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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.

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 αGal 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

Abbreviations: Ac_4ManNAc, peracetylated *N*-acetyl-*D*-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, *N*-acetyl-*D*-glucosamine; GlcNAc-6-P, *N*-acetyl-*D*-glucosamine-6-phosphate; GNE, UDP-*N*-acetyl-*D*-glucosamine 2′-epimerase/*N*-acetylmannosamine kinase; GNPE, *N*-acetyl-*D*-glucosamine-6-phosphate 2′-epimerase; hEPO, Human erythropoietin; ManNAc, *N*-acetyl-*D*-mannosamine; ManNAc-6-P, *N*-acetyl-*D*-mannosamine-6-phosphate; Neu5Ac, *N*-acetylneuraminic acid; PNGase-F, *Flavobacterium meningosepticum* peptide: *N*-glycanase F; PSFM, Protein Sciences Fortified Medium; SAS, Sialic acid-9-phosphate synthase; SNA, *Sambucus nigra* agglutinin

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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.

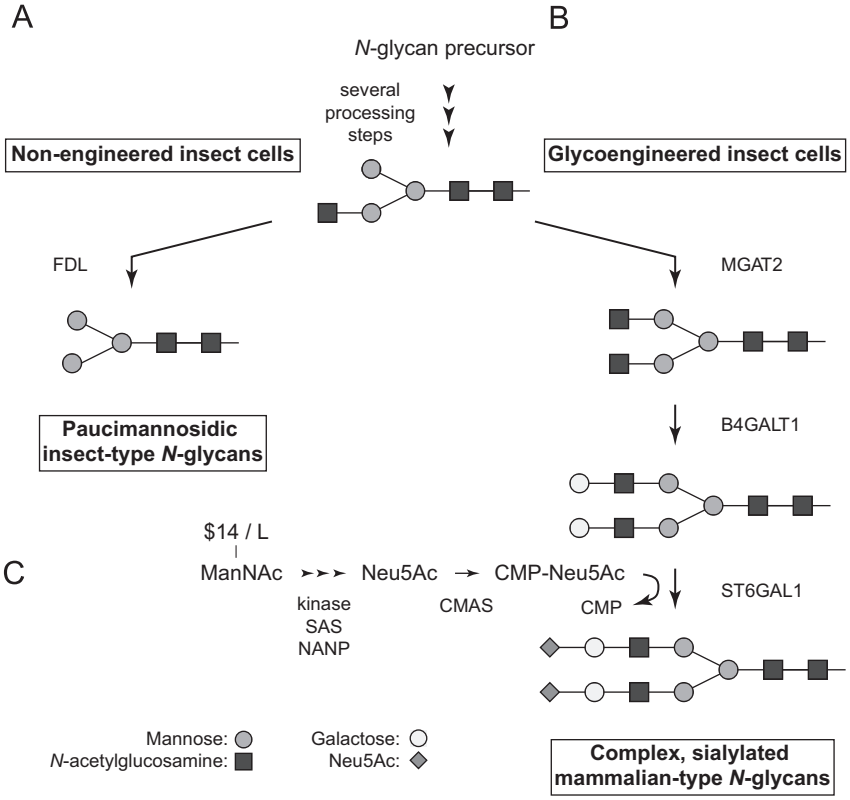

Fig. 1. Recombinant protein glycosylation in non-engineered and glycoengineered baculovirus/insect cell systems: (A) Non-glycoengineered baculovirus/insect cell systems can glycosylate newly synthesized proteins and process their *N*-glycans to produce trimmed, paucimannosidic structures. (B) Glycoengineered 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.