Humanization of Lepidopteran Insect-Cell-Produced Glycoproteins

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ABSTRACT
The insect cell−baculovirus expression vector system, widely used for glycoprotein production, is not ideal for pharmaceutical glycoprotein production due to the characteristics of the *N*-glycans in the expressed products. Insect cells lack several enzymes required for mammalian-type *N*-glycan synthesis and contain a specific *N*-acetylglucosaminidase that stunts the growth of chains and a core α-1,3-fucosyltransferase that yields potentially allergenic glycoforms. Current knowledge on *N*-glycan processing in lepidopteran insect cells is summarized, and strategies to develop better glycoprotein expression systems suitable for pharmaceutical glycoprotein production are discussed.

1. Introduction

Several insect cell lines from lepidopteran insects (see Figure 1) are used for glycoprotein expression with the baculovirus expression vector system because this expression vector system can produce glycoproteins more readily than mammalian cell expression systems. In addition, lepidopteran insect cells grow well in serum-free medium and in suspension culture. However, this system is not yet ideal for the production of pharmaceutical glycoproteins because the insect-produced glycoproteins have significantly different *N*-glycans from those by mammalian cells (Figure 2). They are (i) the inability to synthesize sialylated complex-type *N*-glycans in contrast to mammalian cells[1−3] and (ii) the presence of potentially allergenic structure, Fucα(1,3)GlcNAc-Asn, not seen in glycoproteins from mammalian cells. Insect cells tend to generate paucimannosidic or high mannose structures terminating in Man or occasionally GlcNAc. Paucimannosidic structures have been repeatedly found on glycoproteins produced by lepidopteran insect cells derived from *Spodoptera frugiperda*,[4−17] *Mamestra brassica*,[5,8] *Bombyx mori*,[4,8] *Trichoplusia ni*,[17,18] and *Estigmene acrea*.[9,10] These small glycans consist of one to three Man and one or two GlcNAc residues with or without Fuc attached to the Asn-linked GlcNAc. Differences in *N*-glycan structures may result in different biological activities, pharmacokinetic behavior, and allergenic reactions in human.[19−22]

In the past decade, much effort has been devoted to analysis of *N*-glycan structures, activity measurements and gene cloning of *N*-glycan processing enzymes, and measurements of sugar nucleotide for the baculovirus-insect cell system. It is clear that the inability of most lepidopteran insect cells to produce mammalian-type *N*-glycans are mainly attributable to (i) the lack of certain glycosyltransferases, (ii) the inability to produce CMP-neuraminic acid, and (iii) the presence of a *β*-*N*-acetylglucosaminidase which removes GlcNAc from the Manα-(1,3) branch. Recently, attempts have been made to improve *N*-glycan structures of lepidopteran cell-produced glycoproteins to alleviate these problems. In this Account, we will dissect the *N*-glycan processing pathways in lepidopteran cells with reference to enzyme activities and the levels of donor sugar nucleotides involved in *N*-glycan processing. We also review the status of engineering *N*-glycan processing pathways in this protein expression system. Readers are also referred to other recently published reviews on *N*-glycosylation in insects and insect cells.[2,3]

2. Analytical Methods

2.1. Assay of Glycosyltransferase and Glycosidase Activity. Most common measurement of glycosyltransferase and glycosidase activities is by the use of radiolabeled sugar nucleotides to trace the radiolabeled sugar transferred to the acceptor. Although this method is sensitive, its obstacles are strictly controlled storage, usage, and disposal of radioisotopes. Glycans can be labeled with a fluorescent tag such as pyridylamino (PA) group for structural analysis, glycosyltransferase, and glycosidase assays. 2-D HPLC analysis of PA-glycans enables fine separation and reliable identification of product glycans with subtle structural differences.[23,24]

Many insect cells contain core α-1,3- and core α-1,6-fucosyltransferases, which add Fuc to the Asn-linked GlcNAc via α-1,3- and α-1,6-linkages, respectively. The activities of these enzymes were assayed by measuring the transfer of radiolabeled Fuc from GDP-Fuc to acceptor substrates. However, this assay is unsatisfactory when the sample has both enzyme activities. Since PA-derivatized glycans could not be acceptors for Fuc-T C3,[25] fluorescent

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Latin name	*Spodoptera frugiperda*	*Trichoplusia ni*	*Estigmene acrea*	*Bombyx mori*	*Mamestra brassicae*
Common name	Fall Armyworm	Cabbage looper	Salt marsh moth	Silkworm	Cabbage moth
Family name (Subfamily name)	Noctuidae (Amphipyrinae)	Noctuidae (Plusiinae)	Arctiidae	Bombycidae	Noctuidae (Hadeninae)
Cell line	Sf9/Sf21	BTI-Tn-5B1-4	Ea4	Bm-N	Mb0503
Tissue derived	Pupal ovary	Pupal ovary	Larval hemocytes	Ovary	Larval hemocytes

FIGURE 1. Lepidopteran cell lines often used for glycoprotein production. Pictures of *Spodoptera frugiperda*, *Trichoplusia ni*, and *Estigmene acrea* were obtained from "Web Images of North American Moth Species: http://alpha.furman.edu/~snyder/leplist/". Pictures of *Bombyx mori* and *Mamestra brassicae* were obtained from "Moths and Butterflies of Europe: http://www.leps.it/".

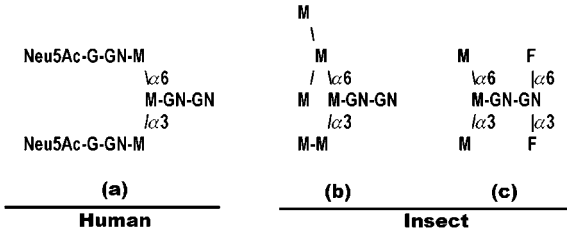

FIGURE 2. Comparison of typical *N*-glycans found in glycoproteins produced by human or lepidopteran cells. (a) Sialylated complex-type *N*-glycan, (b) high mannose-type *N*-glycan, (c) paucimannosidic *N*-glycan. GN, *N*-acetylglucosamine; F, fucose; M, mannose; G, galactose.

derivatized glycopeptides must be used as acceptor substrates, and fucosylated products were analyzed by reversed-phase HPLC[26] and MALDI-TOF mass spectrometry.[27] A recently developed assay of galactosyltransferase (GalT) uses microtiterplate-coated GlcNAc-BSA as the acceptor and Eu-labeled RCA120 lectin as the probe. The newly formed Galβ(1,4)GlcNAc structure is measured as β4GalT activity in both native and engineered lepidopteran insect cells.[28]

2.2. Sugar Nucleotide Assay. Prior methods for measurement of sugar nucleotides in biological samples, using anion-exchange HPLC or reversed-phase HPLC, could not separate UDP-Glc and UDP-GlcNAc from UDP-Gal and UDP-GalNAc, respectively. Our recent method, high-performance anionic exchange chromatography under mildly alkaline condition, can separate all sugar nucleotides in the extracts from lepidopteran insect cells simultaneously.[29]

3. Glycosyltransferases and Glycosidases Involved in *N*-Glycan Processing in Insect Cells

The processing pathway of *N*-glycans in lepidopteran insect cells is shown in Figure 3. A number of studies have suggested that initial processing of *N*-glycans in insect

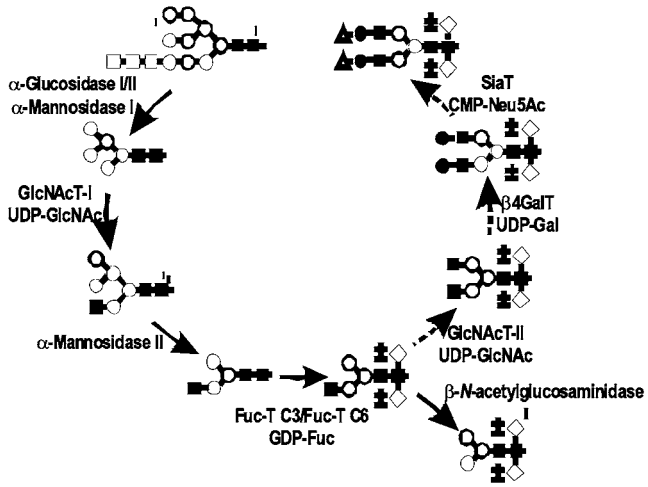

FIGURE 3. *N*-Glycan processing pathway in lepidopteran insect cells. Symbols: ■, GlcNAc; ○, Man; ●, Gal; ◇, Fuc; □, Glc; △, Sia. The dotted arrow indicates pathways which are insufficient in lepidopteran insect cells.

cells is similar or identical to that of mammalian cells. However, insect cells appear to lack some of the processing pathways of mammalian cells but contain additional glycosylation activities absent in mammalian cells.

3.1. α-Glucosidase I, II, and α-Mannosidase I. Glc_3-$Man_9GlcNAc_2$ is processed by α-glucosidase I, II, and α-mannosidase I to generate $Man_5GlcNAc_2$ structure. Many glycoproteins produced by lepidopteran insect cells have high-mannose-type glycans. For example, *N*-glycans on human IgG and hTF produced by Tn-5B1-4 cells included various high mannose-type and paucimannosidic glycans, with some incomplete complex-type glycans.[18,30] Expression of α-glucosidase I and II in several lepidopteran insect cells appears adequate.[31] In addition, α-mannosidase I has been purified from Sf21 cells[32] and cloned from Sf9 cells,[33] and its substrate specificity has been chracterized.[34] These results suggest that lepidopteran insect cells contain ample α-glucosidase I, II, and α-mannosidase I.

3.2. *N*-Acetylglucosaminyltransferase I and α-Mannosidase II. First, GlcNAc is added to Manα(1,3) branch of $Man_5GlcNAc_2$ by *N*-acetylglucosaminyltransferase I (GlcNAcT-I); thereafter, two Man residues are removed by α-mannosidase II. Substantial levels of GlcNAcT-I activities were observed in several insect cell lines including Sf9, Sf21, Mb0503, and Bm-N.[35] Mannosidase-II was active in Sf21, Mb0503, and Bm-N cells[36] and was purified from Sf21 cells.[37] Like its counterpart from mammalian cells, α-mannosidase II from insect cells requires GlcNAc on the Manα(1−3) branch for its activity.[36] These studies suggest that lepidopteran insect cells have high levels of α-mannosidase II and GlcNAcT-I in order to generate the precursor glycan required for the formation of complex-type *N*-glycans.

3.3. *N*-Acetylglucosaminyltransferase II. In mammalian cells, the product *N*-glycan of α-mannosidase II reaction serves as an acceptor for the next reaction catalyzed by *N*-acetylglucosaminyltransferase II (GlcNAcT-II), which adds another GlcNAc to the Manα(1,6) branch. However, lepidopteran insect cells, including Sf9, Sf21, Mb0503, and Bm-N cells, have been shown to have only 1% or less of the endogenous GlcNAcT-II activity present in mammalian cells.

3.4. *β*-1,4-Galactosyltransferase. A terminal GlcNAc on either Man-branch is usually galactosylated by *β*-1,4-galactosyltransferase (GalT) in mammalian cells. In contrast, galactosylated *N*-glycans are rarely found in glycoproteins from lepidopteran cells. In fact, negligible levels of GalT activity were detected in Sf9, Tn-5B1-4, and Mb0503 cells.[38−40] GalT activities in Sf9 and Tn-5B1-4 cells were reexamined using an Eu-fluorescence assay method.[28] 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.[28]

3.5. Core α-1,3- and α-1,6-Fucosyltransferases. *N*-Glycans with one or two GlcNAc on Man_3-core can be further modified by core fucosyltransferases. Both core Fuc-T's require the presence of GlcNAc*β*(1,2) on the Manα(1,3) branch for its action.[41] *N*-Glycans containing either one or both of Fucα(1,3) and Fucα(1,6) attached to the Asn-linked GlcNAc were identified on the membrane glycoproteins from Mb0503, Sf21, and Bm-N cells, in which glycoproteins from Mb0503 cells contained highest levels of α-1,3-fucosylated *N*-glycans.[8] Similar results were reported for *N*-glycans from recombinant IgG,[42] human serum transferrin,[30] neuropsin,[43] and CR3 domain of latent TGF-beta binding protein-1[44] produced by Tn-5B1-4 cells. However, human interferon *ω*1 expressed in Sf9 cells showed no α-1,3-fucosylated *N*-glycan.[6] Activities of core α-1,3-fucosyltransferase (Fuc-T C3) and core α-1,6-fucosyltransferase (Fuc-T C6) were detected in Mb0503 cells, in which the former was much higher than the latter.[41] Fuc-T C6, but not Fuc-T C3, was easily detected in Sf9 cells. Independently, Fuc-T C6 activity was detected in Sf9, Mb0503, and Bm-N cells.[45] Interestingly, in the same study, Fuc-T C3 was detected only in Mb0503 cells and not in Sf9 and Bm-N cells. A gene encoding Fuc-T C3 has been recently cloned from *Drosophila melanogaster*.[27]

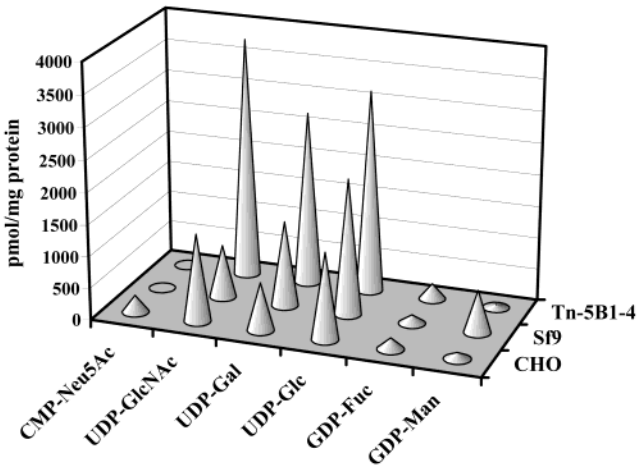

FIGURE 4. Sugar nucleotide contents of Sf9, Tn-5B1-4, and CHO cells (ref 29).

3.6. *β*-*N*-Acetylglucosaminidase. A *β*-*N*-Acetylglucosaminidase specific for the terminal GlcNAc on the Manα(1,3) branch was found in Sf21, Bm-N, and Mb0503 cells,[46] and it was suggested that this enzyme was localized in the microsome-like membrane fraction in Sf21 cells.[46] Similar enzymatic activity was also detected in the cell lysates and cell culture supernatant of insect cells derived from *Spodoptera frugiperda*, *Trichoplusia ni*, *Bombyx mori*, or *Malacosoma disstria*.[47] Structural analysis of *N*-glycans from human IgG[42] and hT*f*[30] expressed in Tn-5B1-4 cells suggested the presence of such a *β*-*N*-acetylglucosaminidase in Tn-5B1-4 cells. The further removal of additional Man residues by α-mannosidase(s) can lead to the generation of structures with fewer than three Man residues, as has been observed in several studies.

3.7. Sialyltransferase. Sialyltransferase (SiaT) adds *N*-acetylneuraminic acid to the terminal Gal residues on *N*-glycans in mammalian cells. However, SiaT activity has yet to be detected in Sf9,[40,48,49] Sf21,[49] Tn-5B1-4,[48] Mb0503,[48] and Ea4[49] cells, even using highly sensitive assays with radiolabeled CMP-NeuAc or fluorescent CMP-Neu5Ac derivatives as the donor substrate.

4. Sugar Nucleotides

4.1. Endogenous Sugar Nucleotide Levels in Lepidopteran Insect Cells. All glycosyltransferases in the synthetic pathway for complex-type *N*-glycans require respective sugar nucleotides as donor substrates. Examination of the sugar nucleotide concentrations in lepidopteran insect cells demonstrated the presence of substantial levels of UDP-hexose, UDP-*N*-acetylhexosamine, GDP-Fuc, and GDP-Man in Sf9, Mb0503, and Tn-5B1-4 cells.[48] However, no CMP-NeuAc was detected in the same study.[48] Similar results were obtained on the sugar nucleotide levels in Sf9 and Tn-5B1-4 cells[29] (Figure 4).

4.2. Enzymes Involved in Sialic Acid and CMP-Sialic Acid Synthesis. Of particular significance is the absence in lepidopteran insect cells of the CMP-NeuAc necessary for sialylation of *N*-glycans. In mammalian cells, sialic

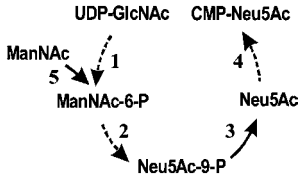

FIGURE 5. CMP-Neuraminic acid synthesis pathway. The dotted arrow indicates pathways which are insufficient in lepidopteran insect cells. 1, bifunctional UDP-GlcNAc 2-epimerase/ManNAc kinase; 2, *N*-acetylneuraminic acid 9-phosphate synthase; 3, *N*-acetylneuraminic acid 9-phosphate phosphatase; 4, CMP-neuraminic acid synthase. Exogenous ManNAc can be converted to ManNAc-6-P by *N*-acetylmannosamine kinase (5).

acids are synthesized from UDP-GlcNAc through multiple enzymatic reactions (Figure 5). The bifunctional enzyme, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase, is believed to be a key enzyme in the biosynthesis of Neu5Ac in rat liver.[50] This enzyme converts UDP-GlcNAc to ManNAc-6P, which is further converted to *N*-acetylneuraminic acids by *N*-acetylneuraminate-9-phosphate synthase (SAS) and *N*-acetylneuraminate-9-phosphate phosphatase. *N*-Acetylneuraminic acid is then converted to CMP-Neu5Ac by CMP-Neu5Ac synthase (CMP-SAS).

Effertz et al.[51] reported that the UDP-*N*-acetylglucosamine-2-epimerase activity in Sf9 cells was about 30 times less (in terms of specific activity) than that in rat liver cytosol fraction. Interestingly, Sf9 cells had 50 times higher *N*-acetylmannosamine kinase activity compared with the 2-epimerase activity.[51] We found that Sf9 cells contained negligible levels of neuraminic acids, and no detectable *N*-acetylneuraminate-9-phosphate synthase activity was present in the lysate of Sf9 cells.[52] We also found that Sf9 cells do not have detectable CMP-sialic acid synthase activity.[53]

5. Engineering of *N*-Glycan Processing Pathway

5.1. Targets. The general strategy for humanizing glycoproteins produced by the insect cell−baculovirus expression system is shown in Figure 6. The goal of engineering *N*-glycan processing is to develop a new insect cell-baculovirus expression vector system(s) that can express human-like sialylated multi-antennary complex-type *N*-glycans. As described in the earlier sections, several lines of evidence suggest that the majority of lepidopteran insect cells currently used for protein expression apparently lack several enzymes for such a goal. Moreover, lepidopteran insect cells contain the undesirable β-*N*-acetylglucosaminidase and Fuc-T C3. The former diminishes the key glycans containing GlcNAcβ(1,2)Manα(1,3) to stunt the normal growth of complex-type *N*-glycans, and the latter generates potentially allergenic *N*-glycans. Therefore, the *N*-glycan processing pathways need to be altered in the insect cells by enhancing or suppressing respective processing pathways.

5.2. Improvement of *N*-Acetylglucosaminylation of the Manα(1,3)-Branch. β-*N*-Acetylglucosaminidase was implicated as a problem in *N*-glycan elongation by its absence of *Estigmene acrea* cells, which produced *N*-glycans containing terminal *N*-acetylglucosamine residues.[54] Sf9 cells are known to contain high levels of β-*N*-acetylglucosaminidase.[54] Using Sf9 cells, Wagner et al. succeeded in *N*-glycan elongation by coexpression of human *N*-acetylglucosaminyltransferase I and fowl plague virus hemagglutinin.[9] Watanabe et al.[55] examined the effect of a β-*N*-acetylglucosaminidase inhibitor, 2-acetamide-1,2-dideoxynojirimycin (2-ADN) on bovine interferon-γ (bIFN-γ) production in Tn-5B1-4 cells. Inhibition of β-*N*-glucosaminidase led to expression of sialylated bIFN-γ. The authors speculated that the inhibitor enhanced accumulation of substrates possessing a β(1,2)-linked GlcNAc, thereby leading to further elongation by GalT and SiaT to form sialylated *N*-glycans. However, the overall increase of *N*-glycans containing β(1,2)-linked GlcNAc was not determined.

5.3. Improvement of Galactosylation. Expression of a mammalian GalT by a baculovirus vector increased galactosylation of glycoprotein,[56] indicating that the mammalian enzyme expressed by baculovirus infection could function in the infected lepidopteran cells and that it could compete with the β-*N*-acetylglucosaminidase activity in insect cells.[56] Similar results were obtained when human serum transferrin (hTf) was expressed by Tn-5B1-4 cells infected with two baculoviruses, one encoding a gene for hTf and the other encoding a gene for a mammalian GalT.[30] In this study, 13% of the total *N*-glycans were galactosylated, and protection of GlcNAc on Manα(1,3) branch against β-*N*-acetylglucosaminidase by galactosylation was confirmed (Figure 7).

In a different strategy, when Sf-9 or Tn-5B1-4 cells stably transformed to include GalT into the host cell genome (Sfβ4GalT[38] from Sf9 or Tn5β4GalT[57] from Tn-5B1-4) were infected with native baculovirus, galactosylated virion glycoprotein, gp64[38,57] was produced (shown by lectin blot). Similarly, the infection of Sfβ4GalT cells with a recombinant baculovirus expression vector encoding human tissue plasminogen activator (t-PA) also produced galactosylated t-PA.[38]

5.4. Production of Biantennary Complex-Type *N*-Glycans. Production of biantennary complex-type *N*-glycans was achieved recently by expressing a mammalian *N*-acetylglucosaminyltransferase II (GlcNAcT-II) in lepidopteran cells using a transgenic insect cell line, SfSWT-1,[58] or using baculovirus expression vector system.[59]

5.5. Formation of Sialylated *N*-Glycans. Sialylation of *N*-glycans was reported in Tn-5B1-4 cells when the cells were cultured in the presence of an hexosaminidase inhibitor (2-ADN).[55] This result is particular intriguing since Tn-TB1-4 cells lack GalT, SiaT, and CMP-NeuAc synthase (see above). Unfortunately, analysis was only by lectin blot and not by quantitative chemical analysis of the exact structures.

Tn-4h is a clonal isolate from Tn-5B1-4[60] and expressed sialylated glycoproteins when cultured in the High Aspect Ratio Vessel (HARV) bioreactor in serum-bearing medium. These findings are especially interesting since closely related cell line (Tn-5B1-4) lack this capability.

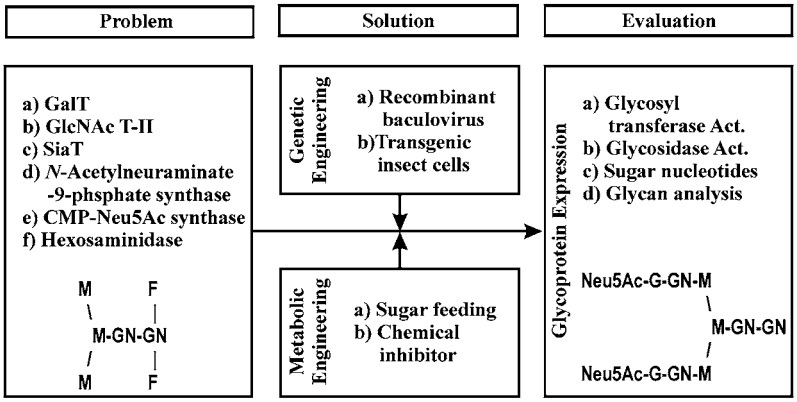

FIGURE 6. General strategy for humanization of glycoproteins produced by lepidopteran cell-baculovirus expression system.

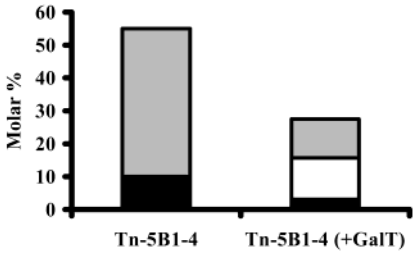

FIGURE 7. Changes in *N*-glycan structures on human serum transferrin expressed in Tn-5B1-4 cells by co-infection of a recombinant baculovirus expression vector encoding the gene for a mammalian GalT. ■, *N*-glycans containing nongalactosylated GlcNAc on the Manα(1,3) branch; □, *N*-glycans containing galactosylated GlcNAc on the same branch; gray shading, Paucimannosidic glycans (ref 30).

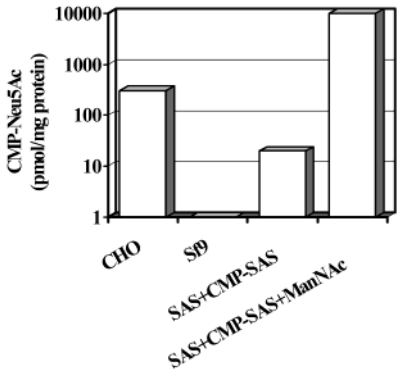

FIGURE 8. Improvement of CMP-Neu5Ac production by expression of *N*-acetylneuraminic acid 9-phosphate synthase (SAS) and CMP-neuraminic acid synthase (CMP-SAS) in Sf9 cells cultured in the medium with or without 10 mM ManNAc supplementation (ref 53).

Sialylation was also detected in virion glycoprotein, gp64, when Sf9 cells were infected with a recombinant baculovirus vector encoding mammalian GalT and α-2,6-sialyltransferase (SiaT6), while no sialylation was detected in the absence of either GalT or SiaT6.[61] Similar results were observed when Sfβ4GalT cells, which constitutively express heterologous GalT, were infected with a recombinant baculovirus encoding SiaT6.[62] Subsequently, two transgenic insect cells, Sfβ4GalT/ST6[40] andTn5β4GalT/ST6[57] cells, each having foreign genes encoding both mammalian GalT and SiaT6 in their genomic DNA, were shown to express both enzymes constitutively. When these cells were infected (in serum-containing medium) with a wild-type baculovirus or a recombinant baculovirus encoding insect α-mannosidase-I, both gp64[40,57] and α-mannosidase-I[40] were found to be sialylated.

Recent study indicated that inclusion of sialylated molecules in serum-free culture medium could increase sialylation.[63] These results suggested the presence of a sialic acid salvaging pathway in lepidopteran cells. The mechanism(s) for increased sialylation is not understood. Sialo-glycoconjugates might may have to be taken up by insect cells, and sialic acid might be released in insect cells by sialidase.[64] However, how the CMP-SAS deficient lepidopteran cells[53] can accomplish substantial sialylation is not clear. Alternatively, sialylation might occur by trans-sialylation, which had been known in *Trypanosoma cruzi*.[65]

5.6. Synthesis of CMP-Neu5Ac. The processing steps catalyzed by UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase, *N*-acetylneuraminate-9-phosphate synthase, and CMP-Neu5Ac synthase represent bottlenecks in the CMP-Neu5Ac synthesis pathway of lepidopteran cells (Figure 5). To overcome this problem, we cloned mammalian *N*-acetylneuraminate-9-phosphate synthase[52] and CMP-Neu5Ac synthase,[53] and expressed these enzymes in Sf9 cells. When Sf9 cells were infected with a recombinant baculovirus expression vector encoding *N*-acetylneuraminate-9-phosphate synthase and were cultured in a medium supplemented with *N*-acetylmannosamine (ManNAc), Sf9 cells produced high levels of *N*-acetylneuraminic acid (Neu5Ac).[52] Furthermore, when Sf9 cells, co-infected with two different baculoviruses, each encoding *N*-acetylneuraminate-9-phosphate synthase, or CMP-Neu5Ac synthase, were cultured in serum-free medium supplemented with ManNAc, Sf9 cells produced high levels of CMP-Neu5Ac (Figure 8). However, without ManNAc supplement, only ca. 1/20 of that of CHO cells was produced (Figure 8), despite the presence of a substantial amount of UDP-GlcNAc (Figure 4). These results are consistent with the observation that Sf9 cells lack UDP-*N*-acetylglucosamine 2-epimerase activity but have high levels of ManNAc kinase activity.[51] This study demonstrated that the metabolic pathway for synthesis of CMP-Neu5Ac could be enhanced by a combination of overexpression of required enzymes and feeding of Man-

NAc, a precursor of Neu5Ac. Our laboratories have shown recently that the supplementation of ManNAc can be superseded by expressing the UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase gene in combination with other sialylation pathway genes (unpublished data).

6. Conclusion and Future Prospects

Many lines of evidence have indicated that the inability of the vast majority of lepidopteran cells to synthesize mammalian-type *N*-glycans. The inability to obtain such *N*-glycans in lepidopteran cells can be attributed to the insufficient levels of GalT, GlcNAcT-II, SiaT, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase, *N*-acetylneuraminate-9-phosphate synthase, and CMP-Neu5Ac synthase activities. β-*N*-Acetylglucosaminidase, which removes GlcNAc on the Manα(1,3) branch, was detected in several lines of lepidopteran cells. This enzyme apparently prevents synthesis of complex-type *N*-glycans by removing the key intermediate glycan containing GlcNAcβ(1,2)-Manα(1,3). In addition, Fuc-T C3 generates the potentially allergenic glycan structure, Fucα-(1,3)GlcNAc-Asn, on glycoproteins expressed in lepidopteran cells. Substantial improvement of galactosylation levels has been achieved by expressing mammalian GalT in lepidopteran cells. Galactosylation prevented degradation of *N*-glycans by β-*N*-acetylglucosaminidase and the generation of truncated paucimannosidic glycans. Synthesis of di-branched, galactosylated complex-type *N*-glycans has also been achieved by coexpression of GalT and GlcNAcT-II. Furthermore, the appearance of sialylation in insect cells grown in serum-containing medium has been observed by expressing SiaT in lepidopteran cells in combination with GalT expression. A strategy to engineer the CMP-neuraminic acid synthesis pathway for cells grown in serum-free medium has been established by a combination of genetic engineering and substrate feeding techniques. Expression of glycoproteins having completely sialylated, branched complex-type *N*-glycans in lepidopteran cells grown in serum-free medium was actually accomplished by using a new transgenic insect cell line, SfSWT-3 with ManNAc feeding.[66] SfSWT-3 is a stably transformed insect cell line derived from Sf9 cells, and these cells express mammalian SAS and CMP-SAS in addition to mammalian GlcNAcT-I, GlcNAcT-II, GalT, and SiaT. Structures that are more complex would require inclusion of additional glycosyltransferases (e.g., GlcNAcT-IV and GlcNAcT-V).

In parallel, several analytical methods have been developed to provide for sensitive, high-throughput, and detailed analysis of enzyme activities, glycan structures, and donor sugar nucleotides. Continuous efforts are also underway to establish better analytical techniques for detecting intracellular metabolites and extracellular structures. By combining cellular engineering with efficient analysis of detailed structures, we should be able to characterize and manipulate glycosylation processing pathways in the future for a wide spectrum of applications.

Abbreviations

CMP-Neu5Ac, cytidine-5′-monopho-*N*-acetylneuraminic acid; Fuc, fucose; Gal, galactose; GalT, galactosyltransferase; GDP-Man, guanosie-5′-diphosphomannose; GlcNAc, *N*-acetylglucosamine; GlcNAc-1-P, *N*-acetylglucosamine-1-phosphate; GlcNAc-6-P, *N*-acetylglucosamine-6-phosphate; Man, mannose; ManNAc, *N*-acetylmannosamine; ManNAc-6-P, *N*-acetylmannosamine-6-phaophte; Neu5Ac, 5-acetamido-3,5-dideoxy-D-glycero-D-galacto-2-nonulosonic acid (*N*-acetylneuraminic acid); Neu5Ac-9-P, *N*-acetylneuraminic acid-9-phosphate; UDP-Gal, uridine-5′-diphophogalactose; UDP-GlcNAc, uridine-5′-diphopho-*N*-acetylglucosamine; SiaT, sialyltransferase.

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