Impact of a human CMP-sialic acid transporter on recombinant glycoprotein sialylation in glycoengineered insect cells

Hideaki Mabashi-Asazuma, Xianzong Shi, Christoph Geisler, Chu-Wei Kuo, Kay-Hooi Khoo, and Donald L. Jarvis

Insect cells are widely used for recombinant glycoprotein production, but they cannot provide the glycosylation patterns required for some biotechnological applications. This problem has been addressed by genetically engineering insect cells to express mammalian genes encoding various glycoprotein glycan processing functions. However, for various reasons, the impact of a mammalian cytosine-5′-monophospho (CMP)-sialic acid transporter has not yet been examined. Thus, we transformed Spodoptera frugiperda (Sf9) cells with six mammalian genes to generate a new cell line, SfSWT-4, that can produce sialylated glycoproteins when cultured with the sialic acid precursor, N-acetylmannosamine. We then super-transformed SfSWT-4 with a human CMP-sialic acid transporter (hCSAT) gene to isolate a daughter cell line, SfSWT-6, which expressed the hCSAT gene in addition to the other mammalian glyogenes. SfSWT-6 cells had higher levels of cell surface sialylation and also supported higher levels of recombinant glycoprotein sialylation, particularly when cultured with low concentrations of N-acetylmannosamine. Thus, hCSAT expression has an impact on glycoprotein sialylation, can reduce the cost of recombinant glycoprotein production and therefore should be included in ongoing efforts to glycoengineer the baculovirus-insect cell system. The results of this study also contributed new insights into the endogenous mechanism and potential mechanisms of CMP-sialic acid accumulation in the Golgi apparatus of lepidopteran insect cells.

Keywords: baculovirus / CMP-sialic acid transport / glycoengineering / glycoprotein sialylation / insect cell glycosylation

Introduction

Insect cells produce N-glycoproteins with relatively simple oligosaccharide side-chains, or glycans, which lack the terminal sialic acid residues often found on the N-glycans produced by mammalian cells (Marz et al. 1995; Altmann et al. 1999; Harrison and Jarvis 2006; Geisler and Jarvis 2009). One reason for this important structural difference between invertebrate and vertebrate N-glycoproteins is that insect cells generally lack adequate levels of the glycosyltransferases needed to elongate trimmed N-glycan processing intermediates and synthesize complex, terminally sialylated end-products (Stollar et al. 1976; Butters et al. 1981; Hooker et al. 1999). Another is that insect cells have an additional N-glycan trimming enzyme that is not found in mammalian cells and that specifically removes terminal β1,2-linked N-acetylgalcosamine residues from the lower branch of a key N-glycan processing intermediate (Altmann et al. 1995; Geisler et al. 2008; Geisler and Jarvis 2010, 2012). Thus, the major processed N-glycans found on insect cell glycoproteins, including recombinant glycoproteins produced by insect cells infected with baculovirus expression vectors, are highly trimmed core structures consisting of Man3GlcNAc2(±Fuc). Substitution of the complex, terminally sialylated N-glycans normally found on native mammalian glycoproteins with these relatively simple, core N-glycans is a major problem associated with the production of recombinant glycoproteins in insect cells because terminal sialic acids influence many biomedical applications of these products. For example, it is well known that the circulatory half-life and, therefore, the pharmacokinetic behavior and clinical efficacy of therapeutic glycoproteins are strongly influenced by terminal sialic acids on their mammalian-type N-glycans (Byrne et al. 2007; Durocher and Butler 2009; Sola and Griebenow 2011).

Previous research has revealed that glycoengineering approaches can be used to address the biotechnological limitations imposed by the relatively simple nature of endogenous insect cell protein glycosylation pathways. Most of this work has involved genetically transforming insect cells with mammalian genes encoding the glycosyltransferases needed to convert trimmed N-glycans into complex, terminally sialylated structures (Breitbach and Jarvis 2001; Hollister and Jarvis 2001; Hollister et al. 2002; Yun et al. 2005). These efforts have yielded various transgenic derivatives of established lepidopteran insect cell lines, including Spodoptera frugiperda.
When cultured in serum-free media supplemented with the
The resulting cell line produced high levels of CMP-sialic acid
and CMP-sialic acid biosynthesis (Aumiller et al. 2003). In addition, given the absence of any detectable
CMP-sialic acid, one would not expect these cells to have the
transporter required to specifically import this nucleotide
cellular pathway that led to the production and import of CMP-sialic acid for
utilization by the mammalian sialyltransferases, which was
able to support recombinant glycoprotein sialylation.

Considering that a salvaging pathway might be less efficient than a de novo biosynthetic pathway, we super-transformed a
transgenic insect cell line that had been previously glycoengineered to encode the requisite mammalian glycosyltransferases
with mammalian sialic acid synthase (SAS) and CMP-sialic acid synthetase (CMAS) genes, which are required for sialic acid and CMP-sialic acid biosynthesis (Aumiller et al. 2003). The resulting cell line produced high levels of CMP-sialic acid when cultured in serum-free media supplemented with the
sialic acid precursor, N-acetylmannosamine. In addition, these
cells were able to produce recombinant glycoproteins with termi-
nally sialylated N-glycans after being infected by baculo-

H Mabashi-Asazuma et al.

Results

Isolation of transgenic Sf9 cell lines glycoengineered with
and without an hCSAT gene

The general approach used in this study was to isolate a
matched pair of transgenic lepidopteran insect cell lines with
and without an hCSAT gene, each capable of producing termi-
nally sialylated N-glycans when cultured in serum-free media supplemented with N-acetylmannosamine. Once these
cell lines were isolated and characterized, we were able to use them to examine the impact of hCSAT gene expression on re-
combant glycoprotein sialylation by glycoengineered insect
cell lines. Based upon previous work, which had established the minimal set of mammalian genes required for this purpose (Aumiller et al. 2003, 2012), we initially transformed Sf9 cells with human N-acetylgalactosaminyltransferase II (hMGAT2), bovine β1,4-galactosyltransferase I (bB4GALT1), mouse α2,3-sialyltransferase (mST3GAL3), rat α2,6-sialyltransferase (rST6GAL1), mouse SAS and mouse CMAS genes, together with a hygromycin B-resistance marker. After selection in growth medium supplemented with hygromycin B, single-cell
clones were isolated by limiting dilution, pre-screened using a
cell surface Sambucus nigra agglutinin (SNA) lectin staining
assay and re-screened by RNA dot-blot hybridization, as
described in Materials and methods. These steps identified a
clone that stained intensely with SNA (data not shown) and
scored positive for all the six genes in the dot-blots (data not
shown), which was designated SfSWT-4 and used for the re-
mainder of this study. We subsequently super-transformed
SfSWT-4 cells with an hCSAT gene and neomycin-resistance
marker and, after selection in growth medium supplemented with
Geneticin®, we re-isolated single-cell clones by limiting
dilution and screened them for hCSAT expression by RNA
dot-blot hybridization. These steps identified a positive clone
(data not shown) that was designated SfSWT-6 and used for
the remainder of this study.

Mammalian transgene expression in SfSWT-4
and SfSWT-6 cells

Reverse transcriptase–polymerase chain reaction (RT–PCR)
assays with total RNA preparations and gene-specific primers
were performed to examine the expression of each mammalian
transgene in uninfected and baculovirus-infected SfSWT-4 and
SfSWT-6 cells. Total RNA from the parental Sf9 cells was
used as a negative control and primers specific to an endogen-
ous Sf9 ribosomal protein (L3) gene were used as positive
controls. Duplicate assays were performed in parallel with and
without the addition of RT to assess possible contamination of
the total RNA preparations with DNA. The results showed that
none of the RT–PCR assays yielded any detectable amplifica-
tion products when performed without any RT, indicating that
glycoprotein production in glycoengineered baculovirus–insect
cell systems and, therefore, that CSAT function should be included in ongoing insect cell glycoengineering efforts. They
also provided new information on the endogenous capacity
and potential mechanisms for the accumulation of CMP-sialic
acid in the Golgi apparatus of lepidopteran insect cell lines.
the RNA preparations were not detectably contaminated with DNA (Figure 1, RT−). The results also showed that all of the RT–PCR assays yielded an amplification product of the expected size when performed with RT and primers specific to the endogenous Sf9 cell ribosomal protein gene, which validated our RT–PCR method and provided an internal standard for the assays (Figure 1, RT+; SfRPL3). None of the RT–PCR assays yielded an amplification product when they included RT, total RNA from the parental Sf9 cells and primers specific to any of the mammalian transgenes, which further validated our RT–PCR method by establishing its specificity (Figure 1, RT+; hMGAT2, bB4GalT1, mST3GAL3, rST6GAL1, mSAS, mCMAS, hCSAT). In contrast, all of the assays yielded an amplification product of the expected size when they were performed with RT, total RNA from either uninfected or baculovirus-infected SfSWT-4 or -6 cells and primers specific to each mammalian transgene except hCSAT (Figure 1, RT+; hMGAT2, bB4GalT1, mST3GAL3, rST6GAL1, mSAS, mCMAS). Finally, the RT–PCR assay that included RT, total RNA from uninfected or baculovirus-infected SfSWT-6 cells and primers specific to the hCSAT gene also yielded an amplification product of the expected size (Figure 1, RT+; hCSAT). Together, these results demonstrated that the two new transgenic insect cell lines produced for this study contained and expressed a common set of mammalian glycogenes at approximately equal levels. In addition, the SfSWT-6, but not the SfSWT-4 cells expressed the hCSAT gene, as expected. Finally, the expression of each transgene appeared to be enhanced by baculovirus infection, as anticipated from this same result in previous studies (Jarvis 1993).

**Analysis of hCSAT function in glycoengineered insect cells**

A valid analysis of the impact of the hCSAT gene on glycoengineered insect cells required evidence that the gene product functions as a CMP-sialic acid transporter when expressed in insect cells. Hence, we assayed hCSAT function in microsomal membranes from SfSWT-4 and SfSWT-6 cells, with membranes from Chinese hamster ovary (CHO) cells as the positive and membranes from Lec 2, a CSA T mutant CHO cell line (Deutscher et al. 1984) with reduced CSA T activity, as the negative control. Upon incubation with radiolabeled CMP-sialic acid, we observed only low levels of transport with the microsomal membranes isolated from SfSWT-4 cells (Figure 2). We were unable to determine whether the level of uptake observed with the SfSWT-4 cell membranes was above background because there is no CSA T null mutant that could have been used to establish the background of the assay for this purpose. Nevertheless, the results clearly demonstrated that the microsomal membranes from SfSWT-6 cells transported CMP-sialic acid at about 4-fold higher levels than microsomes from SfSWT-4 or Lec 2 cells and at about the same level as microsomes from CHO cells (Figure 2). These results showed that hCSAT gene expression leads to the

![Fig. 1](https://academic.oup.com/glycob/article-abstract/23/2/199/1988335/fig1)

**Fig. 1.** Transgene expression in uninfected and baculovirus-infected SfSWT-4 and SfSWT-6 cells. Total RNA isolated from uninfected or baculovirus-infected Sf9, SfSWT-4 and SfSWT-6 cells was used for RT–PCR, as described in Materials and methods. Each reaction was performed in the presence (RT+) or absence (RT−) of reverse transcriptase to assess DNA contamination of the RNA preparations. The primer pairs used for these RT–PCR were specific to an endogenous Sf9 cell gene encoding ribosomal protein L3 or for each of the individual mammalian genes used to transform Sf9 cells and produce the transgenic derivatives, as indicated by the labels on the left-hand side of the figure. hMGAT2, human β1,2-N-acetylgalactosaminyltransferase II; bB4GalT1, bovine β1,4-galactosyltransferase I; rST6GAL1, rat α2,6-sialyltransferase I; mST3GAL3, murine α2,3-sialyltransferase III; mSAS, murine sialic acid synthase; mCMAS, murine CMP-sialic acid synthetase; hCSAT, human CMP-sialic acid transporter; SfRPL3, Spodoptera frugiperda ribosomal protein L3.
production of a functional CMP-sialic acid transporter in glycoengineered insect cells.

Cell surface sialylation levels in SfSWT-4 and SfSWT-6 cells
SfSWT-4 and SfSWT-6 cells were infected with a wild-type baculovirus to increase transgene expression (Jarvis 1993), cultured in serum-free media supplemented with 10 mM \(N\)-acetylmannosamine, which is a standard concentration used for this sialic acid precursor, and then cell surface glycosylation patterns were examined in lectin staining assays with Sf9 cells as controls, as described in Materials and methods. The parental insect cells were not specifically stained, while the glycoengineered insect cells were stained with the sialic acid-specific lectin, SNA, as expected (Figure 3A). A quantitative analysis of the results revealed that SfSWT-6 expressed slightly, but significantly higher levels of cell surface sialic acids than SfSWT-4 cells (Figure 3B).

To extend these results, we performed a cell surface SNA staining assay on SfSWT-4 and -6 cells cultured in serum-free media supplemented with relatively lower (1 mM) and higher (50 mM) concentrations of \(N\)-acetylmannosamine. The SNA staining intensities were about the same when these cells were cultured in high concentrations, but the SfSWT-6 cells were more intensely stained when both were cultured in low concentrations of this sialic acid precursor (Figure 4A and B). These results indicated that the hCSAT gene produced a functional CMP-sialic acid transporter, which had an impact on insect cell sialylation by supporting higher levels of cell surface sialylation, particularly when the cells were cultured in a low concentration of \(N\)-acetylmannosamine.

Impact of hCSAT expression on recombinant glycoprotein sialylation
Finally, we examined the impact of hCSAT expression on sialylation of the biotechnologically relevant recombinant glycoprotein, human erythropoietin (hEPO), during baculovirus infection of the two glycoengineered insect cell lines isolated for this study. SfSWT-4 and SfSWT-6 cells were infected with a recombinant baculovirus encoding a His-tagged version of hEPO (hEPO-His) and then the infected cells were fed with serum-free media supplemented either with 1 or with 50 mM \(N\)-acetylmannosamine. The extracellular growth media were harvested at 48 h after infection and the recombinant hEPO-His was affinity purified and analyzed by SNA lectin blotting. Commercial fetuin was used as the positive and neuraminidase-treated samples of hEPO-His and fetuin were used as the negative controls. The results showed that SNA bound to untreated fetuin and hEPO-His, but not to either of the neuraminidase treated proteins, which validated the specificity of the lectin blotting assay (Figure 5A). The results also showed that SNA bound about equally well to the hEPO-His preparations from SfSWT-4 and -6 cells cultured with the high concentration of \(N\)-acetylmannosamine (Figure 5A). However, when both cell lines were cultured with low concentrations of \(N\)-acetylmannosamine, SNA binding was observed only with the hEPO-His produced by SfSWT-6 cells (Figure 5A). These results were confirmed and extended by a
quantitative analysis of the sialic acids enzymatically released from the hEPO-His preparations purified from SfSWT-4 and -6 cells cultured in low (1 mM) or high (50 mM) concentrations of N-acetylmannosamine (Figure 5B).

To more directly assess the impact of hCSAT expression on hEPO sialylation, we enzymatically released and permethylated the N-glycans from each purified hEPO preparation and determined their structures by MALDI-TOF MS, as described in Materials and methods. The results showed that the hEPO preparations from SfSWT-4 cells cultured with low N-acetylmannosamine concentrations contained the lowest proportion, with only tiny peaks having m/z values corresponding to terminally sialylated N-glycans (Figure 6A). The proportions of sialylated N-glycans became progressively higher with hEPO preparations from SfSWT-4 cells cultured with 50 mM N-acetylmannosamine (Figure 6B), SfSWT-6 cells cultured with 1 mM N-acetylmannosamine (Figure 6C) and from SfSWT-6 cells cultured with 50 mM N-acetylmannosamine (Figure 6D), respectively. The proportions of terminally galactosylated (acceptor) and sialylated N-glycans found on each hEPO preparation are shown in Figure 7. Together, the lectin blotting, quantitative sialic acid determinations and mass spectrometric data all consistently demonstrated that hCSAT expression enhanced recombinant hEPO-His sialylation in glycoengineered insect cells, particularly when the cells were cultured in low concentrations of N-acetylmannosamine.

Discussion

The baculovirus-insect cell expression system is widely used to produce recombinant glycoproteins, but it provides only relatively simple protein glycosylation patterns that are inadequate for some biomedical applications (Marz et al. 1995; Altmann et al. 1999; Harrison and Jarvis 2006; Geisler and Jarvis 2009). This problem has been addressed, at least in part, by engineering commonly used lepidopteran insect cell lines to express mammalian glycosyltransferases (Breitbach and Jarvis 2001; Hollister and Jarvis 2001; Hollister et al. 2002; Yun et al. 2005), as well as certain enzymes involved in the de novo biosynthesis of CMP-sialic acid (Aumiller et al. 2003, 2012). This effort has yielded glycoengineered insect cell lines with modified glycosylation pathways that can produce recombinant glycoproteins with authentic, human-type protein N-glycosylation patterns that include terminal sialic acids. These results imply that Sf9 and High Five™ cells have the endogenous capacity to accumulate CMP-sialic acid in the Golgi apparatus, where it is required as the donor substrate for glycoprotein sialylation. However, neither the precise nature of the infrastructure supporting this endogenous capacity nor the impact of mammalian CSA T gene expression on glycoprotein sialylation in insect cells had been examined. This study focused on the latter issue and provided new insights into the former.

Our basic approach was to isolate a matched set of glycoengineered insect cell lines, both capable of glycoprotein sialylation in the absence of an exogenous source of sialic acids, one with and the other without an hCSAT gene. We successfully isolated both cell lines and demonstrated that they had comparable transgene expression patterns, except for the fact that the hCSAT gene was expressed only in SfWT-6 cells. Further analysis showed that expression of the hCSAT gene led to the production of a functional CSA T in SfSWT-6 cells. This set the stage for a direct comparison of the sialylation capacity of these two matched cell lines in the presence and absence of hCSAT function.

Initially, we used an SNA staining assay to compare cell surface sialylation levels and found that SfSWT-6 cells stained more intensely than SfSWT-4 cells when both were cultured in serum-free growth media containing a standard concentration (10 mM) of N-acetylmannosamine. This new result provided our first indication that hCSAT gene expression had an impact in glycoengineered insect cell lines, leading to a higher level of cell surface sialylation. It is instructive to note that hCSAT expression induced only a relatively small (12%) increase, which was consistent with the relatively small (14%) increase in hEPO sialylation observed when CHO cells were glycoengineered to express higher levels of hCSAT (Son et al. 2011). This small increase was predictable in CHO cells, as it is clear that they have...
endogenous CSAT activity, but not in SF9 cells, as the mechanism used to accumulate CMP-sialic acid in the Golgi apparatus of insect cells remains to be determined.

Subsequently, we examined cell surface sialylation in SISWT-4 and -6 cells cultured in lower (1 mM) and higher (50 mM) concentrations of N-acetylmannosamine and found that the hCSAT gene had little impact (5% increase) when the cells were cultured in high concentrations and a much greater impact (59% increase) when the cells were cultured in low concentrations of this sialic acid precursor. We obtained similar results when we examined how hCSAT gene expression in SISWT-6 cells influenced the sialylation of a His-tagged version of the biotechnologically relevant glycoprotein, hEPO, which was expressed by infecting the glycoengineered insect cells with a baculovirus vector. In these experiments, SISWT-6 cells sialylated hEPO-His at higher levels (35%) than SISWT-4 when the cells were cultured in 50 mM N-acetylmannosamine, but at significantly higher levels (67.5%) when they were cultured in 1 mM N-acetylmannosamine.

Together, the results obtained in this study demonstrated that hCSAT gene expression has an impact on cell surface and recombinant glycoprotein sialylation in glycoengineered lepidopteran insect cells, particularly when the cells are cultured in growth media supplemented with lower concentrations of N-acetylmannosamine. Indeed, SISWT-6 cells provided about the same levels of cell surface and recombinant glycoprotein sialylation when cultured in growth media containing either 1 mM or 50 mM N-acetylmannosamine. Thus, this study also demonstrated that the addition of an hCSAT gene is an approach that can be used to reduce the cost of recombinant glycoprotein production by glycoengineered insect cell systems by reducing the N-acetylmannosamine concentration needed to support sialylation. The addition of 10 mM N-acetylmannosamine adds ~$14/1/L to the cost of the insect cell growth medium even when the sialic acid precursor is purchased at the lowest available, bulk-rate price. Our results showed that the addition of an hCSAT gene reduced the effective concentration of N-acetylmannosamine to at least 1 mM, thereby reducing the cost of media supplementation by about 10-fold.

We should note that the sialylation efficiencies obtained in this study (~0.1–1.0%) were the lowest we have observed to date. In MS analyses of the N-glycan profiles of various recombinant glycoprotein (Toth et al. unpublished) or total

Fig. 5. Sialylation of hEPO-His. SISWT-4 and SISWT-6 cells were infected with a recombinant baculovirus encoding hEPO-His and cultured in serum-free media supplemented with 1 or 50 mM N-acetylmannosamine, and hEPO-His was harvested and affinity-purified at 48 h after infection. The results of SNA lectin blotting and immunoblotting assays of samples treated with neuraminidase buffer alone (−) or buffer plus neuraminidase (+) are shown in (A). Fetuin was used as a positive control. The amounts of sialic acid released by neuraminidase treatment of the purified hEPO-His samples were also determined, as described in Materials and methods, with the results shown in (B). The error bars show the standard deviations calculated using the results obtained with three independent samples.
glycoprotein (Jarvis et al. unpublished) preparations from several different glycoengineered insect cell lines, we have observed sialylation efficiencies ranging from \( \approx 15 \) to \( \approx 40\% \).

Potential reasons for the relatively low sialylation efficiencies observed in this study include the nature of the model glycoprotein, the specific glycoengineered insect cell lines and/or their metabolic condition during the production runs and/or the growth conditions used for those production runs. Nevertheless, we were still able to evaluate the impact of hCSAT expression by using carefully matched cell lines in parallel expression runs, as indicated by the consistency of the results obtained using complementary assays, and to draw a clear conclusion regarding the need to incorporate hCSAT expression into ongoing insect cell glycoengineering efforts.

The results obtained in this study also provide new insights into the endogenous mechanism used to accumulate CMP-sialic acid in the Golgi apparatus of Sf9 cells. The relatively lower impact of hCSAT expression on sialylation by glycoengineered Sf9 cells cultured in high concentrations of \( N\)-acetylmannosamine indicates (i) that there is an endogenous CMP-sialic acid accumulation process in Sf9 cells and (ii) that it is inefficient. One possible model of the endogenous process is that it involves import of CMP-sialic acid into the Golgi apparatus by an insect CSAT with a low substrate affinity, which therefore requires a higher sialic acid precursor concentration to generate adequate intra-organelle CMP-sialic acid concentrations. In this model, the hCSAT, with its higher

---

Fig. 6. \( N\)-glycan profiles of hEPO-His produced under various conditions. hEPO-His preparations were isolated from SISWT-4 and SISWT-6 cells cultured in serum-free media containing 1 or 50 mM \( N\)-acetylmannosamine, as described in the legend to Figure 5, and samples were used for MALDI-TOF MS analysis of enzymatically released, permethylated \( N\)-glycans, as described in Materials and methods. The figure shows the \( N\)-glycan profiles obtained with hEPO-His from SISWT-4 cells grown in 1 mM (A) or 50 mM (B) and SISWT-6 cells grown in 1 mM (C) or 50 mM (D) \( N\)-acetylmannosamine. The insets are magnifications of the sialylated \( N\)-glycan profiles observed from \( m/z \) 1900–2500. All molecular ions were detected as \([M + Na]^+\) and assigned and annotated accordingly using the standard cartoon symbolic representations.
substrate affinity, would amplify CMP-sialic acid import in the presence of the relatively low CMP-sialic acid concentrations produced when the cells are cultured in low concentrations of N-acetylmannosamine. Another possible model of the endogenous process is that it actually involves importing sialic acid, rather than CMP-sialic acid, into the Golgi apparatus. This would require a sialic acid, rather than a CMP-sialic acid transporter, as previously suggested by Koles et al. (2009), and would be followed by the conversion of sialic acid into CMP-sialic acid within the Golgi compartment by an endogenous, Golgi-localized CMAS, as previously suggested by Viswanathan et al. (2006). In this model, hCSAT would amplify the levels of CMP-sialic acid available in the Golgi apparatus by importing the CMP-sialic acid produced by the mCMAS expressed in glycoengineered (SfSWT-6) insect cells. At this time, it is not possible to distinguish between these two models of the endogenous mechanism for CMP-sialic acid accumulation in the Golgi apparatus of Sf9 cells. The first model lacks experimental support for the idea that any insect actually encodes a CSA T. In fact, when the Drosophila ortholog of the human CSA T gene was expressed and the gene product was functionally characterized, the results showed that it was a UDP-galactose/UDP-N-acetylgalactosamine transporter (Aumiller and Jarvis 2002; Segawa et al. 2002). The second model lacks experimental support for the presence of a Golgi sialic acid transporter in any system and is weakened by the inconclusive nature of published data suggesting that the Drosophila CMAS gene product is localized in the Golgi apparatus (Viswanathan et al. 2006). Future studies will be required to address the question of the endogenous CMP-sialic acid accumulation pathway. For now, the addition of an hCSAT gene will provide an effective way of improving glycoprotein sialylation and reducing the cost of sialoglycoprotein production using glycoengineered insect cell systems.

Materials and methods

Plasmid constructions

A cDNA encoding the hCSAT transcript variant 2 (accession no. NM_001168398) was purchased from OriGene Technologies (Rockville, MD). This cDNA was used as a template to amplify the hCSAT open reading frame by PCR with the forward primer 5′-AGATCTATGGCTGCCCGAGAGACTCTCTCC-3′, the reverse primer, 5′-AGGCCTTCACACACGAACTCCTCC-3′ and Phusion High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA). The amplified DNA fragment was cloned into pCR®4TOPO® (Life Technologies, Gaithersburg, MD), an error-free clone was identified by restriction mapping and sequencing and then the fragment was subcloned into the NruI and BglII sites of pIE1HR3 (Jarvis et al. 1996) to create pIE1-hCSAT. pIE1-Hygro, pIE1-Neo and the three piggyBac vectors used in this study, pXLBacII-GnTII/GalT-DsRed1-L TR, pXLBacII-ST6.1/ST3.3-ECFP-L TR and pXLBacII-SAS/CMPSAS-EYFP-L TR, have been described previously (Jarvis et al. 1990; Hollister and Jarvis 2001; Shi et al. 2007).

Cells and viruses

Sf9, SfSWT-4 and SfSWT-6 cells were routinely maintained as shake-flask cultures in ESF 921 medium (Expression Systems, Woodland, CA) at 28°C. SfSWT-4 is a new cell line that was isolated for this study by transforming Sf9 cells with pXLBacII-GnTII/GalT-DsRed1-L TR, pXLBacII-ST6.1/ST3.3-ECFP-L TR, pXLBacII-SAS/CMPSAS-EYFP-L TR and pIE1-Hygro, using previously described transfection and selection methods (Jarvis and Guarino 1995; Harrison and Jarvis 2007a, b). After selection, single-cell clones were isolated by limiting dilution and screened by cell surface staining with SNA, as described below. SNA-positive clones were further screened by RNA dot-blot hybridization using probes specific

Fig. 7. Impact of hCSAT expression on hEPO sialylation. The bar graph shows the proportions of galactosylated (A) and sialylated (B) N-glycans found on the hEPO preparations isolated from SfSWT-4 and SfSWT-6 cells cultured in serum-free media containing 1 or 50 mM N-acetylmannosamine, as calculated from the N-glycan profiles shown in Figure 6.
to each mammalian transgene, as described previously (Jarvis et al. 1996), and a clone expressing all the six transgenes was designated SiSWT-4 and used for the remainder of this study. SiSWT-6 is another new cell line isolated for this study by super-transforming SiSWT-4 cells with pIE1-hCSAT and pIE1-Neo. After selection in medium containing Geneticin® (Life Technologies), single-cell clones were once again isolated by limiting dilution and screened by RNA dot-blot hybridization with an hCSAT probe and a positive clone was designated SiSWT-6 and used for the remainder of this study. CHO and Lec2 (Deutscher et al. 1984) cells were obtained from the American Type Culture Collection and maintained in α-MEM (Life Technologies) containing 10% fetal bovine serum (Thermo Scientific, Logan, UT) at 37°C with 5% CO₂.

The E2 strain of Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV; Smith and Summers 1978) was used as the wild-type baculovirus. A new recombinant baculovirus expression vector encoding a C-terminally His-tagged version of human erythropoietin (AchEPO-His) was isolated for this study. Briefly, an hEPO cDNA (Accession No. BC093628) was obtained from Life Technologies and used together with forward (5′-CACCATGGGGGTGACGAAT GTCC-3′) and reverse (5′-TCTGTCCCCTGTCCTGCAGG-3′) primers to amplify the open reading frame without a stop codon. The product was cloned into pENTR™/D-TOPO® (Life Technologies) and an error-free clone was identified by restriction mapping and sequencing and designated pENTR™/D-TOPO®-hEPO(-stop). The hEPO sequence was then transferred from pENTR™/D-TOPO®-hEPO(-stop) to a baculovirus expression vector encoding a C-terminally His-tagged virus destination vector (BaculoDirect™ LR reaction. The spent LR reaction was used to transfect Sf9 cells with Cellfectin® (Life Technologies) and, after being cultured for 120 h in medium supplemented with gancyclovir to select against the parental virus, the transfected cell-free medium was harvested and progeny baculoviruses were plaque-purified. Recombinant viral clones were presumptively identified by their white plaque phenotypes, several were tested for hEPO-His expression by western blotting, and a positive clone was designated AchEPO-His, amplified, titered and used for the remainder of this study.

**RT-PCR assays**

Total RNA was extracted from 1 × 10⁶ Sf9, SiSWT-4 or SiSWT-6 cells using the TRI Reagent (Life Technologies) according to the manufacturer’s instructions. The RNA preparations were then treated with DNaseI Amplification Grade (Life Technologies) and 3 μg of each was reverse transcribed at 50°C for 90 min with ThermoScript™ Reverse Transcriptase (Life Technologies) and oligo(dT)₁₅-VN (5′-TTTTTTTTTTTTTTTTTTTTVN-3′) as the primer. The resulting cDNA preparations were treated with RNaseH (Life Technologies) and then used for PCRs with Crimson Taq DNA Polymerase (New England BioLabs). The PCR conditions included an initial denaturation step at 95°C for 30 s, followed by 32 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 20 s and extension at 68°C for 30 s, except that the annealing temperatures used for mST3GAL3 and SiRPL3 were 52 and 56°C, respectively. The sequences of all the primers used for the RT-PCR assays are given in Table 1.

**Table 1.** Primer sequences used for RT–PCR assays

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMGAT2-Fw</td>
<td>GGTGCATCAATGCTGAGT</td>
</tr>
<tr>
<td>hMGAT2-Rv</td>
<td>TGCATACCACTGCTCCA</td>
</tr>
<tr>
<td>b84GalT1-Fw</td>
<td>TGGAGTTCACATACCTGGGAC</td>
</tr>
<tr>
<td>b84GalT1-Rv</td>
<td>CCCAGTAGTTAGAAGAIAC</td>
</tr>
<tr>
<td>rsT6GAL1-Fw</td>
<td>TTCCATACCTCGTACC</td>
</tr>
<tr>
<td>rsT6GAL1-Rv</td>
<td>CCAATTAAACTGAGAC</td>
</tr>
<tr>
<td>mST3GAL3-Fw</td>
<td>GTATATCTCCTCGAGAGC</td>
</tr>
<tr>
<td>mST3GAL3-Rv</td>
<td>TCTCAAGGCCCTTCACAG</td>
</tr>
<tr>
<td>mSAS-Fw</td>
<td>GAGTCGACGTCAAGCAC</td>
</tr>
<tr>
<td>mSAS-Rv</td>
<td>GCTATGGTGAAGATGTC</td>
</tr>
<tr>
<td>mCMAS-Fw</td>
<td>CGCACAGACTGGGATGA</td>
</tr>
<tr>
<td>mCMAS-Rv</td>
<td>GACACTTCTGTGCGAG</td>
</tr>
<tr>
<td>hCSAT-Fw</td>
<td>ACTCTCATTCCACAGC</td>
</tr>
<tr>
<td>hCSAT-Rv</td>
<td>CTGAGCACAAATACGCAAGAT</td>
</tr>
<tr>
<td>SiRPL3-Fw</td>
<td>ACATGCGAACCCTCAATG</td>
</tr>
<tr>
<td>SiRPL3-Rv</td>
<td>TCTTGAAACCTGTCATCTT</td>
</tr>
</tbody>
</table>

**SNA cell surface staining assay**

Sf9, SiSWT-4 or SiSWT-6 cells were seeded into 6-well plates at a density of 1 × 10⁶ cells per well and allowed to adhere for 1 h. The media were drained, wild-type baculovirus infected cells were fed with ESF 921 supplemented with 10 mM N-acetylmansosamine (New Zealand Pharmaceuticals, Palmerston North, New Zealand) and incubated for 36 h after infection at 28°C. At that time, the growth media were removed and the cells were rinsed with lectin buffer and incubated with fresh lectin buffer at 4°C for 5 min. The buffer was then removed and biotinylated SNA (Vector Laboratories, Burlingame, CA) was then added to the cells at a concentration of 10 μg/mL in lectin buffer. After another 5 min at 4°C, the lectin was removed and the cells were washed once with lectin buffer and incubated at 4°C for 5 min with fresh lectin buffer containing 5 μg/mL of Texas Red-Streptavidin (Vector Laboratories). Finally, the cells were washed twice with lectin buffer and examined under an Olympus FSX100 microscope (Tokyo, Japan). ImageJ software (Abramoff et al. 2004) was used to measure the average staining intensities of all cells observed in three independent micrographs. The numbers of cells in each micrograph were counted manually and the data were used to calculate the average staining intensity/cell in three independent micrographs, with averages presented as “Relative fluorescence” in the bar graphs shown in Figures 3 and 4.

**CMP-sialic acid transport assays**

The CMP-sialic acid transport assays used in this study have been described previously (Aumiller and Jarvis 2002). Briefly, microsomal fractions were isolated from SiSWT-4, SiSWT-6, CHO and Lec 2 cells and total protein content was determined using a commercial BCA assay kit (Thermo Scientific...
Expression and purification of recombinant hEPO-His

S9, SFSWT-4 and SFSWT-6 cells were seeded into 50 mL shake flask cultures in ESF 921 media at a density of 2 × 10⁶ cells/mL, and then infected with AchEPO-His at a multiplicity of about 5 plaque forming units/cell. The virus was allowed to adsorb for 1 h, then the infected cells were gently pelleted, resuspended in 50 mL of ESF 921 containing 1, 10 or 50 mM N-acetylmannosamine, returned to shake flasks and incubated at 28°C to 48 h after infection. The cells and debris were pelleted by centrifugation at 1000 × g for 10 min, the supernatant was harvested and the budded virus particles were pelleted by centrifugation at 70,000 × g for 30 min. One Complete Protease Inhibitor Cocktail tablet (Roche Diagnostics, Indianapolis, IN) was added to this supernatant and it was dialyzed against 0.1 M NaCl for 6 h and finally desalted using a PD10 desalting column (GE Healthcare, Piscataway, NJ) equilibrated with 10 mM Tris–HCl, pH 7.5.

Lectin blotting analysis of hEPO-His sialylation

Purified hEPO-His and bovine fetuin (Sigma-Aldrich, St. Louis, MO) were treated with neuraminidase in neuraminidase reaction buffer or neuraminidase reaction buffer alone according to the manufacturer’s instructions (New England Biolabs). The proteins were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12.5% polyacrylamide gels and electrophotoethetically transferred to Immobilon-P membranes (Millipore), which were then blocked for 1 h at room temperature with 5% bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline (150 mM NaCl in 50 mM Tris–HCl, pH 7.5) containing 0.5% Tween 20 for immunoblotting or in Tris-buffered saline containing 1% Tween 20 for lectin blotting assays. The immunoblotting assays were completed using rabbit anti-hEPO (U-CyTech, Utrecht, The Netherlands) as the primary antibody, goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma-Aldrich) as the secondary antibody and a standard chromogenic assay for alkaline phosphatase activity (Blake et al. 1984). The lectin blotting assays were completed using biotinylated SNA (Vector Laboratories) as the primary probe for terminal sialic acids, streptavidin-alkaline phosphatase (Sigma-Aldrich) as the secondary reagent and the same chromogenic assay for alkaline phosphatase activity, as described previously (Geisler and Jarvis 2011).

Mass spectrometry

N-glycans were enzymatically released from the various hEPO preparations produced and purified under the conditions described above by exhaustive digestion with PNGase-F (New England Biolabs). The spent reactions were applied to pre-conditioned C18 SepPak cartridges (Waters Corp., Milford, MA) and the flow-through and a 5% (v/v) aqueous acetic acid wash were pooled, evaporated and permethylated, as described previously (Dell et al. 1994). The permethylated N-glycan derivatives extracted into chloroform with several aqueous washes were re-evaporated, then resuspended in acetonitrile and mixed 1:1 with 2,5-dihydroxybenzoic acid matrix (10 mg/mL in 50% acetonitrile in water), and the samples were spotted onto the MALDI-TOF target plate. Data acquisition was performed manually on a Model 4700 Proteomics Analyzer equipped with an Nd:YAG laser (Applied Biosystems, Framingham, MA) and 1000 shots were accumulated in the reflectron positive ion mode.

Funding

This work was supported by Award Number R01GM49734 from the National Institute of General Medical Sciences. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health. The MS data were acquired at the Core Facilities for Protein Structural Analysis at Academia Sinica, supported under the Taiwan National Core Facility Program for Biotechnology, NSC Grant Number 100-2325-B-001-029.

Acknowledgements

The authors thank Jared Aumiller for isolating the recombinant baculovirus encoding human erythropoietin, which was used in this study.
Conflict of interest

Donald L. Jarvis is the founder of a new biotech company, GlycoBac, LLC, that will focus on the glycoengineered insect cell platform as a tool for recombinant glycoprotein production. The other authors have no potential conflicts of interest.

Abbreviations

bB4GALT1, bovine β1,4-galactosyltransferase I; CHO, Chinese hamster ovary; CSAT, CMP-sialic acid transporter; hCSAT, human CMP-sialic acid transporter; hEPO, human erythropoietin; hMGAT2, human β1,2-N-acetylglucosaminyltransferase II; mCMAS, murine CMP-sialic acid synthetase; mSAS, murine sialic acid synthase; mST3GAL3, murine α2,3-sialyltransferase III; rST6GAL1, rat α2,6-sialyltransferase I; RT–PCR, reverse transcription polymerase chain reaction; SNA, Sambus nigra agglutinin.

References


