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Glycoproteins from Insect Cells: Sialylated or Not?

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Abstract

Our growing comprehension of the biological roles of glycan moieties has created a clear need for expression systems that can produce mammalian-type glycoproteins. In turn, this has intensified interest in understanding the protein glycosylation pathways of the heterologous hosts that are commonly used for recombinant glycoprotein expression. Among these, insect cells are the most widely used and, particularly in their role as hosts for baculovirus expression vectors, provide a powerful tool for biotechnology. Various studies of the glycosylation patterns of endogenous and recombinant glycoproteins produced by insect cells have revealed a large variety of O- and N-linked glycan structures and have established that the major processed O- and N-glycan species found on these glycoproteins are (Gal β 1,3)GalNAc-*O*-Ser/Thr and Man3(Fuc)GlcNAc2-*N*-Asn, respectively. However, the ability or inability of insect cells to synthesize and compartmentalize sialic acids and to produce sialylated glycans remains controversial. This is an important issue because terminal sialic acid residues play diverse biological roles in many glyco-conjugates. While most work indicates that insect cell-derived glycoproteins are not sialylated, some well-controlled studies suggest that sialylation can occur. In evaluating this work, it is important to recognize that oligosaccharide structural determination is tedious work, due to the infinite diversity of this class of compounds. Furthermore, there is no universal method of glycan analysis; rather, various strategies and techniques can be used, which provide glyco-cobiologists with relatively more or less precise and reliable results. Therefore, it is important to consider the methodology used to assess glycan structures when evaluating these studies. The purpose of this review is to survey the studies that have contributed to our current view of glycoprotein sialylation in insect cell systems, according to the methods used. Possible reasons for the disagreement on this topic in the literature, which include the diverse origins of biological material and experimental artifacts, will be discussed. In the final analysis, it appears that if insect cells have the genetic potential to perform sialylation of glycoproteins, this is a highly specialized function that probably occurs rarely. Thus, the production of sialylated recombinant glycoproteins in the baculovirus-insect cell system will require metabolic engineering efforts to extend the native protein glycosylation pathways of insect cells.

Keywords

Baculovirus; Glycoproteins; Insect cells; Recombinant glycoproteins; Sialylation

Introduction

Considering the rapid progress in sequencing the genomes of many organisms, including humans, the availability of expression systems that can be used to produce authentic mammalian proteins and glycoproteins has become crucial. One of the most important features of such a system is its glycosylation potential. Indeed, in order to study the biological properties of a recombinant glycoprotein or use it as a therapeutic agent, its glycosylation pattern must closely resemble the *in vivo* glycosylation pattern of the native product. Insect cells are widely used to produce recombinant proteins, as they can synthesize large quantities of a protein of interest when infected with powerful baculovirus-based gene expression vectors, and they can provide post-translational modifications similar to those provided by mammalian cells. Studies have shown that glycoproteins produced by all insect systems studied to date have O- and N-glycans with core structures similar or identical to those produced by all eukaryotes. In contrast, most studies suggest that insect cell-derived glycoproteins typically fail to acquire antennae of the N-acetylglucosamine type or peripheral sugars, especially sialic acids, which are commonly found on native mammalian glycans. Sialic acids play extremely important roles in glycoprotein biology. Because they are typically found as terminal residues on cell-surface glycoconjugates or circulatory components, they are involved in many cell-cell interactions, immunological reactions and in the clearance of circulating glycoproteins. Accordingly, the presence or absence of sialic acids in insect cells is a significant and somewhat controversial issue regarding the use of these cells as recombinant glycoprotein factories.

In addition to its biotechnological significance, the presence or absence of sialic acids in insects is fundamentally interesting from a phylogenetic point of view. According to an early review (Warren, 1963), sialic acids had not been detected among the Coelenterata, Annelida or Sipunculoidea. In contrast, they had been found in all vertebrate species, cephalocords, and echinoderms and were found sporadically among some platyhelminths, molluscs, and arthropods. In some phyla, KDN (ketode-oxynuraminic acid) was found in place of sialic acid. Among the arthropods, lobsters reportedly had sialic acids but, at that time, no sialic acids had been detected in any insect or arachnid species examined.

In this review, we will survey the studies that have contributed to our current view of the presence or absence of sialic acids in endogenous, viral and recombinant glycoproteins synthesized by insect cells, taking into account the methods used to determine glycan structures.

Current Knowledge of Insect Cell Protein Glycosylation

There is evidence that many of the glycoprotein processing events known to occur in mammalian cells also occur in insect cells. For a general review, see März *et al.* (1995).

The insect N-glycosylation pathway parallels the mammalian pathway up until formation of the following structure:

The major insect cell processing pathway downstream of this intermediate involves removal of the GlcNAc residue by a Golgi-associated N-acetylglucosaminidase, which produces a paucimannose type N-glycan (Altmann *et al.*, 1995; Wagner *et al.*, 1996a; Marchal *et al.*,

1999). This end-product is the most highly processed glycan found on most insect cell-derived N-linked glycoproteins. However, this does not appear to be the only N-glycan processing pathway found in insect cells, as various reports indicate that these cells also can produce subpopulations of some glycoproteins containing terminal N-acetylglucosaminyl, galactosyl, and sialyl residues. The O-glycosylation pathway in many lepidopteran insect cell lines produces glycoproteins containing GalNAc-*O*-Ser/Thr and a subpopulation of these structures is further processed to produce the Gal β 1,3GalNAc-*O*-Ser/Thr core-1 structure (März *et al.*, 1995). Both of these O-glycans are potential acceptors for sialyl residues.

Requirements for Glycoprotein Sialylation

The occurrence of sialic acid in a glycoconjugate from a particular cell type implies that that cell can perform the following reaction:

where CMP is cytidine monophosphate.

Thus, the requirements for sialylation of a glycoconjugate are:

The acceptor: acceptors include N-glycoproteins, O-glycoproteins, and glycolipids, and the sialyl residue can be transferred to galactosyl, N-acetylgalactosaminyl or sialyl residues.

The enzyme: the sialyltransferases are a large family of enzymes, which all use CMP-Neu5Ac (CMP-N-acetyl-neuraminic acid) or its derivatives e. g. CMP-Neu5Gc (CMP-N-glycolylneuraminic acid) as a donor. These enzymes have high specificity toward the acceptor and generally recognize not only the monosaccharide to which the sialic acid is transferred, but a more complex glycan motif. For a review, see Harduin-Lepers *et al.* (1995).

The donor substrate: the sialylation reaction requires the activated form of sialic acid, CMP-Neu5Ac, or its derivatives, as mentioned above. The CMP-NeuAc synthase, in contrast to other sugar-nucleotide synthases, is a nuclear resident as shown by biochemical studies (for a review see Kean, 1991) and, more recently, *via* expression of the cloned murine cDNA (Münster *et al.*, 1998).

Proper subcellular localization of both enzyme and substrates: sialylation reactions occur in the *trans*-Golgi. Translocation of donor substrates into the lumen of the Golgi apparatus requires a specific CMP-Neu5Ac/CMP antiporter, which is well characterized in mammals (Eckhardt *et al.*, 1996, Hirschberg *et al.*, 1998).

How to Assess the Sialylation Ability of a Cell

One way to assess the ability of a tissue to sialylate glycoproteins is to measure sialyltransferase activity in that tissue using either endogenous or exogenous acceptors. These assays can be performed by incubating cell extracts, subcellular fractions, or tissue slices with radiolabeled CMP-Neu5Ac. A similar approach is metabolic labeling with radioactive mannosamine or Neu5Ac, although the way by which the latter, anionic precursor, is taken up by cells is not understood.

A totally different approach is to analyze endogenous or recombinant glycoproteins for the presence of sialyl residues. Some methods utilize neuraminidases, which can specifically remove sialic acids, whereas other methods utilize lectins, which can specifically bind sialic acids. Among the lectins, MAA (*Maackia amurensis* agglutinin) and SNA (*Sambucus nigra*

agglutinin) are commonly used to detect α 2,3 and α 2,6 sialic acids, respectively. The use of neuraminidase must be coupled with a subsequent analysis of the biological or biophysical properties of the putatively sialylated molecule; for example, one might compare the electrophoretic mobilities of a glycoprotein before and after neuraminidase treatment. Lectins can be used to stain sialylated glycoproteins after electrophoresis and transfer to a membrane or they can be used for agglutination assays. Lectin-based assays must always be coupled with extensive controls, including endoglycosidase treatments, neuraminidase treatments, and/or the use of competing monosaccharides, to verify the carbohydrate specificity of the lectin binding results. More direct physico-chemical methods also can be used to detect sialic acids. While early work involved histochemical and colorimetric methods, more recent studies have involved the separation of glycan moieties by HPLC (high performance liquid chromatography), HPAEC (high pH anion exchange chromatography), or FACE (fluorophore-assisted carbohydrate electrophoresis), followed by structural determinations using mass spectrometry or NMR (nuclear magnetic resonance).

One factor that can complicate the measurement of sialyltransferase activities or sialic acids is degradation by sialidases, which have been detected in insect cells (Licari *et al.*, 1993), and could lead to false-negative results. The opposite problem is contamination with sialic acids or sialoglycoproteins derived from the animal serum used in the cell growth medium, which can produce false-positive results.

Sialylation of Endogenous Insect Cell Glycoproteins

Endogenous glycoproteins are defined as those that are encoded by the cellular genome, thus excluding any glycoprotein expressed during viral infection, including recombinant glycoproteins.

Detection of Sialyl Residues

In early studies, Vadgama and Kamat (1969) and Kamat (1971) used alcian blue staining, combined with mild acid hydrolysis and neuraminidase digestion to show the presence of sialic acids in the salivary glands of insects from several different orders. These conclusions were inconsistent with the results of Warren (1963), who observed no sialic acids in insects using the thiobarbituric acid method. Gee (1975) also suggested that the diuretic hormone of the tsetse fly *Glossina austeni* contained sialic acid because it lost its biological activity after neuraminidase treatment. A more recent study demonstrated that specific adhesion of *Plasmodium* ookinetes to the midgut epithelium of *Aedes aegypti* involves a midgut carbohydrate ligand and that free N-acetylneuraminic acid competes with the ookinetes in binding assays (Zieler *et al.*, 1999). However, these authors were unable to detect any sialic acids in mosquito midguts using a highly sensitive, HPLC-based fluorometric assay. Scolexin, a bacterially-induced immune protein specifically found in *Manduca sexta* larvae, has been shown to contain N-acetylneuraminic acid using GC-MS (gas chromatography-mass spectrometry) analysis (Kyriakides *et al.*, 1995).

Neu5Ac-specific lectins have been widely used to examine insect cell proteins for sialic acids, but have provided conflicting results. In one study, Davis and Wood (1995) used MAA and SNA blotting to identify sialylated glycoproteins in established insect cell lines from *Spodoptera frugiperda* (Sf21) and *Trichoplusia ni* (TN-368 and BTI-Tn-5B1-4). However, no proper specificity controls were included in their experiments. In another study, McCarthy and Fletcher (1992) were unable to detect sialic acids in seven different lepidopteran insect cell lines, including Sf21 and TN-368, using LPA (*Limulus polyphemus* agglutinin) agglutination assays. Lopez *et al.* (1999) also found no evidence of sialic acids using SNA and PNA (peanut agglutinin) lectin blotting analysis of endogenous glycoproteins from *Spodoptera frugiperda*, *Trichoplusia ni* and *Mamestra brassicae* and, in

this study, the lectin blotting results were confirmed by MALDI-MS (matrix-assisted laser desorption ionization mass spectrometry).

Finally, a precise two-dimensional HPLC and exoglycosidase analysis of membrane glycoprotein glycans from three different lepidopteran insect cell lines (Sf21, IZD-Mb-0503 and Bm-N) revealed no evidence of sialylation (Kubelka *et al.*, 1994).

In summary, the results of most, but not all, of these studies suggest that endogenous insect glycoproteins lack sialic acids. The inconsistency in these results might reflect cell type- and/or developmental stage-specific sialylation events. Indeed, sialic acids have been detected in *Drosophila melanogaster* by cytochemistry using LFA (*Limax flavus* agglutinin) and by a combination of gas-liquid chromatography and electron-impact mass spectrometry (Roth *et al.*, 1992). These authors also used blotting with an anti-polysialic acid antibody to demonstrate that homopolymers of α 2,8 linked sialic acids were expressed only in embryos between 14 and 18 hours of age. These results show that sialic acids do occur in Diptera, and that some forms, *e.g.* polysialic acid, occur in a developmental stage-specific fashion, as shown previously for polysialylation of the mammalian neuronal adhesion molecule N-CAM (Vimr *et al.*, 1984, Seki and Arai, 1993).

Occurrence of Endogenous Sialyltransferase Activity

Some investigators have used *in vitro* assays with radiolabeled or fluorescent CMP-Neu5Ac as the donor substrate to examine insect cell lysates or subcellular fractions for sialyltransferase activities (Butters *et al.*, 1981, Hooker *et al.*, 1999 and Lopez *et al.*, 1999). No significant sialyltransferase activity has ever been detected in these assays, irrespective of the acceptor substrate used. In contrast, Hiruma and Riddiford (1988) reported that the granular phenoloxidase of the tobacco hornworm, *Manduca sexta* was [^{14}C]-labeled when tissue slices were incubated with [^{14}C]Neu5Ac.

The difference in these results might reflect insect type- or tissue-specific differences in sialyltransferase activities, differences in established cell lines *versus* intact insects, or protein-specific differences, as the latter study focused on incorporation of radiolabeled sialic acid into a single insect glycoprotein.

Availability of Endogenous CMP-Neu5Ac

Few studies have examined the occurrence of the sialic acid donor, CMP-Neu5Ac, in insect systems. However, Hooker *et al.* (1999) recently reported that they were unable to detect any CMP-Neu5Ac by anion exchange chromatography of the soluble nucleotides extracted from uninfected Sf9, Sf21, or Ea4 cells or from baculovirus-infected Sf9 cells.

Glycoprotein Sialylation during Viral Infection

The granulosis viruses and the nucleopolyhedroviruses, both of which belong to the family Baculoviridae, can infect numerous insect species and are widely studied because of their potential applications both as biological insecticides and as eukaryotic gene expression vectors. The viruses classified within the genus baculovirus all consist of a nucleocapsid surrounded by an envelope, which includes virus-encoded glycoproteins that are translated and modified by the host cell. These glycoproteins can be used conveniently to study insect cellular protein glycosylation machinery because they are usually synthesized in large quantities. Using the lectin LPA, which binds to sialic acids, Russell and Consigli (1985) detected one sialylated glycoprotein synthesized during infection of *Trichoplusia ni* (TN-368) cells with *Plodia interpunctella* granulosis virus. In contrast, several studies of nucleopolyhedroviruses led to the conclusion that no viral glycoproteins were sialylated. Kretzschmar *et al.* (1994) demonstrated that neuraminidase treatment had no effect on the

HPLC profile of the N-glycans isolated from baculovirus (*Autographa californica* nuclear polyhe-drosis virus; AcMNPV)-infected Sf9 cells. They concluded that viral infection does not alter N-glycosylation and that Sf9 cells are not capable of sialylating glycoproteins. Jarvis and Finn (1995) used lectin blotting analysis to examine N-glycosylation of the major AcMNPV envelope glycoprotein, gp64 and found no evidence of sialic acid when gp64 was expressed in three different lepidopteran insect cell lines. However, gp64 was sialylated when it was expressed in mammalian cells. More recently, Jarvis *et al.* (1998b) constructed several gp64 mutants, each of which had only one of the five potential N-glycosylation sites, and found no evidence of sialic acid in any of the individual N-linked glycans by SNA lectin blotting. Finally, as part of their study, Hooker *et al.* (1999) showed that neither sialyltransferase activity nor CMP-Neu5Ac donor substrate was detectable in baculovirus-infected Sf9 cells, as mentioned above.

In addition to these insect-specific viruses, some animal viruses can grow in a wide variety of cultured cells, including cells of either mammalian or insect origin. This allows for a well-controlled comparison of protein glycosylation by both cell types, as glycosylation of virion proteins is mediated by cellular glycosylation pathways. The sialic acid content of isolated virions can be assayed by simple colorimetric methods. Schloemer and Wagner (1975) showed that sialylation of vesicular stomatitis virus grown in mosquito cells (*Aedes albopictus*) was very low, resulting in markedly reduced hemagglutinating activity. *In vitro* sialylation restored the hemagglutination titre of this virus to levels approaching those of the same virus grown in BHK-21 cells. Impaired sialylation has also been observed for the Semliki forest virus E2 glycoprotein produced by mosquito cells (Stollar, 1980) and purified Sind-bis virus grown in mosquito cells, relative to the same virus grown in BHK cells (Stollar *et al.*, 1976; Hirsch *et al.*, 1981).

Thus, these results provide no evidence for significant induction of glycoprotein sialylation pathways during infection of insect cells by a variety of different viruses.

Sialylation of Recombinant Glycoproteins in Baculovirus-Insect Cell Expression Systems

Recombinant baculoviruses are commonly used as vectors for the high level expression of foreign genes under the transcriptional control of the viral polyhedrin or p10 promoters, which are exceptionally strong promoters [for reviews, see Miller (1988), Luckow and Summers (1988), Jarvis (1997), and Altmann *et al.* (1999)]. Examination of baculovirus-expressed glycoproteins for sialylation has provided conflicting results, which we will discuss according to the methods used for sialic acid detection and characterization.

Use of Lectins to Determine Glycan Structures

Vandenbroeck *et al.* (1994) showed that porcine interferon- γ produced by baculovirus-infected Sf9 cells could be stained with SNA and MAA, indicating that this recombinant protein had acquired α 2,3 and α 2,6-linked sialic acids. Similarly, Davis and Wood (1995) reported that human placental secreted alkaline phosphatase (SEAP) produced in Sf21 and TN-368 cell lines reacted with SNA and concluded that this protein had acquired α 2,6-linked sialic acid. This conclusion was weakened by the lack of suitable controls, as discussed above. Similarly, the conclusion that a bovine leukemia virus glycoprotein expressed by recombinant baculovirus-infected insect cells contains sialic acid was weakened by the fact that it was based on the use of WGA (wheat germ agglutinin), which interacts poorly with sialyl residues and has other specificities (Russo *et al.*, 1998). Moreover, the culture medium used in this study contained 5% fetal calf serum, which could be a source of contaminating sialoglycoproteins, as discussed above. Finally, MAA

competed with antibodies in a competitive ELISA test on the porcine lutropin receptor ectodomain expressed in Sf9 cells, indicating that this recombinant protein contained $\alpha 2,3$ sialic acid (Pajot-Augy *et al.*, 1999).

In contrast to these positive results, many investigators have been unable to detect sialic acid residues using lectin-based analyses of insect cell-derived recombinant glycoproteins. Among them, Lehmann *et al.* (1993) used Con A (concanavalin A) affinity chromatography to separate the N-glycans from a secreted, chimeric form of a human parainfluenza virus type 3 glycoprotein produced in Sf9 cells and their results revealed only oligomannosidic- and paucimannosidic glycans. Sialic acids were not found on human interferon $\omega 1$ expressed in Sf9 cells, as the only positive signal in lectin blots was observed with GNA (*Galanthus nivalis* agglutinin), which recognizes terminal mannose residues (Voss *et al.*, 1993). Sugiyama *et al.* (1993) used lectin blotting with DSA (*Datura stramonium* agglutinin), SNA, MAA and WGA to characterize O-glycosylation of human interferon- $\alpha 2$ synthesized by Sf9 cells and found no evidence of sialylation. Grossmann *et al.* (1997) expressed the human thyroid stimulating hormone (TSH) in Sf9, Sf21, and BTI-TN-5B1-4 (also known as High Five) cells and detected no terminally sialylated complex oligosaccharides using ConA and LFA. Finally, the WGA lectin blotting results of Van de Wiel *et al.* (1998) suggested that the bovine follicular stimulating hormone (FSH) does not acquire terminal sialic acid when expressed in Sf21 cells.

Use of Physico-Chemical Criteria

Electrophoretic Mobility of Glycoproteins—When human melanotransferrin was expressed in baculovirus-infected or stably transfected lepidopteran insect cells (Sf9), neither form of the protein included the higher acid form seen with the native protein produced by human cells. This result was taken by Hegedus *et al.* (1999) as an indication that neither recombinant form of the protein was sialylated by insect cells. The lack of a change in electrophoretic mobility after neuraminidase treatment has also been used to demonstrate the absence of sialic acid in recombinant human complement subcomponent C1s (Luo *et al.*, 1992) and human $\alpha 1$ -microglobulin (Wester *et al.*, 1997), both expressed in Sf9 cells.

Chromatographic Behaviour of Glycans—The structures of the O-linked oligosaccharides from a baculovirus-expressed, truncated pseudorabies virus glycoprotein (gp50) were examined by Thomsen *et al.* (1990) using gel filtration and paper chromatography after metabolic labeling. The major carbohydrate structures found in this study were GalNAc and, to a lesser extent, Gal $\beta 1$,3GalNAc, but both structures were devoid of sialic acid.

Hsu *et al.* (1997) used two-dimensional HPLC mapping to analyze the N-glycans on IgG produced by baculovirus-infected BTI-TN-5B1-4 (High Five) cells. The results of this study showed that *Trichoplusia ni* cells could produce biantennary N-glycans terminating with galactosyl residues; however, there was no evidence of sialylation.

Expression of recombinant human plasminogen has been studied in several insect cell lines, including *Spodoptera frugiperda* IPLB-Sf21AE (Davidson *et al.*, 1990 and Davidson and Castellino, 1991a), *Mamestra brassicae* IZD-MbO503 (Davidson and Castellino, 1991b), and *Manduca sexta* CM-1 (Davidson and Castellino, 1991b). These investigators used HPAEC analyses to examine the structures of the N-glycans on the recombinant plasminogen produced by these cells. The results revealed that the protein contained significant proportions of sialylated, complex N-linked oligosaccharides. The same results were obtained whether the cells were cultured in serum-containing or serum-free media. To date, this remains the only example in which high levels of sialylated complex

oligosaccharides were observed by direct structural analyses of the glycans from an insect cell-derived glycoprotein. Synthesis of these complex-type oligosaccharides was shown to be dependent on the elapsed post-infection time (Davidson and Castellino, 1991a). It has been suggested that human plasminogen might be a particularly good substrate for limited amounts of terminal processing enzymes and/or a particularly poor substrate for the processing N-acetylglucosaminidase found in these cells. These observations imply that insect cells have the glycosyltransferase genes required for the assembly of complex N-linked oligosaccharides and that these genes can be expressed during infection. However, using this same method of glycan analysis (HPAEC), Butters *et al.*, (1998) found no evidence of sialylated species among the N-glycans from HIVgp120 expressed in Sf9 cells.

Glycan Analysis by Fluorophore-Assisted Carbohydrate Electrophoresis

(FACE)—Sialylation of secreted alkaline phosphatase (SeAP) produced in *Trichoplusia ni* larvae, which had been detected by lectin blotting (Davis and Wood, 1995), was reinvestigated by this same group (Kulakosky *et al.*, 1998) using the FACE method. This analysis failed to confirm the presence of sialyl residues on SeAP, irrespective of the host used for production, which included a variety of established cell lines (*Lymantria dispar*, *Heliothis virescens*, *Bombyx mori*) and larvae (*Spodoptera frugiperda*, *Trichoplusia ni*, *H. virescens*, *B. mori* and *Danaus plexippus*). According to the authors, the discrepancy between their FACE and lectin blotting results could have resulted either from coimmunoprecipitation of a contaminating sialylated glycoprotein of the same molecular mass or from non-specific binding in their lectin blots. By adding mannosamine, which can increase intracellular sugar nucleotide pools, Donaldson *et al.* (1999) demonstrated that N-glycosylation of SeAP in Sf21 cells was extended to include terminal N-acetylglucosamine, but neither galactosylated nor sialylated structures were detected.

Glycan Analysis by Mass Spectrometry—Mass spectrometry, which is one of the most powerful tools available for carbohydrate structural determinations, has been used since 1993 to characterize O- and N-linked glycans on insect cell-derived recombinant glycoproteins. Using plasma desorption mass spectrometry Sugiyama *et al.* (1993) found no evidence of sialylated O-glycans on human interferon $\alpha 2$ produced in Sf9 cells. Fast atom bombardment mass spectrometry of glycopeptides allowed Grabenhorst *et al.* (1993) to demonstrate that human interleukin-2 from baculovirus-infected Sf21 cells contained exclusively fucosylated paucimannose type N-glycans. The N-glycans on interferon γ produced by two insect cell lines (Ea4 and Sf9) were studied by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Ogonah *et al.*, 1996). Whereas the recombinant protein produced by Sf9 cells had only paucimannose-type glycans (see also Hooker *et al.*, 1999), the same protein produced by Ea4 cells contained N-glycans with terminal GlcNAc and Gal residues. However, even the Ea4 cells failed to produce N-glycans containing sialic acid. This same method was used to demonstrate that human lactoferrin produced by baculovirus-infected Sf9 cells had only non-sialylated, truncated N-glycans (Salmon *et al.*, 1997). MALDI and electrospray mass spectrometry analyses allowed Lopez *et al.* (1997) to elucidate the structures of the N-glycans on baculovirus-expressed bovine lactoferrin produced in *Mamestra brassicae* cells. Two families of oligosaccharides were found: one consisted of oligo-mannosidic type glycans (Man₆ to Man₅GlcNAc₂) and the other consisted of short, partially fucosylated paucimannose-type glycans, but none were sialylated. Most recently, Rudd *et al.* (2000) used MALDI-TOF to demonstrate the presence of a subpopulation of terminally galactosylated N-glycans on the third cysteine domain of the latent transforming growth factor- β binding protein-1 expressed in baculovirus-infected High Five cells. However, they detected no sialylated structures.

Engineering Glycoprotein Processing Pathways in the Baculovirus-Insect Cell Expression System

Considering that the vast majority of N-glycans on glycoproteins synthesized by insect cells are of the oligoman-nosidic or paucimannosidic type, the relative inability of these cells to produce sialylated glycoproteins could reflect the absence of functional levels of terminal glycosyl-transferases in these cells. This idea led some investigators to attempt to modify the protein N-glycosylation pathway in baculovirus-infected insect cells by introducing exogenous glycosyltransferase activities into the system (for a general review, see Jarvis *et al.*, 1998a).

Wagner *et al.* (1996b) demonstrated that the N-glycans of fowl-plague hemagglutinin expressed in Sf9 cells could be elongated by coexpression of human β 1,2-N-acetylglucosaminyltransferase I (GNT-I). These authors used two different recombinant baculoviruses, one expressing the hemagglutinin and the other expressing the human GNT-I gene, to coinfect Sf9 cells. Glycosylation of hemagglutinin was evaluated by a terminal galactosylation assay and HPAEC analyses. The results indicated that human GNT-I coexpression resulted in the production of hemagglutinin molecules with a four-fold higher content of N-glycans containing terminal GlcNAc residues. However, completely galactosylated and sialylated complex-type oligosaccharide side chains were not observed. In another study, Jarvis and Finn (1996) used a new type of baculovirus vector that can express foreign genes immediately after infection under the control of the promoter from the baculoviral *ie1* gene to express bovine β 1,4-galactosyltransferase. This resulted in galactosylation of the viral glycoprotein, gp64, as demonstrated by RCA (*Ricinus communis* agglutinin) lectin blotting with appropriate controls. The same results were obtained when Sf9 cells were stably transformed to constitutively express bovine β 1,4-galactosyltransferase, then infected with wild type AcMNPV (Hollister *et al.*, 1998). Infection of these stably-transformed cells (Sf β 4GalT) with a conventional recombinant baculovirus expression vector encoding human plasminogen activator also resulted in galactosylation of the recombinant protein, as evidenced by RCA blotting. However, upregulation of galactosyltransferase activity failed to produce any glycoproteins that bound to SNA, indicating that this did not induce production of α 2,6-sialylated glycoproteins. In their review article, Jarvis *et al.* (1998a) reported that infection of Sf β 4GalT cells with an immediate early baculovirus vector encoding a mammalian α 2,6-sialyltransferase under the transcriptional control of the *ie1* promoter resulted in production of gp64 that bound specifically to both RCA and SNA. These findings (Hollister and Jarvis, 2001) suggest that up-regulation of both transferase activities allows Sf9 cells to produce a foreign glycoprotein with N-linked glycans containing both β -linked galactose and terminal α 2,6-linked sialic acid. It is important to note that this conclusion implies that Sf9 cells can produce and transport CMP-sialic acid, which is required for glycoprotein sialylation in addition to the sialyltransferase activity. This implication is not supported by the results of Hooker *et al.* (1999), who were unable to detect CMP-sialic acid in uninfected or infected Sf9 cells, as discussed above. Based on this finding, the latter group concluded that genetic modification of N-glycan processing in Sf9 cells will be constrained to terminal galactosylation. Hopefully, future studies will clarify the discrepancy between these two different viewpoints.

Conclusion

Compared to polypeptides and nucleic acids, primary structural analysis of glycans is far more complicated due to the huge diversity of linkages between monosaccharides.

Methods of glycan structural analysis include colorimetry, endo- and exoglycosidase digestions, permethylation and analysis of methylated monosaccharides by gas-liquid chromatography, the use of lectins with well-defined carbohydrate-binding specificities, and various chromatographic methods, particularly HPLC and HPAEC. However, the application of NMR and mass spectrometry methods has provided a technical leap in glycan structural analysis. These latter methods have been used to establish that insect cells can modify proteins by both O- and N-glycosylation. In addition, these methods have established that the major processed O- and N-glycan species found on endogenous and most baculovirus-expressed recombinant glycoproteins are (Gal β 1,3)GalNAc-*O*-Ser/Thr and Man3(Fuc)GlcNAc2-*N*-Asn, respectively. Among the results obtained using other technical approaches, some suggest that insects can produce sialylated glycoproteins, whereas most suggest that they cannot. There are many potential reasons for the inconsistency in these results, including differences in the sources and nature of materials used for analysis. Some studies have focused on insect cell lines, others on insects. Many different cell lines, derived from different insects, different tissues, and cultured under different conditions have been used for these studies. Some studies have examined glycans from total glycoprotein fractions, some from total membrane glycoprotein fractions, and most have focused on a single, purified model glycoprotein. Among the purified model proteins, some studies have examined endogenous proteins, while others have examined recombinant proteins. Other potential reasons for the different conclusions from different studies could include experimental artifacts, such as the loss of sialic acids, the introduction of sialic acids or sialoglycoproteins, or the absence of proper experimental controls, as discussed above. Finally, it is imperative to recognize that it is extremely difficult to generalize about protein glycosylation pathways in 'insect cells', as insects are an incredibly diverse group of animals. Nonetheless, even with all these caveats, we may still conclude that the production and compartmentalization of sialic acids and glycoprotein sialylation are not major biochemical processes in insect cells. To explain the exceptional observations of sialic acids or sialoglycoproteins in insects, we can speculate that these processes might be highly specialized or might occur only in a tissue- or developmental stage-specific fashion. Similarly, we can speculate that these processes might occur at only nominal, usually undetectable levels in established insect cell lines and, on rare occasions, these low level processes can yield sialylated glycoproteins. The overall conclusion that insect cells generally do not produce sialylated glycoproteins, with rare exceptions among specialized cells or individual model glycoproteins, is consistent with the results of all well-analyzed studies in the current literature.

Based on these conclusions, the development of engineered insect cells able to perform mammalian-type glycosylation can still be considered as a promising field. One can expect that the introduction of mammalian genes encoding not only glycosyltransferases but also the enzymes responsible for sugar-nucleotides (especially CMP-sialic acid, as the endogenous pool must be very reduced) biosynthesis and transport can significantly modify the glycosylation pattern of insect cell-derived glycoproteins. However, since insects seem to possess the genetic potential to perform complex-type glycosylation, which will hopefully be confirmed by future genetic studies, another direction for metabolic engineering of insect cell glycosylation will be to manipulate the expression levels of endogenous glycoprotein-processing enzymes. These new developments will require basic molecular studies to understand the factors that govern the precise regulation of the genes involved in complex-type glycan synthesis.

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