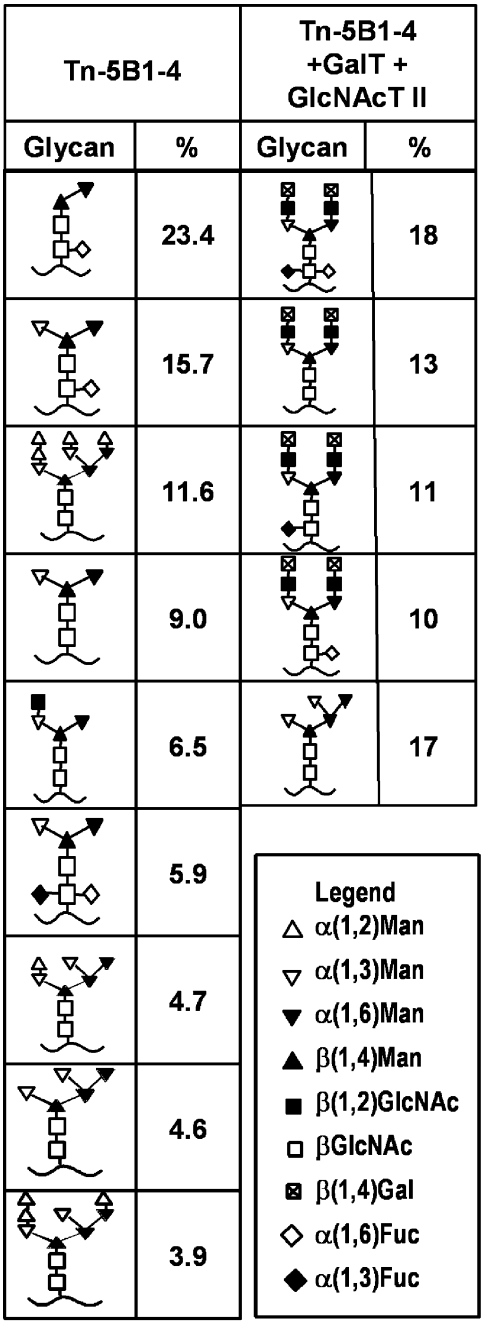

Figure 4. Major N-glycans identified in a recombinant hTf expressed in Tn-5B1-4 cells [95] and in Tn-5B1-4 cells co-expressing mammalian β4GalT and GlcNAcT II [145].

Generation of CMP-NeuAc

Formation of fully sialylated glycoproteins in insect cells requires three components: (1) CMP-NeuAc as a donor substrate, (2) acceptor oligosaccharide substrates terminating in Gal on either one or more branches, and (3) SiaT activity. The presence of fully galactosylated complex-type biantennary N-glycans [144,145] suggested that the proper acceptor substrates for sialylation were now available in engineered insect cells.

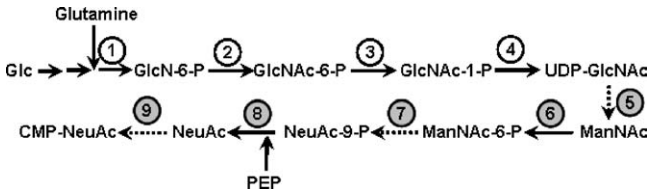

Figure 5. CMP-Neuraminic acid synthesis pathway. The reactions are catalyzed the following enzymes; 1, Glutamine: Fructose-6-phosphate amidotransferase; 2, GlcN-6-P *N*- acetyltransferase; 3, phosphoacetylglucosamine mutase; 4, UDP-*N*-acetylglucosamine diphosphorylase; 5, UDP-GlcNAc 2-epimerase; 6, ManNAc kinase; 7, NeuAc-9-P synthase; 8, NeuAc-9-P phosphatase; 9, CMP-NeuAc synthase. The broken arrows indicate missing processing steps in lepidopteran insect cells. Enzymes corresponding to numbers in shaded circles have been expressed in engineered insect cells. Glc, Glucose; GlcN-6-P, glucosamine-6-phosphate; GlcNAc-1-P, *N*-acetylglucosamine-1-phosphate; UDP-GlcNAc, uridine-5′-diphospho-*N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; ManNAc-6-P, *N*-acetylmannosamine-6-phosphate; PEP, Phosphoenol pyruvate; NeuAc-9-P, *N*-acetylneuraminate-9-phosphate; NeuAc, *N*-acetylneuraminic acid; CMP-NeuAc, cytidine-5′-monophosphate-*N*-acetylneuraminic acid.

However, negligible levels of the endogenous CMP-NeuAc were found in insect cells grown in serum-free media [116,146]. The biosynthetic pathway for generating CMP-NeuAc in mammals is depicted in Figure 5.

While insect cells contain significant amounts of UDP-GlcNAc [146], they lack significant levels of CMP-NeuAc or its precursor, NeuAc [135]. In fact, the NeuAc content of insect cells at 20 fmol/μg total protein was nearly 50 times lower than that of CHO cells at 900 fmol/μg. Unfortunately, several of the genes required for synthesizing CMP-NeuAc from the intermediate ManNAc were unknown in insect cells, mammalian cells, or any other eukaryotes. This prompted a genomic search for human genes in the NeuAc synthesis pathway that could be inserted into the insect cell's metabolic pathway. The genes for NeuAc synthesis (neuB) and CMP-NeuAc synthase (neuA), known in *E. coli* [147,148], were used to query the human genome database in order to identify putative homologs. In order to identify its function, the putative human NeuAc synthase gene selected was inserted into a baculovirus vector and the resulting recombinant baculovirus was found to generate NeuAc when serum-free medium was supplemented with precursor, *N*-acetylmannosamine (ManNAc) [135]. Indeed, the NeuAc levels obtained from insect cells were more 10-fold higher than those obtained from CHO cells [135]. Subsequent experiments revealed that endogenous non-specific kinase activity present in insect cells converted intracellular ManNAc to ManNAc-6-P (substrate of human NeuAc synthase) and non-specific phosphatase activity converted the NeuAc-9-P (direct product of the reaction catalyzed by human NeuAc synthase) to NeuAc. Endogenous insect GlcNAc kinase activity was later found to substitute for ManNAc kinase in phosphorylation of ManNAc but at much lower efficiency [149]. Interestingly, in the absence of ManNAc supplementation, Sf9 cells infected with the recombinant baculovirus containing human NeuAc synthase gene produced an alternative neuraminic acid, KDN (2-keto-3-deoxy-D-glycero-D-galactononic acid), formed from endogenous mannose. Even when insect cells were engineered to express the human NeuAc synthase gene and supplemented with ManNAc, levels of CMP-NeuAc remained 10 times below those of CHO cells. The human CMP-NeuAc synthase was subsequently identified and cloned into a baculovirus vector, and Sf9 cells were co-infected with two viruses containing genes for human NeuAc synthase or human CMP-NeuAc synthase [134]. When the medium was supplemented with ManNAc, the co-infected cells produced CMP-NeuAc levels that were six times higher than those obtained from CHO cells. Thus, metabolic engineering of CMP-NeuAc synthesis pathways has the potential to exceed the endogenous capacity present in normal mammalian cells. However, the elimination of any one of the three components, NeuAc synthase, CMP-NeuAc synthase, or ManNAc nullified the capacity of insect cells to generate significant pools of CMP-NeuAc.

Sf9 cells were also found to lack significant UDP-*N*-acetylglucosamine 2-epimerase activity required for converting UDP-GlcNAc to ManNAc-6-P in the CMP-NeuAc synthesis pathway [133] (Figure 5). The low 2-epimerase activity was also evident by the requirement for ManNAc supplementation in order to generate NeuAc in insect cells expressing a recombinant human NeuAc synthase. Since sugar nucleotide analysis revealed that insect cells could indeed produce UDP-GlcNAc [131], the potential thus existed for metabolic engineering of the pathways for the biosynthesis of CMP-NeuAc from endogenous substrates. In mammalian cells, the enzymatic activities responsible for converting UDP-GlcNAc to ManNAc-6-P are present in a single bifunctional enzyme, UDP-GlcNAc 2-epimerase/ManNAc kinase, and infection of insect cells with a baculovirus containing this gene rescued the enzymatic activity missing from insect cells [133]. In order to determine if insect cells could be engineered to generate NeuAc from endogenous intracellular metabolites, Sf9 cells were co-infected with baculoviruses encoding mammalian versions of both the UDP-GlcNAc 2-epimerase/ManNAc kinase and NeuAc synthase genes [149]. Indeed the co-infected insect cells generated NeuAc from endogenous substrates at levels that were comparable to those obtained with ManNAc supplementation. Addition of GlcNAc to the medium of co-infected cells increased NeuAc to levels that were six-times higher than those obtained with ManNAc feeding. Subsequent experiments revealed metabolic bottlenecks in the uptake of ManNAc from the medium and phosphorylation of ManNAc [149]. Expression of the ManNAc kinase domain relieved the phosphorylation limitation, and the *O*-acetylation of ManNAc overcame the transport bottleneck, although this chemical modification was lethal to the cells at high concentrations. Nonetheless, these baculovirus infections

demonstrated the future potential of generating intracellular CMP-NeuAc completely from endogenous metabolites through the expression of an UDP-GlcNAc 2-epimerase/ManNAc kinase gene in combination with NeuAc synthase and CMP-NeuAc synthase.

Expression of sialyltransferase and sialylation of glycoproteins

The inability of many insect cell lines (Sf9, Tn-5B1-4, and others) to generate sialylated glycoproteins even in the presence of GalT indicated possible limitations in sialyltransferase activity. In order to address this limitation, baculovirus constructs were generated to contain mammalian β4GalT and ST6Gal [150]. Sialylation of a viral glycoprotein, gp64, was detected only when Sf9 cells were infected with a baculovirus containing both transferase genes. Similar results were obtained when Sfβ4GalT cells, which constitutively express heterologous GalT, were infected with a recombinant baculovirus encoding ST6Gal [151]. Subsequently, Sf9 and Tn-5B1-4 cell lines, engineered to include both GalT and ST6Gal (Sfβ4GalT/ST6 [112] and Tn5β4GalT/ST6 [143]) genes in their genomic DNA, were found to express active recombinant enzymes constitutively. These transgenic insect cells were observed to sialylate gp64 when infected with a wild-type baculovirus [112,143]. Sialylation was also observed with a secreted form of glutathione S-transferase tagged α-mannosidase I when the same cells were infected with a recombinant baculovirus containing that gene [112]. It should be noted that cells were cultured in serum-bearing medium in the above studies when sialylation was observed. In this regard, a recent study clearly indicated that some serum components can affect sialylation [152]. In this study, Sfβ4GalT/ST6 cells produced sialylated N-glycans when cultured in the presence but not in the absence of fetal bovine serum. In addition, serum-free media supplemented with purified fetuin but not asialofetuin supported N-glycan sialylation by Sfβ4GalT/ST6 cells [152]. Furthermore, the terminally sialylated N-glycans isolated from fetuin also supported glycoprotein sialylation by Sfβ4GalT/ST6 cells [152]. However, sialylation was very low when the same cells were cultured in serum-free medium supplemented with NeuAc or ManNAc [152]. These results suggest that the presence of a NeuAc salvaging pathway in lepidopteran. Other researchers have shown that sialylation can be improved by expressing ST6Gal and other transferases in the Ea4 insect cells, which lack the high level of β-*N*-acetylglucosaminidase activity found in Sf9 cells [153].

In order to address the limitation in GnT II identified previously [95] as well as possible limitations in GnT I due to hexosaminidase activity, an engineered cell line, Sfβ4GalT [110] was further engineered to include a gene for mammalian GnT II as well as the genes for mammalian β4GalT, ST6Gal I, and ST3Gal IV [144]. These cells, SfSWT-1, generated biantennary complex-type N-glycans with terminal NeuAc residues on one of the branches [144]. Interestingly, however, the cell line required cultivation in serum-containing growth medium in order to deliver sialylated recombinant glycoproteins [144,154,155]. The requirement of capabilities for both SiaT and CMP-NeuAc synthesis identified previously [134] was fulfilled by a single cell line, SfSWT-3, which includes the glycosyltransferases of SfSWT-1 along with NeuAc synthase and CMP-NeuAc synthase foreign genes cloned into a single Sf9 cell line [155]. This cell line, when grown in serum-free medium supplemented with ManNAc, produced predominantly complex-type biantennary N-glycans containing NeuAc on only one branch. These N-glycans were similar to those structures generated by the SfSWT-1 cell line. However, a few of the complex N-glycans generated by SfSWT-3 also observed to include NeuAc on both branches.

As an alternative to engineering sialylation pathways, other researchers have explored alternative insect cell lines with a capacity for sialylation in culture. Tn-4h is a clonal isolate derived from Tn-5B1-4. It has been reported that Tn-4h cells expressed sialylated glycoproteins when cultured in the High Aspect Ratio Vessel (HARV) bioreactor in serum-bearing medium [156]. Cells cultured in T-flasks could also generate sialylated glycoproteins when the serum-bearing medium was supplemented with the CMP-NeuAc precursor, ManNAc. Another clonal isolate, Tn-4s, was also identified that could generate sialylated glycoproteins in spinner flasks [157]. These findings are especially interesting since closely related cell line, Tn-5B1-4, lack this capability when cultured in normal serum bearing medium. However, the addition of silkworm hemolymph (SH) to Tn-5B1-4 cells also enabled the cells to generate fully sialylated glycoproteins but the additive reduced the productivity of this cell line by a factor of five [158]. Another insect cell line adapted from Monarch butterfly, *Danaus plexippus* (DpN1) was also observed to express a recombinant alkaline phosphatase (SEAP) containing sialylated N-glycans when infected with recombinant baculovirus vectors [122].

Why certain cell lines appear to lack the capacity for sialylation while others are capable of sialylation, at least in the presence of serum-containing medium or other culture modifications is unclear. To address this discrepancy, researchers have recently begun to examine genomes for the presence or absence of NeuAc synthesis pathway genes [159]. Indeed, a functional NeuAc phosphate synthase gene has been identified in the genome of *D. melanogaster* although the enzymatic levels are extremely low in Schneider S2 cell line derived from *D. melanogaster* [160]. Recently, a sialyltransferase has been detected also in *D. melanogaster* genome with activity more towards GalNAc acceptor(s) and less towards Gal acceptor(s) [119]. This enzyme appeared to be expressed during particular developmental stages of the embryo to explain the lack of activity observed in many cell lines. Perhaps, the modification of culture conditions results in the isolation of clonal isolates or the stimulation of insect genes responsible for complex glycosylation processing. Either way, many studies remain in order

to understand the N-glycan processing capabilities of insects and insect cells.

Limiting FucT C3 activity in insect cells

Both endogenous and recombinant glycoproteins expressed in many insect cells have been observed to contain Fucα(1,3) linked to the Asn-linked GlcNAc of N-glycans [5,16,95,127,128]. This is not observed in mammalian cells, and may represent a potentially allergic modification [19,21–25,161]. In order to avoid this undesirable modification, the genes responsible for core α(1,3)-fucosylation must be inhibited or else cell lines lacking this processing capability must be used. Recently, a gene encoding FucT C3 was cloned from *D. melanogaster* [130]. Although FucT C3 genes have not yet been cloned from any Lepidopteran species, the potential exists for identifying these enzymes as well. Once the FucT C3 gene is characterized, the gene could be inactivated by site-directed mutagenesis or RNA inhibition technology. Thus, the potential exists for eliminating or inactivating this gene in the production of recombinant glycoproteins. An alternative strategy is to utilize insect cell lines that lack the core α(1,3)-fucosylation capability. Several studies indicated the absence of core α(1,3)-fucosylated N-glycans to suggest that FucT C3 activity may be low in Sf9 cells [3,7,128] and Ea4 cells [7]. More recently, detailed evaluation of the major N-glycans of a recombinant hTf obtained from gypsy moth cells (Ld652Y) also indicated absence of core α(1,3)-fucosylated structures [17]. These studies suggest that multiple insect cell lines may lack or not express significant levels of the FucT C3 activity, and may represent appropriate systems in which to encode additional N-glycan processing capabilities as described above in order to obtain glycoproteins bearing human-type N-glycans from insect cells. Alternatively, the removal of α(1,3)-fucosylation activity from popular cell lines such as Tn-5B1-4 and Schneider S2 cell lines is another potential approach for removing this undesirable glycosylation in insect cells.

Conclusion

A number of studies have revealed the characteristics of N-glycan processing system in insect cells. In contrast to mammalian cells, most insect cell lines appear to lack multiple essential enzymes required for synthesis of sialylated complex-type N-glycans. The lack of these enzymes along with the presence of a unique degradation enzyme, β-*N*-acetylglucosaminidase, and core α(1,3)-fucosyltransferase in several insect cells lead to expression of non-mammalian-type N-glycans represented primarily by paucimannosidic structures, $Man_{1-3}GlcNAc\beta(1,4)GlcNAc$-Asn with or without core α(1,6) and α(1,3) fucosylation. A number of oligomannose-type N-glycans are also observed often in insect-cell produced glycoproteins. Currently, little is known about biological function of such N-glycans in insect cells and insects. However, recent genomic studies in *D. melanogaster* indicate that some insect stages may include more complex oligosaccharide processing events. Indeed, this variability in insect glycosylation may play a key role in dictating the biological activities of the insect.

To address the limitations in insect cell glycosylation for production of biotherapeutics, genetic and metabolic engineering approaches have been applied in order to humanize insect cell N-glycan processing pathways. Insertion of transferase genes and metabolic processing enzymes has revealed the potential for producing glycoproteins bearing complex N-glycans using lepidopteran insect cell - baculovirus expression vector system. These developments represent to our knowledge, the first time that non-mammalian hosts have been engineered in order to produce fully-sialylated, "humanized" glycoproteins. Such improvements in glycosylation processing may have significant technological implications for the production of therapeutic glycoproteins in non-mammalian hosts in the future.

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