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Innovative use of a bacterial enzyme involved in sialic acid degradation to initiate sialic acid biosynthesis in glycoengineered insect cells

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Abstract

The baculovirus/insect cell system is widely used for recombinant protein production, but it is suboptimal for recombinant glycoprotein production because it does not provide sialylation, which is an essential feature of many glycoprotein biologics. This problem has been addressed by metabolic engineering, which has extended endogenous insect cell *N*-glycosylation pathways and enabled glycoprotein sialylation by baculovirus/insect cell systems. However, further improvement is needed because even the most extensively engineered baculovirus/insect cell systems require media supplementation with *N*-acetylmannosamine, an expensive sialic acid precursor, for efficient recombinant glycoprotein sialylation. Our solution to this problem focused on *E. coli* *N*-acetylglucosamine-6-phosphate 2'-epimerase (GNPE), which normally functions in bacterial sialic acid degradation. Considering that insect cells have the product, but not the substrate for this enzyme, we hypothesized that GNPE might drive the reverse reaction in these cells, thereby initiating sialic acid biosynthesis in the absence of media supplementation. We tested this hypothesis by isolating transgenic insect cells expressing *E. coli* GNPE together with a suite of mammalian genes needed for *N*-glycoprotein sialylation. Various assays showed that these cells efficiently produced sialic acid, CMP-sialic acid, and sialylated recombinant *N*-glycoproteins even in growth media without *N*-acetylmannosamine. Thus, this study demonstrated that a eukaryotic recombinant protein production platform can be glycoengineered with a bacterial gene, that a bacterial enzyme which normally functions in sialic acid degradation can be used to initiate sialic acid biosynthesis, and that insect cells expressing this enzyme can produce sialylated *N*-glycoproteins without *N*-acetylmannosamine supplementation, which will reduce production costs in glycoengineered baculovirus/insect cell systems.

Keywords

Sialylation; Glycoengineering; Glycoprotein production; Insect cells; Baculovirus; Epimerase

1. Introduction

The terminal sialic acids on the carbohydrate side chains of glycoproteins have a major impact on their overall properties, including their circulatory half-lives *in vivo* (Ngantung et

al., 2006). Therefore, recombinant glycoprotein biologics intended for human therapeutic applications are usually manufactured in mammalian cell systems, which have protein glycosylation pathways that can provide terminal sialylation (Walsh, 2010). However, one disadvantage of mammalian protein glycosylation pathways is that they sometimes produce recombinant glycoproteins with immunogenic carbohydrate side chains containing either the α Gal epitope (Chung et al., 2008) or the relatively rare sialic acid, *N*-glycolylneuraminic acid (Ghaderi et al., 2010). In contrast, the baculovirus/insect cell system does not produce these immunogenic carbohydrate epitopes. The baculovirus/insect cell system is also generally considered to be safer than mammalian systems because it is less likely to harbor adventitious agents that could infect humans or other mammals (reviewed by Jarvis, 2009). Unfortunately, the unmodified baculovirus/insect cell platform cannot be used to manufacture recombinant glycoproteins for *in vivo* therapeutic applications because it does not provide terminal sialylation.

This limitation has been addressed at the basic research level by metabolic engineering, which has been used to extend the endogenous processing capabilities of the insect cell protein *N*-glycosylation pathway (reviewed by Geisler and Jarvis, 2009; Harrison and Jarvis, 2006; Jarvis, 2009; Shi and Jarvis, 2007). These glycoengineering efforts have yielded genetically modified baculovirus/insect cell systems capable of producing *N*-glycoproteins with complex, mammalian-type carbohydrate side chains (*N*-glycans) terminated with the most common sialic acid, *N*-acetylneuraminic acid (Neu5Ac; Aumiller et al., 2003; Aumiller et al., 2012; Hill et al., 2006; Hollister et al., 2002; Hollister and Jarvis, 2001; Jarvis et al., 2001; Seo et al., 2001). One important step taken to achieve this goal was to metabolically engineer insect cells to produce Neu5Ac and CMP-Neu5Ac, which are required for glycoprotein sialylation, but undetectable in non-engineered insect cell lines (Hooker et al., 1999; Tomiya et al., 2001). This was accomplished by introducing mammalian sialic acid synthase (SAS) and CMP-sialic acid synthetase (CMAS) genes into the baculovirus/insect cell system, which enabled the cells to utilize the key sialic acid precursor, *N*-acetylmannosamine (ManNAc), and produce Neu5Ac and CMP-Neu5Ac (Aumiller et al., 2003; Hill et al., 2006; Fig. 1). While this engineering strategy was successful, the resulting baculovirus/insect cell systems had to be cultured in growth media supplemented with ManNAc in order to efficiently produce Neu5Ac, CMP-Neu5Ac, and sialylated recombinant glycoproteins. This substantially increased the cost of growth media and revealed an acute need for additional glycoengineering to circumvent this expensive media supplementation requirement.

In mammalian cells, a bifunctional enzyme known as UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE) initiates sialic acid production by converting UDP-*N*-acetylglucosamine (UDP-GlcNAc) to *N*-acetylmannosamine-6-phosphate (ManNAc-6-P; Hinderlich et al., 1997; Stäsche et al., 1997). Thus, the most obvious way to circumvent the ManNAc supplementation requirement was to introduce mammalian GNE. In fact, it had already been shown that insect cells infected with recombinant baculovirus vectors encoding human GNE and SAS could produce Neu5Ac in the absence of any media supplements (Viswanathan et al., 2003). However, in the context of our broader goal of glycoengineering insect cells for glycoprotein sialylation, we were concerned that GNE overexpression might deplete intracellular UDP-GlcNAc, thereby reducing one or more *N*-acetylglucosaminyltransferase activities and diminishing the overall efficiency of *N*-glycan processing. This concern was heightened by a report showing that the level of UDP-GlcNAc is a critical factor regulating *N*-acetylglucosaminyltransferase V activity *in vivo* (Sasai et al., 2002).

With this in mind, we conceived a more innovative way to circumvent the ManNAc supplementation requirement for recombinant glycoprotein sialylation in glycoengineered

baculovirus/insect cell systems. Though it initially seemed counterintuitive, our approach focused on *E. coli* GlcNAc-6-P 2'-epimerase (GNPE), which normally converts ManNAc-6-P to GlcNAc-6-P in a bacterial sialic acid degradation pathway (Vimr et al., 2004). Although insect cells have no detectable ManNAc-6-P, they convert glucose to GlcNAc-6-P as a part of their basal metabolism. Realizing that the GNPE reaction might be reversible, we hypothesized that GNPE could drive the reverse of its normal reaction in insect cells, producing ManNAc-6-P from GlcNAc-6-P and initiating sialic acid biosynthesis in the absence of exogenous ManNAc.

In this study, we tested this hypothesis and demonstrated that a eukaryotic recombinant protein production platform can be glycoengineered with a bacterial gene, that a bacterial enzyme normally involved in sialic acid degradation can be innovatively used to initiate sialic acid biosynthesis, and that insect cells expressing this enzyme can produce sialylated glycoproteins in the absence of media supplementation, which will reduce the cost of recombinant glycoprotein sialylation in glycoengineered insect cell systems.

2. Materials and methods

2.1. Immediate early expression plasmids

The transgenic insect cells described in this study were produced using various immediate early expression plasmids, which can be used to express foreign genes constitutively in uninfected insect cells under the transcriptional control of the baculovirus *ie1* promoter and *hr5* enhancer elements (Table 1; [Jarvis, 1996 #60; Jarvis, 1990 #312]. pIE1Hygro, pIE1GlcNAcTII, pIE1HRGalT, pIE1ST6, and pIE1-hCSAT are described in the references given in Table 1. pIE1MmSAS and pIE1MmCSAS are new immediate early expression plasmids encoding mouse SAS (Genbank Acc. No. BC003307) and CSAS (Genbank Acc. No. BE689556), respectively. Finally, pIE1EcGNPE is a new immediate early expression plasmid encoding *E. coli* K12 GNPE (GenBank Acc. No. AP012306.1 nucleotides 2838888 – 2838199). The structures of each of the plasmids used in this study were verified by restriction mapping and/or nucleotide sequencing and each was extracted from a culture of the relevant *E. coli* strain using the alkaline lysis method and purified by equilibrium ultracentrifugation in continuous CsCl-EtBr density gradients, as described previously (Sambrook et al., 1989).

2.2. Insect cells, cell culture, and viruses

In this study, we used *expresSF+* cells (Protein Sciences Corporation, Meriden, CT; Sf900+ in United States Patent 6,103,526), which are a derivative of Sf9 cells (Summers and Smith, 1987) for our experiments. We chose this cell line because it is a thoroughly characterized, commercially relevant insect cell line used as a host for baculovirus expression vectors. *ExpresSF+* and SfSWT-19 and -21 (this study) cells were routinely maintained as shake flask cultures at 125 rpm and 28°C in Protein Sciences Fortified Medium (PSFM; Protein Sciences Corporation) at densities ranging from 0.5 to 6.0 × 10⁶ cells/mL. For media supplementation experiments, PSFM was supplemented with 200 μM peracetylated ManNAc (Ac₄ManNAc) in lieu of ManNAc because the peracetylated form is more readily taken up by cells (Jones et al., 2004) and, therefore, can be used at much lower concentrations.

SfSWT-19 and SfWT-21 are transgenic variants of *expresSF+* cells that were isolated in this study. Briefly, *expresSF+* cells were transfected with immediate early expression plasmid DNA mixtures using a modified calcium phosphate method and selected in PMSF containing 1 mg/mL hygromycin, as described previously (Harrison and Jarvis, 2007a; Harrison and Jarvis, 2007b). The plasmid DNA mixtures used to produce SfSWT-19 and SfSWT-21 cells included 5 μg each of pIE1HRGlcNAcTII, pIE1HRGalT, pIE1HRST6,

pIE1mSAS, pIE1mCSAS, pIE1-hCSAT, and pIE1Hygro. In addition, the mixture used to produce SfSWT-21 cells included 5 μ g of pIE1EcGNPE.

Human erythropoietin (hEPO) and a murine IgG2a-Fc were used to assess the recombinant glycoprotein sialylation capabilities of the new transgenic insect cell lines isolated for this study. We used these molecules as models for this study because EPO and recombinant antibodies represent the vast majority of the recombinant glycoprotein biologics market (Walsh, 2010). AchEPO-His is a recombinant baculovirus encoding a C-terminally 6X-HIS-tagged version of hEPO, as described previously (H. Mabashi-Asazuma, X. Shi, C. Geisler, and D.L. Jarvis, submitted for publication). AcmlgG2a-Fc is a recombinant baculovirus encoding an N-terminally 8X-HIS-tagged version of the soluble domain of a mouse IgG2a-Fc fragment (Genbank Acc. No. S37483; amino acids 237 – 469). This recombinant baculovirus was produced using Gateway® technology with a pENTR™/D-TOPO® TA (Life Technologies Corporation, Grand Island, NY) entry vector kindly provided by Dr. Henrik Olson (GlikNik, Baltimore, MD) and a modified baculovirus destination vector, Ac6.9-GT, previously isolated in our lab (Toth et al., 2011). The spent LR reaction was used to transfect *expresSF+* cells, as described above, the transfected cells were cultured in PSFM containing ganciclovir to select against the parental (Ac6.9-GT) virus, and then viral progeny were resolved by plaque assay, as described previously (Toth et al., 2011). A recombinant virus identified by its white plaque phenotype was amplified, characterized, and then a working virus stock was prepared, titered by plaque assay, and used for the remainder of this study, as described previously (Summers and Smith, 1987).

Growth curves were produced by seeding 1×10^6 exponential phase cells in 50 mL of PSFM media in 50 mL shake flasks. Samples were taken daily for 5 days and counted manually in an improved Neubauer hemocytometer in triplicate. For photography, 5×10^6 mid-log phase cells were seeded in 35-mm glass bottom culture dishes (number 1.5, MatTek, Ashland, MA), allowed to attach for 5 min at 28 °C, and then the media were aspirated and photomicrographs were taken at 40X magnification using an Olympus FSX100 microscope.

2.3 Cell surface staining

Insect cells were seeded into 6-well plates at a density of 1×10^6 cells/well in PSFM with or without 200 μ M peracetylated ManNAc (Ac₄ManNAc; New Zealand Pharmaceuticals). After 24 h at 28°C, the media were removed and the cells were washed with lectin buffer (10 mM HEPES pH 7.5, containing 150 mM NaCl, 0.08% NaN₃, and 1 mM each of CaCl₂, MgCl₂, and MnCl₂), covered with the same buffer, and incubated for 10 min at 4°C. The buffer was then removed and the cells were incubated with fresh lectin buffer containing 10 μ g/mL of FITC-conjugated Concanavalin A (ConA) or *Sambucus nigra* agglutinin (SNA) for 10 min at 4°C. Each of the FITC-conjugated lectins was purchased from Vector Laboratories (Burlingame, CA). After this incubation period, the lectins were removed and the cells were washed twice with fresh lectin buffer, covered with the same, and imaged using an Olympus FSX-100 microscope (Tokyo, Japan) with identical exposures for all samples.

2.4 Recombinant glycoprotein expression and purification

Insect cells were seeded into 50 mL shake flask cultures at a density of 2×10^6 cells/mL in PSFM medium and infected with AchEPO-His or AcmlgG2a-Fc at a multiplicity of infection of about 2 plaque-forming units/cell. After a 1 h adsorption period, the cells were pelleted by centrifugation at $200 \times g$ for 5 min, resuspended in PSFM supplemented with antibiotics (1.25 μ g/mL amphotericin B and 25 μ g/mL gentamicin) and with or without 200 μ M Ac₄ManNAc, transferred to fresh shake flasks, and incubated for 48 h. The cultures were then harvested and cells and debris were removed by centrifugation at $1,000 \times g$ for 10

min at 4°C. The supernatants were harvested and budded virus particles were removed by ultracentrifugation at $100,000 \times g$ for 30 min at 4°C. mIgG2a-Fc supernatants were dialyzed in 12–14 kDa molecular weight cut off membranes (Spectrum Labs, Rancho-Dominguez, CA) against 0.05 M Na_2HPO_4 (pH 7.5) containing 0.5 M NaCl. hEPO-His supernatants were buffer-exchanged on a Sephadex G25 column equilibrated with 10 mM Tris (pH 7.5) containing 0.5 M NaCl. Subsequently, each protein was affinity-purified using ProBond nickel affinity resin (Life Technologies) according to the manufacturer's instructions and, after elution with 10 mM Tris (pH 7.5) containing 0.5 M NaCl and 0.5 M imidazole, each was desalted on Sephadex G25 columns equilibrated with 50 mM Tris (pH 7.5) containing 0.15 M NaCl. Finally, each protein was concentrated using an Amicon® Ultra-4 centrifugal filter with a 5 kDa molecular weight cutoff (EMD-Millipore; Merck, Darmstadt, Germany).

2.5 Sialic acid and CMP-sialic acid determinations

Sialic acid and CMP-sialic acid determinations were performed in triplicate using AchEPO-His-infected cells in order to assess sialic acid metabolism and recombinant hEPO-HIS sialylation in parallel. Cell pellets were isolated from AchEPO-His-infected cell cultures as described in section 2.4, washed once with phosphate buffered saline (pH 7.4), and then SDS was added to a final concentration of 1% (w/v), the cell suspension was vortexed, and the DNA was sheared by repeatedly passing the extracts through a 22 ga syringe. Triplicate 100 μL aliquots of these cell extracts were used to measure the total sialic acid and CMP-sialic acid content in a modified thiobarbituric acid assay. CMP-sialic acid content was measured independently using a separate set of triplicate 100 μL aliquots of the same extracts after treatment with 7.5 μL of 10% (w/v) NaBH_4 to destroy the free sialic acids. After adding 7.5 μL acetone and 55 μL of 0.2 M NaIO_4 in 57% concentrated H_3PO_4 , the extracts were incubated for 1 h at room temperature, and then 0.55 mL of 0.1 M H_2SO_4 containing 10% (w/v) NaAsO_2 and 0.5 M Na_2SO_4 was added and the extracts were vortexed and incubated for another 10 min at room temperature. Finally, 1.65 mL of TBA reagent (0.5 M Na_2SO_4 , 0.6% 2-thiobarbituric acid, pH 9) was added slowly with mixing and the extracts were incubated overnight at 4°C. The extracts were then transferred to an 80°C water bath, incubated for 1 h, cooled in a 25°C water bath, and then 0.75 mL of cyclohexanone was added, the extracts were vortexed vigorously, and the organic phase was used to measure absorbance at 549 and 532 nm. The number of picomoles of sialic acid or CMP-sialic acid in each sample was calculated by subtracting the absorbance contributed by the 2-deoxyribose chromophore using the formula $750 \times (21 \times A_{549} - 7.6 \times A_{532})$, as described previously (Warren, 1959). Triplicate samples of each lysate also were used to measure total protein concentrations in a commercial bicinchoninic acid assay with bovine serum albumin as the standard, according to the manufacturer's recommendations (Pierce, Rockford, IL). The final average sialic acid values obtained with the untreated extracts were presented as pmol sialic acid/ μg total protein in Fig. 2A. The final average sialic acid values obtained with the NaBH_4 -treated extracts were presented as pmol CMP-sialic acid/ μg total protein in Fig. 2B. Confidence intervals were calculated using Microsoft® Office Excel 2010.

2.6 Recombinant glycoprotein analyses

To determine protein concentrations, aliquots of the purified protein and known amounts of bovine serum albumin (Sigma Aldrich, St. Louis, MO) were resolved by SDS-PAGE in adjacent lanes of the same gel. The gels were then stained with Coomassie Brilliant Blue (CBB) and staining intensities were measured using Quantity One version 4.6.5 software (Bio-Rad, Hercules, CA). Replicate samples containing equal amounts of protein were then treated with buffer alone, *Flavobacterium meningosepticum* peptide:N-glycanase F (PNGase F; New England BioLabs, Ipswich, MA), or *Clostridium perfringens* neuraminidase (New England BioLabs), according to the manufacturer's instructions. Commercial human

transferrin and bovine serum albumin (Sigma-Aldrich) were used as sialylated and non-glycosylated protein controls, respectively. After the appropriate treatment, each sample was resolved by SDS-PAGE with 12% (w/v) polyacrylamide gels, a gel containing one set of replicates was stained with CBB, and the other three were transferred to Immobilon-P PVDF membranes (Immobilon, Billerica, MA) for immunoblotting or lectin blotting assays, as described previously (Geisler and Jarvis, 2011). In the immunoblotting assays, mIgG2a-Fc was detected using alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) and hEPO was detected using rabbit anti-human EPO (U-CyTech, Utrecht, The Netherlands) and alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma). In the lectin blotting assays, mannose or sialic acid were detected using alkaline phosphatase-conjugated ConA or SNA, respectively, each of which was obtained from EY Laboratories (San Mateo, CA). Immunoblotting and lectin blotting signals were visualized using a standard alkaline phosphatase-based chromogenic detection method, as described previously (Leary et al., 1983).

Cell-free supernatants were prepared from AcmIgG2a-Fc-infected *expresSF+* and SfSWT-21 cultures and equal aliquots were resolved by SDS-PAGE, transferred onto Immobilon-P membranes, probed with anti-mouse IgG, and developed, as described above. Recombinant glycoprotein production levels were compared by analyzing the band intensities using Quantity One version 4.6.5 software (Biorad) and confidence intervals were calculated using Microsoft® Office Excel 2010.

3. Results

3.1. Glycoengineering the baculovirus/insect cell system

The most commonly used hosts for baculovirus-mediated recombinant glycoprotein production are established lepidopteran insect cell lines, such as IPLB-SF-21 (Sf21; Vaughn et al., 1977), Sf9 (Summers and Smith, 1987), *expresSF+* (Protein Sciences Corporation), and BTI Tn 5B1-4 (High Five™; Wickham et al., 1992). Each of these cell lines has metabolic pathways that support recombinant glycoprotein biosynthesis and processing. However, these insect cell pathways are simpler than the corresponding mammalian cell pathways and, as a result, none of these insect cell lines can produce recombinant glycoproteins with complex, terminally sialylated *N*-glycans (Fig. 1A). This problem has been addressed through glycoengineering efforts, in which mammalian genes have been incorporated into baculovirus expression vectors and/or lepidopteran insect cell lines (reviewed by Geisler and Jarvis, 2009; Harrison and Jarvis, 2006; Jarvis, 2009; Shi and Jarvis, 2007). These efforts have yielded modified baculovirus/insect cell systems with more extensive protein *N*-glycosylation pathways and the capacity to sialylate recombinant glycoproteins (Fig. 1B). However, as noted above, even the most extensively glycoengineered baculovirus/insect cell systems require supplementation with ManNAc for efficient sialylation (Fig. 1C), which substantially increases media costs.

To determine if *E. coli* GNPE could be used to initiate sialic acid biosynthesis and overcome the ManNAc supplementation requirement, as hypothesized, we isolated a matched pair of transgenic *expresSF+* cell derivatives designated SfSWT-19 and SfSWT-21. Each was genetically transformed in parallel with the suite of mammalian genes required to enable sialylated recombinant glycoprotein production in the presence of ManNAc, which included genes encoding *N*-acetylglucosaminyltransferase II, β 4-galactosyltransferase I, α 2,6-sialyltransferase I, SAS, CMAS, and a CMP-sialic acid transporter, together with a bacterial hygromycin phosphotransferase (Hygro^R) gene as the selectable marker, as described in Materials and methods. SfSWT-21, but not SfSWT-19 cells were additionally transformed with the *E. coli* GNPE gene. As detailed above, *E. coli* GNPE is normally involved in sialic acid degradation, but we hypothesized that this enzyme might drive the reverse reaction in

insect cells, effectively converting pre-existing pools of GlcNAc-6-P to ManNAc-6-P, and initiating sialic acid biosynthesis in the absence of exogenous ManNAc.

After transfection and selection in growth media containing hygromycin B, we examined SfSWT-19 and -21 cells in preliminary cell surface SNA staining assays designed to detect the presence of sialylated glycoconjugates, as described in Materials and methods. The results showed that SNA intensely stained both SfSWT-19 and -21 cells that had been cultured in PSFM supplemented with Ac₄ManNAc (data not shown). Despite being transformed with multiple unlinked markers, SNA stained virtually 100% of each cell type under these growth conditions, although there was variation in the staining intensities observed among individual cells (data not shown). Most importantly, SNA also intensely stained SfSWT-21 cells, which had been additionally transformed with the *E. coli* GNPE gene, when these cells were cultured in PSFM lacking Ac₄ManNAc. This preliminary result supported the hypothesis that *E. coli* GNPE could be used to circumvent the ManNAc supplementation requirement in glycoengineered *expresSF*⁺ cells and prompted us to examine the properties of SfSWT-19 and -21 cells in further detail.

3.2. Sialic acid and CMP-sialic acid production

Our first set of formalized experiments was designed to examine the ability of the transgenic insect cells to produce sialic acid and CMP-sialic acid when cultured in the presence or absence of ManNAc. These assays were performed using AchEPO-His-infected cells so that the results could be coupled with a subsequent assessment of recombinant hEPO sialylation. Hence, *expresSF*⁺, SfSWT-19, and SfSWT-21 cells were infected with AchEPO and cultured for 48 h in PSFM or PSFM supplemented with Ac₄ManNAc. The extracellular growth media were harvested and used to affinity-purify hEPO-His for lectin blotting assays (see below), and then the infected cell pellets were extracted and assayed for total sialic acid and CMP-sialic acid, as described in Materials and methods.

The results of these assays showed that *expresSF*⁺ cells cultured in the presence or absence of Ac₄ManNAc contained no detectable sialic acid (Fig. 2A). SfSWT-19 cells cultured in the absence of Ac₄ManNAc also contained no detectable sialic acid, but the same cells contained substantial amounts of sialic acid when cultured in the presence of Ac₄ManNAc. This was consistent with the fact that SfSWT-19 cells express mammalian SAS, which would convert ManNAc obtained from the growth medium to sialic acid. SfSWT-21 cells contained equally high amounts of sialic acid when cultured in the presence of Ac₄ManNAc, as expected. Strikingly, however, SfSWT-21 cells also contained high amounts of sialic acid when cultured in the absence of Ac₄ManNAc. Nearly identical results were obtained when CMP-sialic acid levels were measured in extracts of each cell type cultured in the presence or absence of ManNAc (Fig. 2B). In each case, the observed CMP-sialic acid content reflected the presence or absence of intracellular sialic acid (Fig. 2A) and the mammalian CMAS gene.

These results showed that *E. coli* GNPE can efficiently initiate sialic acid biosynthesis in insect cells cultured in medium lacking ManNAc and that ManNAc supplementation does not augment sialic acid or CMP-sialic acid levels in insect cells glycoengineered to express this enzyme. Thus, these results supported the hypothesis that the bacterial GNPE can be used to circumvent the ManNAc supplementation requirement for recombinant glycoprotein sialylation by glycoengineered insect cells.

3.3. Cell surface sialylation

The next set of experiments was designed to determine if SfSWT-21 cells can sialylate endogenous glycoproteins in the absence of ManNAc supplementation. *expresSF*⁺,

SfSWT-19, and SfSWT-21 cells were cultured for 24 h in PSFM with or without Ac₄ManNAc, and then cell surface staining assays were performed with ConA or SNA, as described in Materials and methods. ConA binds to mannose and, therefore, stains all cell surface *N*-glycoproteins irrespective of their *N*-glycan structures. SNA binds to terminal sialic acids and, therefore, stains only terminally sialylated cell surface glycoconjugates. The results of these assays showed that ConA intensely stained *expresSF*⁺, SfSWT-19, and SfSWT-21 cells cultured in PSFM with (Fig. 3C) or without Ac₄ManNAc (Fig. 3A), with no obvious differences in staining intensities among the different cell lines or culture conditions. This was consistent with the expectation that all three cell lines would display endogenous cell surface *N*-glycoproteins containing mannose under both culture conditions. SNA stained *expresSF*⁺ cells cultured under either condition at only very low, background levels (Fig. 3B and 3D), which was expected because these cells cannot produce terminally sialylated glycoconjugates and were the negative controls for the SNA staining assays. In contrast, SNA intensely stained both SfSWT-19 and -21 cells cultured in PSFM supplemented with Ac₄ManNAc (Fig. 3D). This was expected because both had been glycoengineered with a suite of mammalian glycogenes known to support sialylate *N*-glycoprotein production in the presence of this sialic acid precursor. Most importantly, SNA stained SfSWT-21 cells cultured in PSFM with or without Ac₄ManNAc at about the same intensity, while this sialic acid-specific lectin stained SfSWT-19 cells grown in PSFM without Ac₄ManNAc at much lower levels (Fig. 3B).

These results indicated that *E. coli* GNPE can not only initiate sialic acid biosynthesis, but also can support glycoprotein sialylation in glycoengineered insect cells. Furthermore, the fact that there was no augmentation of cell surface sialylation with Ac₄ManNAc supplementation suggests that GNPE-mediated sialic acid production is not a bottleneck in glycoprotein sialylation by these cells. These results provided further support for the hypothesis that GNPE can circumvent the ManNAc supplementation requirement for recombinant glycoprotein sialylation by glycoengineered insect cells.

3.4. Recombinant glycoprotein sialylation

We also directly examined the ability of *E. coli* GNPE to support recombinant glycoprotein sialylation by glycoengineered insect cells cultured without Ac₄ManNAc supplementation. *expresSF*⁺, SfSWT-19, and SfSWT-21 cells were infected with recombinant baculoviruses encoding HIS-tagged forms of mIgG2a-Fc or hEPO, the infected cells were cultured for 48 h in PSFM with or without Ac₄ManNAc, the recombinant glycoproteins were affinity purified, and their glycosylation patterns were compared in tightly controlled lectin blotting assays. One set of samples was stained with CBB to demonstrate that approximately equal amounts of purified protein had been loaded into each lane (Figs. 4A and 5A). Another was used for immunoblotting with antibodies specific for mIgG or hEPO to verify their identities and document their banding patterns (Figs. 4B and 5B). A third set was stained with ConA, the mannose-specific lectin, to identify the *N*-glycosylated forms of each purified recombinant protein (Figs. 4C and 5C). Finally, a fourth set of samples was stained with SNA, the terminal sialic acid-specific lectin, to determine if the two different recombinant glycoproteins were sialylated when produced in the three different cell types under the two different growth conditions (Figs. 4D and 5D).

The results of these experiments showed that *expresSF*⁺ cells failed to detectably sialylate either mIgG2a-Fc or hEPO when cultured in PSFM with or without Ac₄ManNAc. This was expected, as this parental cell line does not produce sialylated glycoproteins and was a negative control for the lectin blotting assays. SfSWT-19 cells also failed to detectably sialylate mIgG2a-Fc when cultured in PSFM. When cultured in PSFM supplemented with Ac₄ManNAc, SfSWT-19 cells sialylated this recombinant protein, albeit at low levels, as

expected. SfSWT-21 cells cultured in PSFM with or without Ac₄ManNAc sialylated mIgG2a-Fc at a much higher level, with no augmentation in the presence of Ac₄ManNAc.

Unexpectedly, SfSWT-19 cells sialylated hEPO at low levels when cultured in PSFM without Ac₄ManNAc. The addition of Ac₄ManNAc strongly augmented hEPO sialylation by these cells. In fact, when cultured in PSFM containing Ac₄ManNAc, SfSWT-19 cells sialylated hEPO at about the same levels as SfSWT-21 cells grown in PSFM without Ac₄ManNAc. Again, the addition of Ac₄ManNAc did not augment recombinant protein sialylation by SfSWT-21 cells.

Importantly, the right hand panels of Figs. 4 and 5 were dedicated to an extensive set of control reactions that clearly document the specificity of the lectin blotting assays. These panels show CBB staining, immunoblotting, and lectin blotting of samples of each protein after pretreatment with PNGase-F, which removes all *N*-linked glycans, or sialidase, which removes terminal sialic acids, as described in Materials and methods. The absence of ConA and SNA staining after PNGase treatment and the absence of SNA staining after sialidase treatment in each and every case demonstrates that the lectins bound specifically to *N*-glycans or terminal sialic acids, respectively, under the lectin blotting assay conditions used in this study.

3.5. Properties of SfSWT-21 cells

Finally, we investigated the biotechnologically relevant properties of SfSWT-21, including growth, gross morphology, and recombinant glycoprotein productivity, in comparison to *expresSF*⁺ cells. The growth curves obtained with both cell lines showed that they grew at similar rates and achieved similar peak densities (Fig. 6A and B). The calculated cell doubling times (*T*_D) over the first two days of growth were very similar; 19.8 and 21.7 h for *expresSF*⁺ and SfSWT-21, respectively (Fig. 6C and D). *expresSF*⁺ and SfSWT-21 morphologies also were quite similar, as both were mostly spherical cells of about the same size with a somewhat mottled appearance (Fig. 6E and F). Both cell types grew as single cells in suspension without appreciable clumping. Finally, the amounts of mIgG2a-Fc secreted by both cell types were statistically indistinguishable when measured by immunoblotting (Fig. 6G). Thus, the expression of a large set of transgenes, including GNPE, did not appreciably alter any of the biotechnologically relevant cell properties of SfSWT-21 cells examined in this study.

Our combined results directly demonstrated that *E. coli* GNPE can not only initiate sialic acid biosynthesis, but also can support recombinant glycoprotein sialylation in glycoengineered insect cells cultured in a growth medium lacking ManNAc. In addition, while Ac₄ManNAc supplementation augmented recombinant glycoprotein sialylation by glycoengineered insect cells lacking GNPE, it did not augment sialylation by glycoengineered insect cells expressing GNPE. Thus, all of the results obtained in this study showed that *E. coli* GNPE can be used to drive one step in a sialic acid degradation pathway in reverse, thereby initiating sialic acid biosynthesis and circumventing the requirement for ManNAc supplementation for recombinant glycoprotein sialylation by glycoengineered insect cells.

4. Discussion

Terminal sialic acids are required for the therapeutic efficacy of many recombinant glycoprotein biologics, such as erythropoietin and some antibodies (Ngantung et al., 2006). This requirement restricts the utility of the unmodified baculovirus/insect cell system as a glycoprotein biologics production platform, which does not support recombinant glycoprotein sialylation (reviewed by Geisler and Jarvis, 2009; Harrison and Jarvis, 2006;

Jarvis, 2009; Marchal et al., 2001; Shi and Jarvis, 2007). The inability of the unmodified baculovirus/insect cell platform to support recombinant glycoprotein sialylation is due to the fact that lepidopteran insect cell lines lack functional levels of late acting glycosyltransferases, as well as the pathways needed for sialic acid biosynthesis and utilization (reviewed by Geisler and Jarvis, 2009; Harrison and Jarvis, 2006; Jarvis, 2009; Marchal et al., 2001; März et al., 1995; Shi and Jarvis, 2007). Previous studies have shown that these deficiencies can be corrected by metabolically engineering baculovirus vectors and/or their lepidopteran insect cell hosts with mammalian genes encoding these functions (reviewed by Geisler and Jarvis, 2009; Harrison and Jarvis, 2006; Jarvis, 2009; Shi and Jarvis, 2007). However, even the most extensively glycoengineered baculovirus/insect cell systems developed to date require media supplementation with the sialic acid precursor, ManNAc. This would increase production costs to non-competitive levels for scaled-up recombinant glycoprotein biologics manufacturing. Thus, it was necessary to further glycoengineer the baculovirus/insect cell system to enable sialic acid biosynthesis from endogenous cellular precursor pools and circumvent the ManNAc supplementation requirement.

An obvious approach was to engineer insect cells to express GNE, which naturally initiates sialic acid biosynthesis in mammalian cells (Hinderlich et al., 1997; Stäsche et al., 1997; Fig. 7A). However, we believed this would be suboptimal for several reasons, one of which was that GNE overexpression might reduce intracellular UDP-GlcNAc pools and decrease the overall efficiency of *N*-glycan processing by limiting the activities of one or more *N*-acetylglucosaminyltransferases (Sasai et al., 2002). With this in mind, we examined other metabolic pathways to identify an enzyme that could be used to initiate sialic acid biosynthesis in insect cells, preferably one that could directly produce ManNAc-6-P, the substrate for SAS. Counter-intuitively, this search led us to *E. coli* GNPE, which normally converts ManNAc-6-P to GlcNAc-6-P as part of a bacterial sialic acid degradation pathway (Plumbridge and Vimr, 1999; Ringenberg et al., 2003; Vimr et al., 2004, Fig. 7B). Realizing that insect cells have endogenous pools of GlcNAc-6-P, but no detectable ManNAc-6-P, we hypothesized that GNPE might be able to use the former metabolite to initiate sialic acid biosynthesis in insect cells. To test this hypothesis, we isolated two closely matched transgenic insect cell lines by transforming *expresSF+* cells with a suite of mammalian genes known to support the production of sialic acid, CMP-sialic acid, and sialylated *N*-glycoproteins. One of these lines, designated SfSWT-21, was additionally transformed with the *E. coli* GNPE gene, while the other, designated SfSWT-19, was not.

SfSWT-21, but not SfSWT-19 cells were able to produce sialic acid and CMP-sialic acid, as well as sialylated endogenous cell surface glycoconjugates and recombinant mammalian glycoproteins, with no requirement for ManNAc supplementation. ManNAc supplementation did not augment the levels of glycoprotein sialylation and sialic acid and CMP-sialic acid levels in SfSWT-21 cells cultured without supplementation were comparable to those in insect cells infected with a recombinant baculovirus encoding GNE (Viswanathan et al., 2005). Thus, this study clearly demonstrated that GNPE can be used to initiate sialic acid biosynthesis and circumvent the requirement for ManNAc supplementation for recombinant glycoprotein sialylation in glycoengineered insect cells (Fig. 7C). Importantly, expression of a large set of transgenes, including GNPE, did not affect any biotechnologically relevant cell property examined, indicating that the newly derived SfSWT-21 cell line has the same potential as the parental *expresSF+* cell line for recombinant glycoprotein production (Fig. 6).

We emphasize that the utility of this *E. coli* enzyme for initiating sialic acid production is not limited to the baculovirus/insect cell system. GNPE could be used for this same purpose in many other recombinant glycoprotein production platforms that cannot produce sialic

acid, such as yeast, plants, and other insect systems. In previous studies, GNE was used to initiate sialic acid biosynthesis in yeast, plant, and baculovirus/insect cell systems (Castilho et al., 2010; Hamilton et al., 2006; Viswanathan et al., 2003). However, using GNPE presents an alternative, potentially better approach because it would not consume UDP-GlcNAc, as discussed above. GNPE also could be used to enhance sialic acid levels in expression systems that can produce sialic acid, such as the widely used Chinese hamster ovary (CHO) cell and other mammalian production platforms. Previous efforts to use GNE to increase sialic acid, CMP-sialic acid, and protein sialylation levels in CHO cells failed because GNE is feedback-inhibited by CMP-sialic acid (Bork et al., 2005). This problem was cleverly overcome by using a mutant GNE that is insensitive to feedback inhibition by CMP-sialic acid (Seppala et al., 1999) and CHO cells expressing this enzyme had higher levels of sialic acid, which enhanced recombinant glycoprotein sialylation (Bork et al., 2005; Bork et al., 2007; Son et al., 2011). Engineering CHO cells with *E. coli* GNPE would also avoid feedback inhibition by CMP-sialic acid and an additional advantage is that this enzyme would not consume UDP-GlcNAc and, therefore, would have no potentially adverse impact on recombinant glycoprotein processing by the *N*-acetylglucosaminyltransferases.

While sialic acid is clearly critical for the clinical efficacy of recombinant glycoprotein biologics, it also serves as an important precursor for the chemical synthesis of other drugs, such as zanamivir, which is a neuraminidase inhibitor used to treat influenza virus infections. Sialic acid is typically produced in bacteria by enzymes including sialic acid lyase or SAS, each of which use ManNAc as the substrate (reviewed by Tao et al., 2010). Insect or other cell types engineered to constitutively express *E. coli* GNPE, mammalian SAS, and *N*-acetylneuraminic acid phosphatase would present an attractive alternative production platform for industrial production of sialic acids.

In summary, we used *E. coli* GNPE to initiate sialic acid biosynthesis in the absence of an exogenous sialic acid precursor in the baculovirus/insect cell system. This innovative approach can be used to metabolically engineer a variety of systems to enable or enhance recombinant glycoprotein sialylation and it also could be used to create new systems for the production of sialic acid as a precursor for chemical synthesis.

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Abbreviations

Ac₄ManNAc	peracetylated <i>N</i> -acetyl- <i>D</i> -mannosamine
CBB	Coomassie Brilliant Blue
CHO	Chinese hamster ovary
CMAS	CMP-sialic acid synthetase
CMP	cytidine monophosphate
ConA	Concanavalin A

CSAT	CMP-sialic acid transporter
GlcNAc	<i>N</i> -acetyl- <i>D</i> -glucosamine
GlcNAc-6-P	<i>N</i> -acetyl- <i>D</i> -glucosamine-6-Phosphate
GNE	UDP- <i>N</i> -acetyl- <i>D</i> -glucosamine 2'-epimerase/ <i>N</i> -acetylmannosamine kinase
GNPE	<i>N</i> -acetyl- <i>D</i> -glucosamine-6-phosphate 2'-epimerase
hEPO	human erythropoietin
ManNAc	<i>N</i> -acetyl- <i>D</i> -mannosamine
ManNAc-6-P	<i>N</i> -acetyl- <i>D</i> -mannosamine-6-phosphate
Neu5Ac	<i>N</i> -acetylneuraminic acid
PNGase-F	<i>Flavobacterium meningosepticum</i> peptide: <i>N</i> -glycanase F
PSFM	Protein Sciences Fortified Medium
SAS	Sialic acid-9-phosphate synthase
SNA	<i>Sambucus nigra</i> agglutinin

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Highlights

- Bacterial GNPE can function in reverse to initiate sialic acid synthesis.
- Insect cells glycoengineered with GNPE can produce sialylated glycoproteins.
- Using GNPE circumvents an expensive media supplementation requirement.

Fig. 1.

Recombinant protein glycosylation in non-engineered and glycoengineered baculovirus/insect cell systems: (A) Non-glycoengineered baculovirus/insect cell systems can glycosylate newly synthesized proteins and process their *N*-glycans to produce trimmed, paucimannosidic structures. (B) Glycoengineered baculovirus/insect cell systems have extended *N*-glycan processing capabilities and can produce recombinant glycoproteins with complex, terminally sialylated *N*-glycans. (C) All glycoengineered baculovirus/insect cell systems described to date require media supplementation with ManNAc, an expensive sialic acid precursor, for efficient sialylation.

Fig. 2. Bacterial GNPE increases sialic acid and CMP-sialic acid content of insect cells cultured in the absence of ManNAc: (A) Total sialic acid content of *expresSF+*, SfSWT-19, and SfSWT-21 cells cultured in PSFM medium with or without Ac₄ManNAc. (B) CMP-sialic acid content of *expresSF+*, SfSWT-19, and SfSWT-21 cells cultured in PSFM medium with or without Ac₄ManNAc. Error bars: 95% confidence interval (n = 3).

Fig. 3. Bacterial GNPE increases sialylation of endogenous insect cell surface glycoconjugates without ManNAc supplementation: ConA staining reveals mannose-containing glycoconjugates on the surfaces of *expresSF+*, SfSWT-19, and SfSWT-21 cells cultured in PSFM medium without (A) or with (C) $Ac_4ManNAc$. SNA staining reveals sialylated glycoconjugates on the surfaces of *expresSF+*, SfSWT-19, and SfSWT-21 cells cultured in PSFM medium without (B) or with (D) $Ac_4ManNAc$.

Fig. 4. Insect cells expressing bacterial GNPE sialylate mIgG2a-Fc without ManNAc supplementation: (A) CBB staining, (B) immunoblotting, (C) ConA lectin blotting, and (D) SNA lectin blotting of recombinant mIgG2a-Fc produced by *expresSF+*, SfSWT-19, and SfSWT-21 cells cultured in PSFM medium with or without Ac₄ManNAc. Some samples of the protein produced under each condition were treated with sialidase or PNGase-F, as indicated by the labels in the Figure and as described in Materials and methods.

Fig. 5.

Insect cells expressing bacterial GNPE sialylate hEPO without ManNAc supplementation: (A) CBB staining, (B) immunoblotting, (C) ConA lectin blotting, and (D) SNA lectin blotting of recombinant mIgG2a-Fc produced by *expresSF+*, SfSWT-19, and SfSWT-21 cells cultured in PSFM medium with or without $Ac_4ManNAc$. Some samples of the protein produced under each condition were treated with sialidase or PNGase-F, as indicated by the labels in the Figure and as described in Materials and methods.

Fig. 6.

Transgene expression does not alter biotechnologically relevant properties of SfSWT-21 cells. (A) *expresSF+* and (B) SfSWT-21 cell growth curves. (C) *expresSF+* and (D) SfSWT-21 logarithmic relative cell densities during exponential phase. Different symbols are used to distinguish repeat experiments. Phase contrast micrographs of exponential phase *expresSF+* (E) and SfSWT-21 cells (F). Production levels of secreted mIgG2a-Fc (G). The production level of *expresSF+* was set at 100%, error bars: 95% confidence interval (n = 3).

Fig. 7. Sialic acid biosynthetic and degradative pathways. (A) GNE initiates sialic acid metabolism in mammals. (B) GNPE functions in sialic acid degradation in bacteria. (C) Hybrid bacterial/mammalian sialic acid pathway in glycoengineered insect cells.

Table 1

Genes used for insect cell glycoengineering in this study

Plasmid	Enzyme	HGNC* Abbreviation	Species	Reference
pIE1HRE<GNPE	GlcNAc-6-P 2'-epimerase	GNPE	<i>E. coli</i>	This study
pIE1HRMmSAS	Sialic acid-9-P synthase	SAS	Mouse	This study
None	Sialic acid-9-P phosphatase	NANP	Endogenous	Not applicable
pIE1HRMmCSAS	Sialic acid synthetase	CMAS	Mouse	This study
pIE1-hCSAT	Golgi CMP-sialic acid transporter	CMAT	Human	Mabashi-Asazuma et al, submitted
pIE1/GlcNAcTII	N-acetylglucosaminyltransferase II	MGAT2	Human	Hollister et al., 2002
pIE1HRGalT	β 1-4 galactosyltransferase	B4GAL.T1	Bovine	Hollister et al., 1998
pIE1ST6	α 2-6 sialyltransferase	ST6GAL.I	Rat	Hollister and Jarvis, 2001
pIE1Hygro	Hygromycin phosphotransferase	Hygro	<i>E. coli</i>	Hollister and Jarvis, 2001

* Human Gene Nomenclature Committee (<http://www.genenames.org/>)