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Engineering β 1,4-galactosyltransferase I to reduce secretion and enhance *N*-glycan elongation in insect cells

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Abstract

β 1,4-galactosyltransferase (B4GALT1) is a Golgi-resident enzyme that elongates glycoprotein glycans, but a subpopulation of this enzyme is secreted following proteolytic cleavage in its stem domain. We hypothesized that engineering B4GALT1 to block cleavage and secretion would enhance its retention and, therefore, its function. To test this hypothesis, we replaced the cytoplasmic/transmembrane/stem (CTS) domains of B4GALT1 with those from human α 1,3-fucosyltransferase 7 (FUT7), which is not cleaved and secreted. Expression of FUT7-CTS-B4GALT1 in insect cells produced lower levels of secreted and higher levels of intracellular B4GALT1 activity than the native enzyme. We also noted that the B4GALT1 used in our study had a leucine at position 282, whereas all other animal B4GALT1 sequences have an aromatic amino acid at this position. Thus, we examined the combined impact of changing the CTS domains and the amino acid at position 282 on intracellular B4GALT1 activity levels and *N*-glycan processing in insect cells. The results demonstrated a correlation between the levels of intracellular B4GALT1 activity and terminally galactosylated *N*-glycans, *N*-glycan branching, the appearance of hybrid structures, and reduced core fucosylation. Thus, engineering B4GALT1 to reduce its cleavage and secretion is an approach that can be used to enhance *N*-glycan elongation in insect cells.

Keywords

Glycosylation; Glycoengineering; Galactosyltransferase; Glycosyltransferase; Insect cells; Secretion

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1 Introduction

β 1,4-galactosyltransferase I (B4GALT1) is a type II transmembrane glycoprotein that resides in the Golgi apparatus of higher eukaryotic cells (reviewed in Amado et al., 1999; Berger and Rohrer, 2003; Furukawa and Sato, 1999; Kornfeld and Kornfeld, 1985; Qasba et al., 2008). Like other Golgi glycosyltransferases, the structure of B4GALT1 consists of an N-terminal cytoplasmic domain, followed by transmembrane, stem, and C-terminal catalytic domains (Fig. 1; Paulson and Colley, 1989). A major function of B4GALT1 is the addition of β 1,4-linked galactose residues to oligosaccharide acceptors with terminal *N*-acetylglucosamine residues, which is a late elongation step in the *N*-glycan processing pathway (Kornfeld and Kornfeld, 1985).

In a broader context, B4GALT1 presents a potential bottleneck in *N*-glycan sialylation. This is functionally significant because *N*-glycan sialylation enhances the circulatory half-lives of glycoproteins, including therapeutic glycoproteins like recombinant human erythropoietin (hEPO), by blocking recognition and binding of underlying sugars by the Ashwell-Morell and other receptors (Fukuda et al., 1989; Liu et al., 2013). Binding of nonsialylated therapeutic glycoproteins to these receptors results in their removal from the circulation, which reduces their clinical efficacy. Thus, it is desirable to have high levels of B4GALT1 activity in any system used to produce recombinant glycoproteins for therapeutic applications. For this reason, yeast (Bobrowicz et al., 2004; Vervecken et al., 2004), insect (Hollister et al., 1998; Jarvis and Finn, 1996), plant (Bakker et al., 2001; Palacpac et al., 1999) and mammalian (Lee et al., 1989; Weikert et al., 1999) systems have all been engineered to overexpress B4GALT1.

A subpopulation of B4GALT1 is proteolytically cleaved in its stem region, which releases the catalytic domain from the transmembrane anchor and allows it to be secreted (D'Agostaro et al., 1989; Kitazume-Kawaguchi et al., 1999; Weinstein et al., 1987). We recognized that this cleavage event reduces the intracellular pool of B4GALT1 available for *N*-glycan processing, effectively causing a partial loss of function in the Golgi apparatus. This led us to hypothesize that cleavage diminishes the ability of B4GALT1 to elongate *N*-glycans, and thereby impacts the *N*-glycosylation profiles of native and recombinant glycoproteins.

To test this hypothesis, we replaced the cytoplasmic/transmembrane/stem (CTS) domains of B4GALT1 with the CTS domains of human α 1,3-fucosyltransferase 7 (FUT7), which does not undergo cleavage and secretion in CHO-K1 cells (El-Battari et al., 2003). We then examined the impact of this exchange on the intracellular and extracellular levels of B4GALT1 activity. We found that insect cells expressing the FUT7-CTS-B4GALT1 chimera had higher levels of intracellular and lower levels of secreted B4GALT1 activity, as compared to insect cells expressing a native B4GALT1. During the course of this study, we also noted that a previously described bovine B4GALT1 cDNA (Shaper et al., 1986), which we have used widely for insect cell glycoengineering (Ailor et al., 2000; Aumiller et al., 2003; Aumiller et al., 2012; Breitbach and Jarvis, 2001; Hollister et al., 2002; Hollister and Jarvis, 2001; Hollister et al., 1998; Jarvis and Finn, 1996; Jarvis et al., 2001; Mabashi-Asazuma et al., 2013; Seo et al., 2001; Tomiya et al., 2003; Wolff et al., 1999), encodes a

leucine at position 282, whereas an aromatic amino acid residue is conserved at this position in all other animal B4GALT1's. Thus, we also examined the impact of this difference and found that bovine B4GALT1 Leu282 has substantially lower specific activity than the Phe282 variant. Finally, we examined the individual and combined impacts of the polymorphism at position 282 and the CTS exchange on the *N*-glycan processing function of B4GALT1. We found a direct correlation between the levels of intracellular B4GALT1 activity and terminally galactosylated *N*-glycans on a recombinant glycoprotein co-expressed in insect cells. We also found that increased B4GALT1 activity correlated with enhanced *N*-glycan branching, reduced core fucosylation, and the production of hybrid *N*-glycans. We conclude that increasing intracellular levels of B4GALT1 activity enhances *N*-glycan elongation in insect cells and suggest that this approach might also be an effective glycoengineering approach for other recombinant glycoprotein production platforms.

2 Materials and Methods

2.1 Protein alignments

Protein sequences were aligned using Invitrogen Vector NTI version 10.3.0 AlignX with the default settings and Figures were created using Accelrys Discovery Studio Gene 1.5.

2.2 DNA constructions

All PCRs for DNA constructions were performed with high-fidelity Phusion DNA polymerase (New England BioLabs, Ipswich, MA) and the manufacturer's HF buffer. Transfer plasmids encoding the bovine B4GALT1 Leu282 variant were produced by PCR using B4GALT1 CTS SP and B4GALT1 Cat ASP primers (Table 1) with pIE1HRGalT (Hollister et al., 1998) as the template. The gel-purified amplicon was cloned into pCR4® Blunt TOPO® (Invitrogen) according to the manufacturer's instructions. A sequence-verified clone was subsequently used as the template for PCR mutagenesis with Mut SP and Mut ASP primers (Table 1) to obtain a plasmid encoding the bovine B4GALT1 Phe282 variant. Finally, the ORFs encoding the bovine B4GALT1 variants were excised with *Bg*III and *Not*I and subcloned into the same sites of pAcP(+)IE1TV3 (Jarvis et al., 1996).

The sequence encoding the human FUT7 CTS domains (Genbank accession number NP_004470 amino acids 1–48) was codon-optimized for *S. frugiperda* (Nakamura et al., 2000) using the online version of OPTIMIZER (Puigbò et al., 2007). The codon-optimized DNA sequence was then synthesized by PCR with the Syn1, Syn2A, Syn3, Syn4A and Syn5 primers (Table 1). A portion of the reaction was used as the template to amplify the FUT7 CTS coding region with FUT7 CTS SP and FUT7 CTS ASP primers (Table 1). The catalytic domains of the B4GALT1 Phe282 and Leu282 variants (Genbank accession number B4GT1_BOVIN amino acids 125–402) were amplified with B4GALT1 Cat SP and B4GALT1 Cat primers (Table 1). The resulting amplicons were gel-purified, joined by overlap PCR, and the products were gel-purified and subcloned into pCR4® Blunt TOPO®. The ORFs encoding the chimeric FUT7-CTS-B4GALT1's were then excised from sequence-verified clones with *Bg*III and *Not*I and subcloned into the same sites of pAcP(+)IE1TV3 (Jarvis et al., 1996).

The human FUT7 CTS domains were amplified using primers designed to introduce a 5' *Bgl*III site and 3' *Age*I site (see Table 1), with a plasmid encoding the chimeric FUT7-CTS-B4GALT1 as the template. In parallel, the ORF encoding the Phe282 variant of FUT7-CTS-B4GALT1 minus the stop codon was amplified with primers designed to retain a 5' *Bgl*III site and introduce a 3' *Age*I site. Both PCR amplicons were gel-purified, digested with *Bgl*III and *Age*I, and re-purified on a silica spin column. Each was then cloned into the corresponding sites of pIE1HR3-SfGNT-I-eGFP, a plasmid encoding a C-terminally eGFP-tagged form of the *S. frugiperda* N-acetylglucosaminyltransferase I (MGAT1; Geisler and Jarvis, 2012), which had been digested with *Bgl*III and *Age*I to excise the MGAT1 coding sequence. The inserts in the resulting plasmids, which encoded either the human FUT7 CTS domain or FUT7-CTS-B4GALT1 (Phe282) in-frame with a C-terminal eGFP tag, were sequence-verified.

2.3 Isolation of recombinant baculoviruses encoding full-length B4GALT1 or FUT7-CTS-B4GALT1 variants

Recombinant baculoviruses encoding each full-length B4GALT1 variant were produced by homologous recombination with the pAcP(+)/IE1TV3-based transfer plasmids described above plus *Bsu*36I-digested Ac6.9GT viral DNA (Toth et al., 2011), as described previously (Kitts and Possee, 1993). The resulting recombinants lack the endogenous baculoviral cathepsin/chitinase genes and encode a full-length B4GALT1 variant under the control of a baculovirus *ie1* promoter (Guarino and Summers, 1987). The viruses were plaque-purified once and occlusion positive white clones were picked, amplified, and titered, as described previously (Summers and Smith, 1987). The viruses encoding the full-length bovine B4GALT1 Phe282 or Leu282 variants were designated AcIE1BtB4GALT1F282 and AcIE1BtB4GALT1L282, respectively, while those encoding the full-length FUT7-CTS-B4GALT1 Phe282 or Leu282 variants were designated AcIE1HyB4GALT1F282 and AcIE1HyB4GALT1L282, respectively.

2.4 Isolation of recombinant baculoviruses encoding secreted, affinity-tagged B4GALT1 catalytic domains or hEPO

The sequences encoding the secretable catalytic domains of the bovine B4GALT1 Phe282 and Leu282 variants (nts 373–1209 of the ORF) were amplified using solB4GALT1 SP and ASP primers (Table 1) with plasmids encoding the respective full-length enzymes as the templates. The sequence encoding mature human hEPO (nts 82–582 of the ORF) was amplified using hEPO SP1, SP2, and ASP (Table 1) primers with pENTRTM/D-TOPO®-hEPO(-stop) (Mabashi-Asazuma et al., 2013) as the template. The amplicons were gel-purified and cloned into pENTRTM/D-TOPO® (Invitrogen) according to the manufacturer's instructions. The resulting plasmids were sequence verified and used for Gateway® recombination reactions with *Bsu*36I-digested Ac6.9GT viral DNA, as described previously (Toth et al., 2011). The resulting recombinants lack the endogenous viral cathepsin/chitinase genes and encode secretable catalytic domains of the bovine B4GALT1 Phe282 or Leu282 variants or hEPO, each N-terminally tagged with 8xHis and StrepTag®II, under the control of the baculovirus p6.9 promoter (Hill-Perkins and Possee, 1990). All viruses were plaque-purified once and white clones were picked, amplified and titered as described previously (Summers and Smith, 1987). The recombinant baculovirus encoding the secretable catalytic

domains of the bovine B4GALT1 Phe282 or Leu282 variants were designated Acp6.9solB4GALT1F282 and Acp6.9solB4GALT1L282, respectively, while the recombinant virus encoding hEPO was designated Acp6.9hEPO.

2.5 hEPO expression and purification

hEPO was co-expressed with various forms of B4GALT1 by infecting 10^8 logarithmic phase Sf9 cells in ESF 921 medium (Expression Systems, Woodland, CA) with Acp6.9hEPO plus AcIE1BtB4GALT1F282, AcIE1BtB4GALT1L282, AcIE1HyB4GALT1F282, or AcIE1HyB4GALT1L282 at a multiplicity of infection of 2 plaque-forming units/cell for each virus. The cells were gently tumbled at room temperature for 1 h, pelleted at 400 x g for 4 min, the supernatant was aspirated, and the cells were resuspended in 50 ml of fresh medium. The cells were then incubated for another 48 h at 28°C in a shaker incubator, pelleted at 1000 x g for 5 min and the supernatant was further clarified at 100,000 x g in a Beckman Ti45 rotor for 30 min at 4°C. This virus-free supernatant was then dialyzed overnight in 12–14 kDa cutoff dialysis membranes (Spectrum) against dialysis buffer (500 mM NaCl, 10 mM Tris pH 7.5, 0.02% NaN_3). After changing the dialysate, dialysis was extended for another 6 h. Imidazole was added to the dialyzed sample to a final concentration of 10 mM, followed by the addition of 1 ml of HisPur Ni-NTA resin (Thermo) equilibrated with dialysis buffer. The suspension was tumbled for 1 h at RT and the resin was gently pelleted at 50 x g for 5 min. The supernatant was aspirated and the resin was transferred to a 5 ml disposable plastic column (Pierce), washed with 5 ml of dialysis buffer, and washed twice with 5 ml of dialysis buffer containing 30 mM imidazole. Bound proteins were eluted with 2.5 ml of dialysis buffer containing 0.5 M imidazole. Finally, the eluate was buffer-exchanged with 3.5 ml of Tris-buffered saline (TBS) containing 0.02% NaN_3 on a PD-10 column (GE Healthcare, Piscataway, NJ) and concentrated to a final volume of 0.5 ml using Amicon® Ultra-4 filters with a 5 kDa molecular weight cutoff (EMD-Millipore; Merck, Darmstadt, Germany).

2.6 Expression and purification of soluble B4GALT1 variants

The soluble B4GALT1 variants described above were produced by infecting 2×10^8 logarithmic phase Sf9 cells in ESF 921 medium (Expression Systems, Woodland, CA) with the appropriate recombinant baculovirus at a multiplicity of infection of 2 plaque-forming units/cell. After allowing the virus to adsorb for 1 h, cells were pelleted, the supernatant was aspirated, and the cells were resuspended in 100 ml of fresh medium. Cell- and virus-free supernatants were prepared at 48 h post-infection and nickel-affinity purifications were performed as described above, except B4GALT1 was buffer-exchanged against TBS (pH 7.5) with 0.5% ammonium sulfate and 10% glycerol (Fraser et al., 1980).

2.7 Glycosyltransferase assays

Log phase Sf9 cells were seeded in ESF921 medium at a density of 5×10^6 cells/flask and allowed to adhere for 1 h at 28°C. The cells were then infected with recombinant baculoviruses encoding various forms of B4GALT1 at a multiplicity of infection of 2 plaque forming units/cell and the cells were incubated for 1 h at 28°C. The supernatant was aspirated, the cells were washed once with fresh medium, and 5 ml of fresh medium were added. After a 24 h incubation at 28°C, the cell-free supernatants were harvested,

concentrated to a final volume of 540 μ l using Amicon® Ultra-4 filters with a 5 kDa molecular weight cutoff (EMD-Millipore), and supplemented with 60 μ l of 10% BSA in B4GALT1 storage buffer (100 mM MES pH 6.5 containing 0.5% ammonium sulfate, 10% glycerol, 1% Triton X-100, and 10 mM MnCl_2) to stabilize the enzyme. In parallel, the cell pellets were lysed with 1 ml of B4GALT1 storage buffer and the clarified supernatants were supplemented with BSA at a final concentration of 2% to stabilize the enzyme. The concentrated cell-free supernatants and lysates were frozen at -80°C for up to one week before 50 μ l aliquots were assayed in triplicate. In addition to the crude enzyme preparations, each assay contained 50 μ g of chicken ovalbumin (Sigma) in a total volume of 50 μ l of B4GALT1 buffer (100 mM MES pH 6.5, 10 mM MnCl_2 , 1% Triton X-100) as the acceptor substrate and 0.3 μ Ci of uridine diphosphate [$6\text{-}^3\text{H}$]galactose (15 Ci/mMol; American Radiolabeled Chemicals, St. Louis, MO) as the donor substrate. The reaction mixtures were prepared on ice, then incubated for 1 h at 37°C and quenched by the addition of an equal volume of 10% w/v trichloroacetic acid (TCA). The mixtures were spotted onto Whatman GF/D filters, which were then washed for 30 min each with 10% TCA, 5% TCA (twice), and 95% ethanol (twice). The filters were air-dried and then 7 ml of Ultima Gold F scintillation cocktail (Perkin Elmer) were added and radioactivity was measured in a Beckman model LS6500 liquid scintillation counter.

2.8 Live cell co-localization

Sf9 cells were co-transfected with 10 μ g of plasmids encoding the human FUT7 CTS domains or chimeric FUT7-CTS-B4GALT1 (Phe282) C-terminally tagged with eGFP, respectively, plus 10 μ g of pIE1HR3-SfGNT-I-RFP, which encodes an RFP-tagged form of the full length *S. frugiperda* MGAT1 (Geisler and Jarvis, 2012). After transfection, the cells were incubated for 2 days at 28°C , seeded onto concanavalin A-coated microscope dishes (No. 1.5, MatTek, Ashland, MA), allowed to attach for 2 h, and imaged using an Olympus FSX100 microscope. The images were processed with Adobe Photoshop CS3 to remove background and adjust the signal intensities to comparable levels.

2.9 Western and lectin blotting

Samples of various purified hEPO and B4GALT1 preparations were normalized for equal Coomassie brilliant blue staining intensities, and then separated on 12% SDS-PAGE gels and either stained with Coomassie brilliant blue or transferred to PVDF membranes (Millipore). Western blots were performed as described previously; hEPO was detected with an anti-EPO primary antibody (U-CyTech; Mabashi-Asazuma et al., 2013), and 8xHis-tagged B4GALT1 was detected with an anti-penta-His primary antibody (Invitrogen; Toth et al., 2011). The probe used for lectin blotting was *Ricinus communis* agglutinin (RCA-I), which specifically binds to glycans containing terminal $\text{Gal}\beta 1\text{-4GlcNAc}\beta$ (Cummings, 1994), directly conjugated to alkaline phosphatase (EY laboratories). The lectin blots were performed by blocking the PVDF membrane for 1 h in TBS containing 1% (v/v) Tween-20, probing for 1 h with the RCA-I conjugate diluted 1:10,000 in blocking buffer, washing three times with blocking buffer, and developing the signal using a standard method, as described previously (Blake et al., 1984).

2.10 MALDI-TOF mass spectrometry

The 8xHis-tagged form of hEPO was expressed in the presence or absence of recombinant baculoviruses encoding a specific B4GALT1 variant and the product was harvested and affinity-purified, as described above. Samples of the purified hEPO preparations were reduced, alkylated, and trypsinized, then total *N*-glycans were released by exhaustive digestion with PNGaseF (New England Biolabs). The released *N*-glycans were purified, derivatized, and analyzed by MALDI-TOF-MS as described previously (Mabashi-Asazuma et al., 2014; Toth et al., 2014). Structures were assigned to peaks based on knowledge of the *N*-glycan processing pathway in glycoengineered insect cells. Quantification involved dividing the combined peak intensities from isotopic clusters of individual permethylated *N*-glycan structures by the total intensity of all annotated *N*-glycan peaks.

3 Results

3.1 Designing a chimeric FUT7-CTS-B4GALT1

The first step in designing a construct encoding a chimeric form of B4GALT1 containing the non-cleavable FUT7 CTS domains was to delineate the relevant functional domains of these enzymes. Glycosyltransferase CTS domains are poorly conserved among species, but their catalytic domains are relatively highly conserved and, therefore, more readily distinguished (Breton et al., 2001). We aligned each enzyme with its respective orthologs and found that the more highly conserved amino acid sequences in B4GALT1 and FUT7 began at Leu131 (bovine sequence numbering, Fig. 2) and Ile47 (human sequence numbering, Fig. 3), respectively. We assigned these as the approximate N-termini of the catalytic domains and presumed that each extended to the C-termini of the respective full-length enzymes. We also noted that the proteolytic cleavage sites previously identified in B4GALT1 (D'Agostaro et al., 1989; Kitazume-Kawaguchi et al., 1999; Weinstein et al., 1987) are located at positions 79 and 106, which clearly precede the approximate N-terminus of the catalytic domain at position 131 (Fig. 1). This information was used to design an expression construct encoding a chimeric form of B4GALT1 consisting of the N-terminal FUT7 CTS domains (human FUT7 amino acids 1 to 46) joined to the conserved B4GALT1 catalytic domain (bovine B4GALT1 amino acids 131 to 402) through a short, flexible linker consisting of two additional amino acids from FUT7 and six additional amino acids from B4GALT1 (Fig. 1).

Our animal B4GALT1 sequence alignments also highlighted a polymorphism in the bovine B4GALT1 encoded by a previously described cDNA (Shaper et al., 1986), which has been used widely to glycoengineer the baculovirus-insect cell system (Ailor et al., 2000; Aumiller et al., 2003; Aumiller et al., 2012; Breitbach and Jarvis, 2001; Hollister et al., 2002; Hollister and Jarvis, 2001; Hollister et al., 1998; Jarvis and Finn, 1996; Jarvis et al., 2001; Mabashi-Asazuma et al., 2013; Seo et al., 2001; Tomiya et al., 2003; Wolff et al., 1999). This B4GALT1 variant has a leucine at position 282, in striking contrast to other animal B4GALT1's, including bovine B4GALT1 sequences reported by other groups (D'Agostaro et al., 1989; Narimatsu et al., 1986), which all have an aromatic amino acid residue at this position (Figs. 4A and 2). In fact, except for the enzyme encoded by the cDNA described by Shaper et al. (Shaper et al., 1986), all other animal B4GALT1's have either a phenylalanine

or tyrosine residue at this position (Fig. 2). The conservation of these residues in most other B4GALT1's suggests that the substitution of a leucine residue for Phe282 in the bovine B4GALT1 variant described by Shaper and coworkers (Shaper et al., 1986) might have a functional impact.

3.2 B4GALT1 Phe282 and Leu282 enzyme activities

To examine this possibility, we expressed the Leu282 and Phe282 variants of a soluble, secreted form of B4GALT1 in insect cells and compared their activities using an *in vitro* assay. SDS-PAGE and immunoblotting showed that both variants were secreted from insect cells at similar levels (Fig. 4A), indicating that the polymorphism did not detectably impact their expression levels or physical stability. However, enzyme activity assays of crude insect cell culture supernatants showed that B4GALT1 Phe282 had about 6.5-fold higher activity than the Leu282 variant (Fig. 4B). We subsequently purified 8x-His-tagged, secreted versions of both B4GALT1 variants to >95% homogeneity (Fig. 4C) and found that B4GALT1 Phe282 had about 5-fold higher specific activity than the Leu282 variant (Fig. 4D). These results support the idea that conservation of an aromatic amino acid residue at position 282 of B4GALT1 impacts its structure and function.

3.3 Impact of FUT7 CTS on B4GALT1 retention

Having explored the impact of the polymorphism in the bovine B4GALT1 variant encoded by the cDNA described by Shaper and coworkers (Shaper et al., 1986), we refocused on the hypothesis that replacing the native CTS domains with the CTS domains of human FUT7 would reduce or eliminate B4GALT1 cleavage and secretion and, therefore, increase the amount of enzyme activity retained by the cell. We also chose to extend our original plan to include an assessment of the individual and combined effects of the polymorphism and CTS domain swap on intracellular levels of B4GALT1 activity.

Insect cells were infected with recombinant baculoviruses encoding various forms of B4GALT1 and the enzyme activity levels in crude cell-free supernatants and cell lysates were compared using an *in vitro* assay. The results confirmed that expression of bovine B4GALT1 Phe282 induced higher levels of intracellular activity than expression of the Leu282 variant (Fig. 5A). In addition, expression of both bovine B4GALT1 variants, when engineered to have the FUT7 instead of the native B4GALT1 CTS domains, induced higher levels of intracellular (Fig. 5A) and lower levels of secreted (Fig. 5B) enzyme activities than the corresponding native B4GALT1 variants. Thus, replacing the native B4GALT1 CTS with the FUT7 CTS domains increased the levels of intracellular enzyme activity by reducing secretion, as expected. In combination, engineering bovine B4GALT1 to have both the phenylalanine residue at position 282 and the FUT7 CTS increased the level of intracellular enzyme activity by nearly 6-fold, relative to the Leu282 variant with the native B4GALT1 CTS domain (Fig. 5A).

3.4 Golgi targeting by FUT7-CTS

Golgi-resident glycosyltransferases, such as B4GALT1, are targeted to the Golgi apparatus by signals within their CTS domains (Reynders et al., 2011; Tu and Banfield, 2010). In previous studies, the bovine B4GALT1 Leu282 variant contributed to *N*-glycan processing,

indicating that its native CTS domains can mediate at least partial Golgi localization in insect cells (Breitbach and Jarvis, 2001; Hollister et al., 1998; Jarvis and Finn, 1996). To determine if the isolated FUT7 CTS domains can drive Golgi localization, we performed living cell co-localization experiments using a FUT7-CTS-eGFP reporter and an RFP-tagged form of the full-length *S. frugiperda* MGAT1 (also known as Sf-GNT-I), which is an endogenous Sf9 cell Golgi marker (Geisler and Jarvis, 2012). The results revealed substantial overlap between the green and red fluorescence observed in insect cells co-expressing these two proteins (Fig. 6A). These results, together with the punctate staining pattern, which is typical of the insect cell Golgi apparatus, strongly suggested that the FUT7 CTS domains had targeted eGFP to the Golgi compartment. To determine if the FUT7 CTS domains could also target the B4GALT1 catalytic domain to the Golgi compartment, we constructed a C-terminally eGFP-tagged form of FUT7-CTS-B4GALT1 and examined its distribution in living cell co-localization experiments with RFP-tagged Sf-GNT-I. Again, the results revealed substantial overlap between the green and red fluorescence observed in Sf9 cells expressing both proteins (Fig. 6B), indicating that FUT7-CTS-B4GALT1 is at least partially localized in the Golgi apparatus.

3.5 Impact of intracellular B4GALT1 activity levels on N-glycan processing in Sf9 cells

At this point in the study, we turned our attention to the impact of intracellular B4GALT1 activity levels on *N*-glycan processing in Sf9 cells. To address this issue, we produced 8x-His-tagged hEPO in Sf9 cells co-expressing the individual full-length B4GALT1 variants used for the *in vitro* enzyme activity assays shown in Fig. 5. We then purified the recombinant hEPO from the cell-free media and analyzed samples of each by SDS-PAGE. Previous studies have shown that Sf9 cells produce recombinant glycoproteins with mainly paucimannose-type *N*-glycans plus a small subpopulation of hybrid structures with a single terminal *N*-acetylglucosamine residue (Altmann et al., 1999; Geisler and Jarvis, 2009; Harrison and Jarvis, 2006; März et al., 1995). In addition, previous studies have shown that Sf9 cells expressing bovine B4GALT1 can produce mono-antennary, terminally galactosylated *N*-glycans (Hollister et al., 1998; Jarvis and Finn, 1996). Thus, we expected hEPO would be terminally galactosylated in Sf9 cells expressing the various forms of B4GALT1 and that the relative degree of terminal galactosylation would reflect the relative levels of intracellular B4GALT1 activity. Furthermore, because hEPO is a small *N*-glycoprotein containing up to three *N*-glycans, we expected the addition of terminal galactose residues to alter its electrophoretic mobility. Thus, we initially used an electrophoretic mobility shift assay to indirectly assess the relative ability of various forms of B4GALT1 to extend *N*-glycan processing in insect cells and add terminal galactose residues to the insect-type *N*-glycans on hEPO. The results demonstrated that there was a direct correlation between an electrophoretic mobility shift in hEPO (Fig. 7, A and B) and the level of intracellular enzyme activity associated with each B4GALT1 variant (Fig. 5B). To verify that the most slowly migrating form(s) of hEPO actually had terminally galactosylated *N*-glycans, we performed lectin blotting assays with RCA-I, which specifically binds to glycans containing terminal Gal β 1-4GlcNAc β (Cummings, 1994). The results showed that RCA-I only stained the hEPO produced by insect cells co-expressing B4GALT1 (Fig. 7C). In addition, they showed that RCA-I stained only the most slowly migrating form(s) of hEPO, indicating this form has terminally galactosylated *N*-glycans.

Finally, and most importantly, the data demonstrated a direct correlation between the intensity of RCA-I staining of this upper band (Fig. 7C) and the level of intracellular enzyme activity induced by the various forms of B4GALT1 (Fig. 5A).

3.6 Impact of modified B4GALT1 on N-glycan processing in glycoengineered insect cells

Next, we examined the impact of increasing levels of intracellular B4GALT1 activity on *N*-glycan processing in SfSWT-6, a glycoengineered insect cell line that expresses a suite of mammalian glycosyltransferases including MGAT1, MGAT2, and B4GALT1 (Mabashi-Asazuma et al., 2013). SfSWT-6 cells were particularly useful for this purpose because they have very low levels of B4GALT1 activity and produce only very small proportions of terminally galactosylated *N*-glycans. Accordingly, these cells produce high proportions of human-type *N*-glycans with terminal *N*-acetylglucosamine residues, which are acceptors for B4GALT1 and sensitive indicators of B4GALT1 function. hEPO was produced in SfSWT-6 cells alone or with a co-expressed B4GALT1 variant, purified from the cell-free media, and the *N*-glycans were enzymatically removed, permethylated, and profiled by MALDI-TOF-MS. The annotated MALDI-TOF-MS profiles are shown in Fig. 8 and a quantitative analysis of those data is shown in Fig. 9. The results demonstrated that the levels of terminally galactosylated *N*-glycans recovered from hEPO (Figs. 8 and 9) were directly proportional to the levels of intracellular enzyme activity induced by the co-expressed form of B4GALT1 (Fig. 5A). In addition, the levels of *N*-glycans with unsubstituted terminal *N*-acetylglucosamine residues (Figs. 8 and 9) were inversely proportional to the levels of intracellular activity induced by the co-expressed form of B4GALT1 (Fig. 5A). The MALDI-TOF-MS profiles also demonstrated that the levels of monoantennary and paucimannose *N*-glycans were inversely proportional and the levels of biantennary *N*-glycans were directly proportional to the levels of intracellular enzyme activity induced by the co-expressed B4GALT1 variants (Figs. 8, 9, and 5A). Due to this latter impact of intracellular B4GALT1 activity levels on *N*-glycan processing in insect cells, we also examined the prevalence of fucosylated and hybrid *N*-glycans. The results revealed a slight inverse correlation between the levels of fucosylated structures and intracellular B4GALT1 activity (Figs. 5A, 8, and 9C). In addition, no hybrid *N*-glycans were detected on the hEPO produced by SfSWT-6 cells expressing no additional B4GALT. However, hybrid *N*-glycans were present on hEPO produced by SfSWT-6 cells expressing additional B4GALT1, at levels that were directly proportional to their relative activities.

4 Discussion

Proteolytic cleavage in the stem region of most Golgi glycosyltransferases, including fucosyltransferases (Grabenhorst et al., 1998), *N*-acetylglucosaminyltransferases (Nakahara et al., 2006), *N*-acetylgalactosaminyltransferases (El-Battari et al., 2003), xylosyltransferases (Ponighaus et al., 2010), galactosyltransferases (Cho et al., 1997; D'Agostaro et al., 1989), and sialyltransferases (El-Battari et al., 2003; Weinstein et al., 1987), produces subpopulations of soluble, catalytic domains of each enzyme that retain activity. These cleavages reduce Golgi *N*-glycan processing function by releasing subpopulations of elongating enzymes from Golgi membranes for secretion from the cell (Cho and Cummings, 1997; Grabenhorst et al., 1998; Zhu et al., 1998). This partial loss of processing function is

undesirable for biotechnological applications, for which it is important to achieve a high efficiency of elongation leading to terminal sialylation.

B4GALT1 is required to produce sialylated *N*-glycans, which enhance the clinical efficacy of recombinant glycoproteins used for human therapeutic applications. Accordingly, B4GALT1 has been engineered in many different systems used for recombinant glycoprotein production, as noted above. These previous efforts involved knocking-in B4GALT1 genes and/or engineering the native CTS domains to influence localization of the enzyme to the Golgi compartment. In a related study, it was shown that cleavage and secretion of FUT6, an enzyme that produces Lewis^X and sialyl-Lewis^X *O*-glycans, can be essentially eliminated by replacing its CTS domains with those of human FUT7, which is not cleaved and secreted (El-Battari et al., 2003; Grabenhorst and Conradt, 1999). However, there have been no efforts to enhance B4GALT1 activity by preventing the partial loss of function imposed by its cleavage and secretion. Thus, the overall purpose of the present study was to determine how replacing the native B4GALT1 CTS domains with the FUT7 CTS domains would impact the intracellular levels of B4GALT1 activity and *N*-glycan processing in insect cells.

To design the expression construct needed for this study, we delineated the CTS and catalytic domains in multiple amino acid sequence alignments of FUT7's and B4GALT1's from various species. The results indicated that the FUT7 CTS domains extended from the N-terminus to about Thr46 and the B4GALT1 catalytic domain extended from about Leu131 to the C-terminus of these enzymes. These results were consistent with the previously reported bovine B4GALT1 structure determined by crystallography (Ramakrishnan et al., 2002) and a previous definition of the FUT7 CTS domains by other investigators (Grabenhorst and Conradt, 1999).

Our B4GALT1 alignment also highlighted a polymorphism between the first (Shaper et al., 1986) and two subsequently reported (D'Agostaro et al., 1989; Narimatsu et al., 1986) bovine B4GALT1 sequences. Specifically, the alignment shows the B4GALT1 sequence reported by Shaper and coworkers (Shaper et al., 1986) has a leucine, while the others have a phenylalanine residue at position 282 (Fig. 2). While this and other polymorphisms have been noted before (D'Agostaro et al., 1989; Narimatsu et al., 1986), none had been investigated. We were particularly intrigued by the polymorphism at position 282 because we had used the cDNA encoding the B4GALT1 Leu282 variant for many previous insect glycoengineering projects (Ailor et al., 2000; Aumiller et al., 2003; Aumiller et al., 2012; Breitbach and Jarvis, 2001; Hollister et al., 2002; Hollister and Jarvis, 2001; Hollister et al., 1998; Jarvis and Finn, 1996; Jarvis et al., 2001; Mabashi-Asazuma et al., 2013; Seo et al., 2001; Tomiya et al., 2003; Wolff et al., 1999), our alignment showed that an aromatic amino acid residue is highly conserved at this position in all other animal B4GALT1 sequences (Fig. 2), and the bovine genome also encodes the Phe282 variant (Zimin et al., 2009). Thus, we wondered if this change had a structural and/or functional impact. We found that B4GALT1 Phe282 has an average of ~6.5-fold more activity than the B4GALT1 Leu282 variant (Fig. 3).

The results of subsequent experiments confirmed our original hypothesis that replacing the native B4GALT1 CTS domains with those from human FUT7 would increase intracellular enzyme activity levels by reducing cleavage in the stem domain and secretion of the catalytic domain (Fig. 5). There was a clear correlation between increased intracellular and decreased extracellular activity, which supported the conclusion that the former was due to reduced cleavage and secretion of the chimeric form of B4GALT1. It is also possible that the increased intracellular activity might reflect other effects of the CTS domain swap, such as higher transcription and/or translation, which were not investigated here. However, this would not explain the impact on B4GALT1 secretion, which was reduced by about 78%, from an average of 12.5% to an average of 2.7%, when the native B4GALT1 CTS was replaced with the FUT7 CTS domains.

In order to function in *N*-glycan processing, the B4GALT1 catalytic domain must be targeted to the Golgi apparatus (Colley et al., 1992; Nilsson et al., 1991; Roth, 1987; Roth et al., 1986; Watzele et al., 1991). The intracellular distribution of human FUT7 had not been determined. However, it was reasonable to assume the FUT7 CTS domains would target the B4GALT1 catalytic domain to the Golgi apparatus considering that human FUT7 functions in the final step of sialyl-Lewis^X biosynthesis. Moreover, previous studies suggest that the transmembrane domain targets native FUT7 to the Golgi or trans-Golgi compartment (Natsuka et al., 1994; Sasaki et al., 1994) and the FUT7 CTS was previously shown to target eGFP (Zerfaoui et al., 2002) and a chimeric protein complex (Kohler and Bertozzi, 2003) to the mammalian cell Golgi apparatus. We confirmed and extended these results by demonstrating that the FUT7 CTS can target eGFP and the B4GALT1 catalytic domain to the insect cell Golgi apparatus (Fig. 6).

We subsequently sought to determine the impact of higher levels of intracellular B4GALT1 activity on *N*-glycan processing in insect cells. We examined terminal galactosylation of recombinant hEPO produced by insect cells co-expressing no heterologous B4GALT1 or B4GALT1 variants that induced different levels of intracellular B4GALT1 activity (Fig. 6A). Sf9 cells have no detectable B4GALT1 activity (Jarvis and Finn, 1996), but can produce monoantennary *N*-glycans with a terminal *N*-acetylglucosamine residue that is an acceptor substrate for B4GALT1 (Altmann et al., 1993; Hollister et al., 2002). Our results revealed a direct relationship between the levels of hEPO galactosylation and intracellular levels of B4GALT1 activity (Fig. 7). Finally, we extended these results by examining the impact of increased levels of intracellular B4GALT1 activity on *N*-glycan processing in a glycoengineered insect cell line, SfSWT-6 (Mabashi-Asazuma et al., 2013). These cells encode and express mammalian MGAT1 and MGAT2, but only low levels of B4GALT1, which enables them to produce high proportions of bi-antennary *N*-glycans with terminal *N*-acetylglucosamine residues. In this context, SfSWT-6 cells were an excellent tool for analyzing the impact of increased intracellular B4GALT1 activity on *N*-glycan processing in insect cells with a partially humanized processing pathway. Again, we found there was a direct relationship between the levels of intracellular B4GALT1 activity and the proportions of terminally galactosylated *N*-glycans on recombinant hEPO (Figs. 8 and 9A). We also found an inverse relationship between the levels of B4GALT1 activity and the proportions of *N*-glycans with terminal *N*-acetylglucosamine residues on recombinant hEPO.

Higher levels of intracellular B4GALT1 activity correlated with the production of a higher proportion of biantennary *N*-glycans and lower proportions of monoantennary and paucimannose *N*-glycans (Figs. 8 and 9B). This was somewhat unexpected because MGAT1, MGAT2, and FDL are the enzymes considered to influence the trimming versus elongation branch point in the insect cell *N*-glycan processing pathway (Geisler and Jarvis, 2012). One likely explanation for the decreasing proportions of paucimannose *N*-glycans observed with increasing levels of intracellular B4GALT1 activity is that the addition of galactose protects the α 3-branch terminal *N*-acetylglucosamine on MGn (Fig. 10) from cleavage by FDL (Altmann et al., 1995; Geisler et al., 2008). The α 6-branch of this protected intermediate can then be elongated by MGAT2 to produce GnGal (Fig. 10), which would lead to a higher proportion of biantennary *N*-glycans at the expense of the monoantennary structures. This model suggests that the final structures of the *N*-glycans produced by insect cells are determined not only by the relative levels of MGAT1, MGAT2, and FDL, but also by the relative levels of downstream glycosyltransferases, such as B4GALT1. A previous report indicating that B4GALT1 expression in insect cells reduces the abundance of paucimannose glycans supports this model (Ailor et al., 2000).

In addition to the impact on the production of paucimannose, monoantennary, and biantennary *N*-glycans, we observed correlations between higher levels of intracellular B4GALT1 activity, slight reductions in the level of core fucosylated *N*-glycans, and higher proportions of hybrid *N*-glycans (Figs. 8 and 9C). The impact on core fucosylation might reflect enhanced galactosylation of MGn (boxed in Fig. 10), which is the preferred substrate for the core α 6-fucosyltransferase of Sf9 cells (Longmore and Schachter, 1982; Mabashi-Asazuma et al., 2014; Paschinger et al., 2005; Voynow et al., 1991). Higher levels of intracellular B4GALT1 activity could reduce fucosylation by depleting this substrate. Similarly, the impact on hybrid *N*-glycan production might reflect a higher level of galactose addition to the terminal *N*-acetylglucosamine residue of M5Gn (Fig. 10), which is the preferred substrate for Golgi α -mannosidase II (Shah et al., 2008; Zhong et al., 2008). In this case, higher levels of intracellular B4GALT1 would deplete the preferred substrate for Golgi α -mannosidase II trimming, which would increase the proportion of hybrid *N*-glycan structures.

Finally, the results of this study provide insights that might help to explain the results of another study published while the present manuscript was in preparation (Hesselink et al., 2014). This study examined the production of galactosylated *N*-glycans in transgenic tobacco plants expressing native B4GALT's from different species and B4GALT1 variants engineered to have altered CTS domains. The results showed that the prevalence of *N*-glycans with terminal *N*-acetylglucosamine residues, hybrid *N*-glycans with terminal galactose residues, and biantennary *N*-glycans with terminal galactose residues varied according to the specific B4GALT variant used to transform tobacco plants. The authors suggested that the impact of different B4GALT variants on *N*-glycan processing reflected differences in their subcellular distributions, which were determined by their CTS domains. However, this speculation was not supported by an analysis of the subcellular distributions of the different B4GALT variants. Our results suggest that the differences in *N*-glycan processing observed in this study might be explained by differences in intracellular levels of

B4GALT1 activity induced by the different B4GALT1 variants. This interpretation is supported by the fact that the primary sequences of the B4GALT1 variants, as well as codon usages and the 5'- and 3'-UTR sequences of the expression constructs used in this study were all quite different and, therefore, likely to induce different levels of intracellular B4GALT1 activity. However, because these activity levels were not determined, our interpretation is also speculative and remains open to further investigation.

5 Conclusions

In conclusion, we suggest that cleavage and secretion of glycosyltransferases can be reduced by replacing their native CTS domains with the FUT7 CTS domains. This, in turn, should enhance the intracellular levels these enzyme activities and their *N*-glycan processing functions, thereby enhancing the elongation of *N*-glycans in insect and, perhaps, other recombinant glycoprotein production platforms.

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Abbreviations

AcMNPV	<i>Autographa californica</i> multiple nucleopolyhedrosis virus
CTS	cytoplasmic tail/transmembrane domain/stem region
FDL	Fused Lobes, the insect cell <i>N</i> -glycan processing β - <i>N</i> -acetylglucosaminidase
FUT7	GDP-fucose: β -galactoside: α 1,3-fucosyltransferase 7
B4GALT1	UDP-galactose: β - <i>N</i> -acetylglucosaminide: β 1,4-galactosyltransferase 1
MGAT	α -mannoside: β - <i>N</i> -acetylglucosaminyltransferase
hEPO	human erythropoietin
RCA	<i>Ricinus communis</i> agglutinin

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Highlights

B4GALT1 is a resident Golgi enzyme with a key role in N-glycan elongation

The natural process of B4GALT1 cleavage and secretion attenuates its Golgi function

B4GALT1 was engineered to block its secretion and enhance its specific activity

Engineered B4GALT1 had higher intracellular activity levels and Golgi localization

Engineering B4GALT1 for increased intracellular activity enhanced *N*-glycan elongation

Fig. 1.

Functional domains of glycosyltransferases. The functional domains of B4GALT1, FUT7, and chimeric FUT7-CTS-B4GALT1 are shown, including the cytoplasmic tail (C), transmembrane (T), stem (S), and catalytic (B4GALT1 and FUT7) domains. The previously reported proteolytic cleavage sites in the stem region of B4GALT1 are marked by the scissors. The lengths of the bars are to scale and differential shading is used to indicate the origin of each domain in the chimeric FUT7-CTS-B4GALT1 protein used in this study.

Fig. 2.

Multiple sequence alignment of bovine (*B. taurus*, P08037.3) B4GALT family members with cabbage looper (*T. ni*, AAT11926.1) and fruit fly (*D. melanogaster*, NP_610946.1) galactosyltransferases, and human (*H. sapiens*, NP_001488.2), clawed frog (*X. tropicalis*, NP_001016664.1), zebrafish (*D. rerio*, NM_001077259.2), chicken (*G. gallus*, NP_990534.1), yellow sea squirt (*C. intestinalis*, XP_002129444.2), and Oikopleura (*O. dioica*, CBY06895.1) B4GALT1's. The arrow marks the start of the catalytic domain at Leu131 (bovine sequence numbering).

Fig. 3.

Multiple sequence alignment of human (*H. sapiens*, NP_004470.1), rat (*R. norvegicus*, EDL93588.1), chicken (*G. gallus*, BAB82491.1), zebrafish (*D. rerio*, XP_002667770.2), clawed frog (*X. tropicalis*, NP_001165371.1), coelacanth (*L. chalumnae*, XP_005994407.1), and elephant shark (*C. millii*, XP_007898545.1) FUT7's. The arrow marks the start of the catalytic domain at Ile47 (human sequence numbering).

Fig. 4.

Activities of recombinant bovine B4GALT1 Phe282 and Leu282 expressed in insect cells. Sf9 cells were infected with recombinant baculoviruses encoding 8X-His-tagged secretable forms of the bovine B4GALT1 Phe282 and Leu282 catalytic domains. Cell-free supernatants were harvested after infection and the B4GALT1's were analyzed by (A) SDS-PAGE with Coomassie brilliant blue staining or western blotting and (B) *in vitro* assays of relative B4GALT1 activities in crude extracts. The 8x-His-tagged forms of B4GALT1 were affinity-purified and normalized samples were analyzed by (C) SDS-PAGE with Coomassie brilliant blue staining or western blotting and (D) *in vitro* assays of specific B4GALT1 activities in the purified enzyme preparations.

Fig. 5. Intracellular and secreted enzyme activities induced by expressing various forms of B4GALT1 in insect cells. The Figure shows (A) the levels of enzyme activity in crude cell lysates and (B) the percentages of enzyme activity in crude cell-free media isolated Sf9 cells infected with recombinant baculoviruses encoding various forms of B4GALT1.

Fig. 6.

Golgi targeting by FUT7 CTS in insect cells. Sf9 cells were co-transfected with expression plasmids encoding GFP-tagged FUT7 CTS or FUT7-CTS-B4GALT1 (Phe282) and an RFP-tagged form of *S. frugiperda* MGAT1 (Sf MGAT1). The first column shows the green fluorescence induced by expression of the eGFP-tagged (A) FUT7 CTS or (B) FUT7-CTS-B4GALT1 (Phe282). The second column shows the red fluorescence induced in the same cells by co-expression of *S. frugiperda* MGAT1. The third column shows the overlap between the green and red fluorescence. The fourth column shows a phase-contrast image of the co-transfected Sf9 cells.

Fig. 7.

N-glycan processing in insect cells expressing various forms of B4GALT1. Sf9 cells were co-infected with recombinant baculoviruses encoding an 8x-His tagged form of hEPO and individual forms of B4GALT1. The cell-free media were harvested and hEPO was affinity purified and normalized samples were analyzed by SDS-PAGE with (A) Coomassie brilliant blue staining, (B) western blotting with anti-hEPO antibody, and (C) lectin blotting with RCA-I.

Fig. 8.

N-glycan processing in glycoengineered insect cells expressing various forms of B4GALT1. SfSWT-6 cells were co-infected with recombinant baculoviruses encoding an 8x-His tagged form of hEPO plus baculoviruses encoding (A) no B4GALT1, (B) bovine B4GALT1 Leu282, (C) FUT7-CTS-B4GALT1 Leu282 (D), B4GALT1 Phe282 (E), and FUT7-CTS-B4GALT1 Phe282. hEPO was affinity-purified from the cell-free media harvested from each infected cell culture, used to produce permethylated *N*-glycans, and samples of those preparations were used for MALDI-TOF-MS analysis, as described in Experimental Procedures. Peaks matching *N*-glycans produced by glycoengineered insect cells are annotated with predicted structures and calculated molecular weights.

Fig. 9.

Quantitative analysis of MALDI-TOF-MS results. The relative proportions of various types of *N*-glycans were calculated by dividing the combined peak intensities from isotopic clusters of the relevant *N*-glycans (Fig. 9) by the total intensity of all annotated *N*-glycan peaks. (A) Percentages of *N*-glycans containing galactose (left, dark grey) or terminal, unsubstituted *N*-acetylglucosamine (right, light grey) residues, (B) percentages of biantennary (left, dark grey), monoantennary (middle, light grey), and paucimannose *N*-glycans (right, medium grey), and (C) percentages of fucosylated (left, dark grey) and hybrid *N*-glycans (right, light grey) on the recombinant hEPO produced by SfSWT-6 cells co-expressing various forms of B4GALT1. Monoantennary *N*-glycans are defined as glycans with one *N*-acetylglucosamine residue on either the upper or lower branch mannose residues of the *N*-glycan core. Hybrid *N*-glycans are defined as glycans with one or two mannose residues attached to the upper branch mannose residue of the *N*-glycan core.

Fig. 10.

An expanded model of the branch point in the insect cell *N*-glycan processing pathway. The early steps in the insect cell *N*-glycan processing pathway produce a pentamannose *N*-glycan with a terminal *N*-acetylglucosamine residue on the α 1,3 branch, M5Gn. B4GALT1 competes with Golgi α -mannosidase II for this substrate and can transfer a galactose residue to the terminal *N*-acetylglucosamine. The reaction product is a less preferred substrate of Golgi α -mannosidase II and, therefore, higher levels of B4GALT1 activity increase the prevalence of hybrid *N*-glycans. Moreover, B4GALT1 competes with core fucosyltransferase(s) for its preferred *N*-glycan substrate (MGn, boxed) and, as a result, higher levels of B4GALT1 activity also decrease the prevalence of core fucosylated *N*-glycans. Finally, B4GALT1 competes with FDL for *N*-glycan substrates with a terminal *N*-acetylglucosamine residue on the α 1,3 branch (MGn and GnGn) and, therefore, higher levels of B4GALT1 activity lead to increased branching. In summary, the net outcome of the insect cell *N*-glycan processing pathway is unexpectedly influenced by the relative intracellular levels of a late processing enzyme, B4GALT1, in context of the relative levels of MGAT1, MGAT2, FDL, Golgi α -mannosidase II, and core fucosyltransferases, which are more obvious functions determining the outcome of this pathway. Symbols are drawn according to guidelines of the Consortium for Functional Glycomics Nomenclature Committee.

Table 1

Sequences of primers used in this study

Primer	Sequence (5' -> 3')
B4GALT1 CTS SP	AGATCTCACCATGAAGTTTCGGGAG
B4GALT1 Cat ASP	CTAGCTCGGCGTCCCGAT
Mut SP	AAGTTTGGATTAGCCTACCTT
Mut ASP	TAGGCTAAATCCAAACTTATCC
EPO SP1	CACCGAAAACCTGTACTTCCAGGGAGCCCCACCACGCCTCATCTG
EPO SP2	CACCGAAAACCTGTACTTCC
EPO ASP	TCATCTGTCCCCTGTCCTGC
Syn1	AGATCTCACCATGAACAATGCAGGACACGGACCCACTCGTAGATTACGCG
Syn2A	ACAAGGCCACGCCGCAACACGCCCAAGCCGCGTAATCTACGAGTGGGT
Syn3	GTTGGCCGCGCTGGCCTTGTGGCAGCGTTGTGGTTGCTATGGTTGCTTG
Syn4A	TGGGGCGCAGGGGTTCTCTGCGGGCAGAGCCAAGCAACCATAGCAACCAC
Syn5	AGAGGAACCCCTGCGCCCCAACCCACGATAACCTCCACCACAACACGCTCGCTG
FUT7 CTS SP	AGATCTCACCATGAACAATGC
FUT7 CTS ASP	CAGCGAGCGTGTGTGGT
B4GALT1 Cat SP	TCCACCACAACACGCTCGC
solB4GALT1 SP	CACCGAAAACCTGTACTTTCAAGGCTCCACCACAACACGCTCG
solB4GALT1 ASP	CTAGCTCGGCGTCCCGAT
Hs FT7 CTS f FP SP	GTCAGATCTCACCATGAACAATGC
Hs FT7 CTS f FP ASP	ATACCGGTGGTTATCGTGGGTTGGG