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SHORT COMMUNICATION

Electrophoretic analysis of glycoprotein glycans produced by lepidopteran insect cells infected with an immediate early recombinant baculovirus encoding mammalian β1,4-galactosyltransferase

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Glycosylation, the most extensive co- and post-translational modification of eukaryotic cells, can significantly affect biological activity and is particularly important for recombinant glycoproteins in human therapeutic applications. The baculovirus-insect cell expression system is a popular tool for the expression of heterologous proteins and has an excellent record of producing high levels of biologically active eukaryotic proteins. Insect cells are capable of glycosylation, but their *N*-glycosylation pathway is truncated in comparison with the pathway of mammalian cells. A previous study demonstrated that an immediate early recombinant baculovirus could be used to extend the insect cell *N*-glycosylation pathway by contributing bovine β-1,4 galactosyltransferase (GalT) immediately after infection. Lectin blotting assays indicated that this ectopically expressed enzyme could transfer galactose to an *N*-linked glycan on a foreign glycoprotein expressed later in infection. In the current study, glycans were isolated from total Sf-9 cell glycoproteins after infection with the immediate early recombinant baculovirus encoding GalT, fluorescently conjugated and analyzed by electrophoresis in combination with exoglycosidase digestion. These direct analyses clearly demonstrated that Sf-9 cells infected with this recombinant baculovirus can synthesize galactosylated *N*-linked glycans.

Keywords: **glycosylation, baculovirus, galactosyltransferase, *N*-linked glycans, insect cell glycosylation**

Introduction

The baculovirus-insect cell expression system has an excellent record for producing high levels of biologically active eukaryotic proteins, including glycoproteins. Glycosylation is the most complex post- and co-translational modification performed by eukaryotic cells. *N*-glycosylation of insect cells differs from the pathway found in mammalian cells (Marz et al. 1995; Jarvis et al. 1998). Insect cells can add $Glc_3Man_9GlcNAc_2$ to the appropriate recognition site in polypeptides and convert this precursor to a high mannose glycan by trimming the glucose residues. Some *N*-linked glycans are not further processed, remaining in this high mannose form, whereas others are further processed by the action of one or more class I α-mannosidases to Man_5-$GlcNAc_2$. The next step in the *N*-glycosylation pathway is the addition of *N*-acetylglucosamine by the action of *N*-acetylglucosaminyltransferase I yielding $GlcNAcMan_5GlcNAc_2$. *N*-acetylglucosaminyltransferase I activity has been found in lepidopteran insect cells (Altmann et al. 1993; Velardo et al. 1993). The addition of *N*- acetylglucosamine enables insect cells to trim two more mannose residues to produce $GlcNAcMan_3GlcNAc_2$, an intermediate that can be converted to complex glycans by various glycosyltransferases found in mammalian cells. However, the majority of recombinant glycoproteins produced by insect cells, including the Sf-9 cell line, have $Man_3GlcNAc_2$, with or without fucose added to the innermost GlcNAc as their most highly processed *N*-linked glycan (Marz et al. 1995; Jarvis et al. 1998). Presumably, these structures reflect the absence of high levels of terminal glycosyltransferases, including *N*-acetylglucosaminyltrans-

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ferases, galactosyltransferases (GalT), and sialyltransferases, in insect cells (Marz et al. 1995; Jarvis et al. 1998). In addition, some insect cells have an unusual membrane-bound *β-N*-acetylglucosaminidase, which can convert GlcNAc-$Man_3GlcNAc_2$ to $Man_3GlcNAc_2$ and preclude elongation of the trimmed intermediate (Altmann et al. 1995; Wagner et al. 1996). Hence, it should be possible to enhance the *N*-glycosylation capabilities of the baculovirus-insect cell expression system by increasing the levels of terminal glycosyltransferase activities and/or reducing the levels of *N*-acetylglucosaminidase activity (Jarvis and Finn, 1996; Jarvis et al., 1998).

In this study, we used electrophoresis in combination with exoglycosidase digestion to more directly and definitively examine the structures of glycans derived from total proteins isolated from Sf-9 cells after infection with AcP(+)IEHRGalT.

Materials and Methods

Fermentation. Spodoptera frugiperda (Sf-9) cells were obtained from Gibco BRL (Grand Island, NY). The cells were maintained at 27°C in shake flasks with Sf-900 II SFM (Gibco BRL). A 2 L Biostat A bioreactor (B. Braun Biotech International, Allentown, PA) with a working volume of 1.0 L, equipped with a marine impeller, was seeded with $\sim 5 \times 10^5$ cells/ml. The temperature was controlled at 27°C, the pH between 6.20 and 6.30 and the dissolved oxygen (DO) concentration at $50 \pm 15\%$ air saturation. At a cell density of $\sim 4 \times 10^6$ cells/ml, the cells were transferred to a 10 L BioFlo 3000 bench top fermentor (New Brunswick Scientific Co., Edison, N.J.) running under essentially the same conditions. After reaching an early exponential growth phase (2.5×10^6 cells/ml), the cells were infected with the recombinant baculovirus AcP(+)IE1HRGalT, encoding bovine *β*1,4 GalT under the control of an *ie1* promoter (Jarvis and Finn, 1996), at a multiplicity of infection (MOI) of ~ 10. The cells were harvested after the viability dropped to $\sim 75\%$, centrifuged and stored at −80°C.

Preparation and analysis of glycans. Total glycoprotein was recovered, glycans removed through hydrazinolysis, purified by chromatography and labeled with monopotassium 7-amino-1,3-naphthalenedisulfonic acid, AGA (Aldrich, Milwaukee, WI) as previously described (Wolff et al., 1999). Polyacrylamide gel electrophoresis (PAGE) analysis was on an isocratic 22% gel as previously described (Lee et al., 1991). Capillary electrophoresis (CE) analysis at 25 kV with fluorescent detection (Dionex, Sunnyvale, CA) and exoglycosidase digestion were as described previously (Wolff et al., 1999).

Results and Discussion

The biological role of glycans ranges from trivial to crucial for the development, growth, function, or survival of an organism (Dwek, 1996; Jenkins et al. 1996; Sears and Wong, 1998; Varki, 1993). For the production of human therapeutic recombinant glycoproteins, it is essential to determine if the expression system being used can synthesize the necessary glycan structures required for full biological activity *in vivo*. The degree of glycosylation of recombinant proteins depends mainly on the host cell type and the cell culture environment. The baculovirus-insect cell system has an excellent track record for high level expression of biologically active eukaryotic proteins and this system is used routinely for foreign glycoprotein production (O'Reilly et al. 1992; Jarvis, 1997). However, glycoproteins produced by insect cells usually lack fully elaborated complex *N*-linked glycans (Marz et al. 1995; Jarvis et al. 1998). Most recombinant glycoproteins produced in the baculovirus-insect cell system have highly trimmed glycans consisting of Man^3-$GlcNAc^2$-(+/−)Fuc in place of the fully extended complex glycans found on some native mammalian glycoproteins. Presumably, this difference reflects the absence of high levels of terminal glycosyltransferase activities in insect cells and, in some cases, the presence of a competing, membrane-bound *N*-acetylglucosaminidase activity (Altmann et al. 1995; Wagner et al. 1996).

Previous studies have demonstrated that metabolic engineering can be used to extend the glycoprotein processing capabilities of lepidopteran insect cells (Jarvis and Finn, 1996; Hollister et al. 1998). An immediate early recombinant baculovirus was used to express bovine *β*-1,4 GalT in Sf-9 cells during the immediate early phase of infection. Lectin blotting assays demonstrated that expression of higher levels of active *β*-1, 4 GalT in these cells led to the production of a galactosylated foreign protein, gp64, during the late phase of infection. gp64 is a major baculovirus envelope glycoprotein that acquires mannose and fucose, but no detectable galactose when it is produced during infection of various insect cell lines with wild type baculovirus (Jarvis and Finn, 1995). The lectin blotting assays reported in this study were coupled with competing sugar controls and endoglycosidase treatment to demonstrate specificity. However, lectin blotting analyses are indirect, are not quantitative, and they cannot provide detailed information on glycan structures. Therefore, a more direct and quantitative method was sought to examine the structures of the glycans produced by AcP(+)IEHRGalT-infected Sf-9 cells.

In the present study, we used PAGE and CE to accomplish this goal. PAGE analysis (not shown) of the fluorescently conjugated glycans derived from the total glycoprotein of AcP(+)IE1HRGalT infected Sf-9 cells revealed seven major glycan structures ranging in size from monosaccharide to nonasaccharide. *O*-linked glycans of non-mucin secreting cells are generally shorter (monosaccharides or disaccharides) than *N*-linked glycans. Several investigators have identified non-sialylated *O*-glycans, composed of GalNAc or Gal-GalNAc, on insect cell-derived recombinant glycoproteins (Chen et al. 1991; Grabenhorst et al. 1993; Sugyiama et al. 1993; Thomsen et al. 1990; Wathen et al. 1991). Our results suggest that the

glycans released by hydrazinolysis include both small *O*-linked glycans and larger *N*-linked glycans.

CE of the fluorescently conjugated glycans from uninfected cells show five major peaks that are all α-mannosidase sensitive (Figure 1A). CE of the glycans from AcP(+)IE1HRGalT infected Sf-9 cells revealed more than 20 different structures (Figure 1B). Coinjection of the glycans from AcP(+)IE1HRGalT infected Sf-9 cells with fluorescently conjugated glycan standards (Wolff, 1999) ranging from a monosaccharide to an undecasaccharide, showed that the major glycans of infected cells comigrated between the excess AGA (3.72 min) to just behind the nonamannosylated undecasaccharide (14.41 min), as shown in Figure 1C. This size distribution was confirmed by PAGE analysis (not shown).

The percentage of AGA conjugated glycans having terminal galactose and mannose residues was next determined by exoglycosidase digestion in combination with CE. Digestion with α-mannosidase revealed numerous susceptible glycans throughout the entire electropherogram, corresponding to more than 60% of the total glycans. All peaks migrating after 13 min (Figure 1B), corresponding to larger glycan structures, were degraded by α-mannosidase. A small number of oligosaccharides migrating between 5 and 13 min were also susceptible to both α-mannosidase and β-*N*-acetylhexosaminidase digestion (data not shown). Two glycans (Figure 1B at 9.07 min and 12.27 min) from AcP(+)IE1HRGalT infected Sf-9 cell were β-galactosidase susceptible corresponding to 6.9% of the total glycan pool. The peak eluting at 9.07 min migrated between disaccharide and trisaccharide standards. The size of this galactosylated structure suggests that it is an *O*-linked glycan, most likely Gal-GalNAc, which was previously observed on a recombinant glycoprotein produced by insect cells infected with a conventional recombinant baculovirus that did not encode a GalT (Thomsen et al. 1990). The second galactosylated structure eluted at 12.27 min, earlier than an AGA-labeled biantennary complex *N*-linked nonasaccharide standard (Figures 1B & 1C). Coinjection studies demonstrated that this glycan migrated between a hexa- and heptasaccharide. The glycans isolated from total uninfected Sf-9 cells do not show this galactosylated structure (Figure 1A). Based on our current knowledge of the biosynthetic pathways responsible for *N*-glycosylation, this structure is probably a non-fucosylated, galactosylated heptasaccharide (Gal GlcNAc Man_3 $GlcNAc_2$). In support of this interpretation, the galactosylated glycan also was susceptible to α-mannosidase, suggesting that it has both terminal galactose and terminal mannose residues. Finally, the single galactose residue in this heptasaccharide is probably on the Man α1–3 branch of the glycan because Sf-9 cells have much higher levels of *N*-acetylglucosaminyltransferase I than *N*-acetylglucosaminyltransferase II activity (Altmann et al. 1993).

Thus, our CE studies have directly confirmed the previous conclusion, based on lectin blotting assays, that AcP(+)IEHRGalT-infected Sf-9 cells can produce galactosylated glycans. However, only a small proportion of the glycans

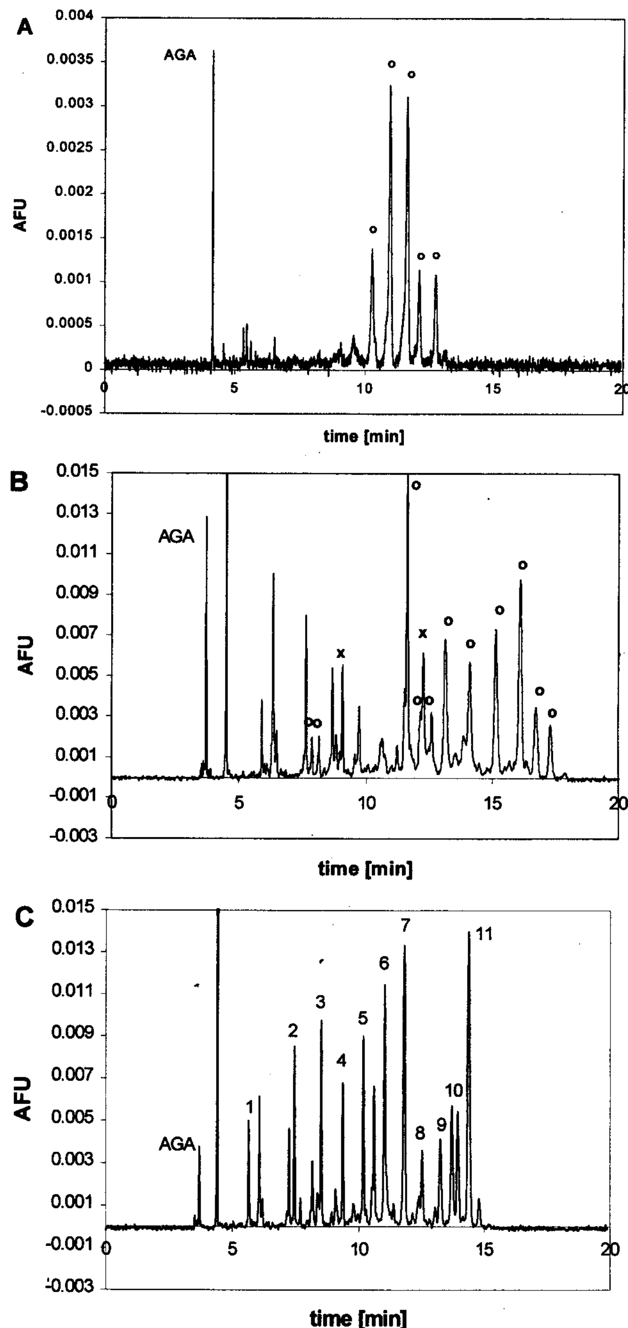

Figure 1. Electropherogram of fluorescently conjugated glycans derived from the total protein of uninfected and AcP(+)IE1HRGalT infected Sf-9 cells. **A**: Uninfected cells, where (○) indicates α-mannosidase sensitivity. **B**: Glycans derived from AcP(+)IE1HRGalT infected Sf-9 cells, where (○) indicates α-mannosidase susceptible peaks and (×) β-galactosidase susceptible peaks. **C**: Sample as shown in B coinjected with a mixture of AGA labeled saccharide standards ranging in size from a monosaccharide (**1**) to a undecasaccharide (**11**) as indicated next to the peaks. The migration times of individual peaks vary due to the presence of buffer salts. The analysis was performed using a fused silica capillary (55 cm in effective length and 50 µm i.d.) at 22 kV. The separation buffer contained 20 mM sodium phosphate pH 3.5. Detection of the analytes was carried out by fluorescent detection (λ_{ex} of 250 nm and λ_{em} of 450 nm).

released from total proteins isolated from AcP(+)IEHRGalT-infected Sf-9 cells contained galactose. The α-mannosidase sensitivity of numerous glycans, particularly the larger ones (Figure 1B), indicated that most of the glycans produced by AcP(+)IEHRGalT-infected Sf-9 cells had terminal mannose residues. In fact, the α-mannosidase susceptible structures represented >60% of the total glycan structures derived from these cells. This probably reflects a major contibution of nongalactosylated glycans from Sf-9 cellular proteins, which were synthesized before the cells were infected and β-GalT was available at high enough levels to functionally extend the host cell *N*-glycosylation pathway. In future studies we will address this issue by analyzing the galactose content of glycans isolated from total proteins of Sf-9 cells stably-transformed to constitutively express bovine β1,4-GalT (Hollister et al. 1998).

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