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N-glycan patterns of human transferrin produced in *Trichoplusia ni* insect cells: effects of mammalian galactosyltransferase

Eric Ailor, Noriko Takahashi[2], Yoshinori Tsukamoto[2], Katsuyoshi Masuda[3], Badarulhisam Abdul Rahman[5], Donald L.Jarvis[4], Yuan Chuan Lee[5] and Michael J.Betenbaugh[1]

Department of Chemical Engineering, The Johns Hopkins University, Baltimore, MD 21218, USA, [2]GlycoLab, Central Research Institute, Nakano Vinegar Co. Ltd., 2–6 Nakamura-cho, Handa-City Aichi 475–8585, Japan, [3]Suntory Institute for Bioorganic Research, Wakayama-dai, Shimamoto-cho, Mishima-gun, Osaka 618, Japan, the [4]Department of Microbiology, University of Wyoming, Laramie, WY 82071, USA, and [5]Department of Biology, The Johns Hopkins University, Baltimore, MD 21218, USA

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The *N*-glycans of human serum transferrin produced in *Trichopulsia ni* cells were analyzed to examine *N*-linked oligosaccharide processing in insect cells. Metabolic radiolabeling of the intra- and extracellular protein fractions revealed the presence of multiple transferrin glycoforms with molecular weights lower than that observed for native human transferrin. Consequently, the *N*-glycan structures of transferrin in the culture medium were determined using three-dimensional high performance liquid chromatography. The attached oligosaccharides included high mannose, paucimannosidic, and hybrid structures with over 50% of these structures containing one fucose, α(1,6)-, or two fucoses, α(1,6)- and α(1,3)-, linked to the Asn-linked *N*-acetylglucosamine. Neither sialic acid nor galactose was detected on any of the *N*-glycans. However, when transferrin was coexpressed with β(1,4)-galactosyltransferase three additional galactose-containing hybrid oligosaccharides were obtained. The galactose attachments were exclusive to the α(1,3)-mannose branch and the structures varied by the presence of zero, one, or two attached fucose residues. Furthermore, the presence of the galactosyltransferase appeared to reduce the number of paucimannosidic structures, which suggests that galactose attachment inhibits the ability of hexosaminidase activity to remove the terminal *N*-acetylglucosamine. The ability to promote galactosylation and reduce paucimannosidic *N*-glycans suggests that the oligosaccharide processing pathway in insect cells may be manipulated to mimic more closely that of mammalian cells.

Key words: baculovirus/glycosylation/glycosyltransferase/oligosaccharide/expression

Introduction

The Baculovirus Expression Vector System (BEVS) has become increasingly popular for the expression of recombinant proteins (Luckow and Summers, 1988; Luckow, 1995). One of the key factors that has contributed to the wide spread use of this system is its ability to produce relatively large quantities of posttranslationally modified eukaryotic proteins (O'Reilly *et al.*, 1992; Klenk, 1996; Jarvis, 1997) often including peptide glycosylation. Unlike mammalian cells, however, the oligosaccharide processing pathway in insect cells is not well characterized (Marz *et al.*, 1995; Altmann, 1996). Insects represent the majority of the known animal species (Kubelka *et al.*, 1994) and are widely used in developmental and recombinant protein studies. Therefore, identifying and detailing the differences in oligosaccharide processing between mammalian and insect cells is of scientific interest. Furthermore, the ability to manipulate the oligosaccharide processing pathway of insect cells may be useful for exploiting the benefits of the baculovirus system across a wider spectrum of applications.

Experimental evidence suggests that glycoproteins produced in insect cells possess *N*-linked oligosaccharides that are principally comprised of high mannose and truncated low mannose (paucimannosidic) structures (Butters and Hughes, 1981; Hsieh and Robbins, 1984; Kuroda *et al.*, 1990; Chen and Bahl, 1991; Kulakosky *et al.*, 1998). Even though activities for β(1,2)-*N*-acetylglucosaminyltransferase I (GlcNAcTI) (Altmann *et al.*, 1993; Velardo *et al.*, 1993), β(1,2)-*N*-acetylglucosaminyltransferase II (GlcNAcTII) (Altmann *et al.*, 1993), and β(1,4)-galactosyltransferase (GalT) (van Die *et al.*, 1996) have been detected in insect cells, *N*-glycan structures containing terminal *N*-acetylglucosamine (GlcNAc) and galactose (Gal) residues are not widely observed on native or heterologous glycoproteins. However, recent studies of both homologous (Hard *et al.*, 1993; Kubelka *et al.*, 1994) and heterologous (Davidson *et al.*, 1990; Ogonah *et al.*, 1996) proteins obtained from insect cell sources have revealed the presence of a limited number of hybrid and complex type *N*-glycan structures.

We previously reported on the variability of *N*-glycan structures that exist between the intracellular and secreted forms of a recombinant immunoglobulin G (IgG) (Hsu *et al.*, 1997). The presence of GlcNAc at the terminal position of some *N*-glycans indicates that insect cells produce the acceptor substrate for galactosylation. However, the processing of the *N*-linked oligosaccharides of IgG is highly variable (Radmacher *et al.*, 1986; Tandai *et al.*, 1991) so a secreted protein with more defined carbohydrate structures was chosen as a candidate for further study. As a result, human serum transferrin (hTf) has been expressed and purified from insect cells for oligosaccharide analysis of the attached *N*-glycans.

[1]To whom correspondence should be addressed

Transferrin is a serum glycoprotein found in the physiological fluids of vertebrates (Aisen, 1989; Thorstensen and Romslo, 1990) and insect larva (Bartfeld and Law, 1990) that is responsible for carrying Fe^{+3} to all cells in the body. When bound to iron the circulating transferrin is recognized by a specific surface receptor on cells and internalized to release iron into the cytoplasm (Trowbridge *et al.*, 1984). Other members of the 80 kDa glycoprotein family include lactoferrin, melanotransferrin, and ovotransferrin (Montreuil *et al.*, 1997). Serum transferrin also plays a role in host defense by depriving any circulating microorganisms of essential iron (Bullen *et al.*, 1990).

Substantial information exists about the carbohydrate structure of hTf and other transferrins. HTf has two potential *N*-linked glycosylation sites in its carboxy-terminal domain at Asn_{413} and Asn_{611} (MacGillivray *et al.*, 1983). Previous studies have shown that the transferrin glycoforms present in human serum are comprised of species having terminally sialylated bi-, tri-, and, tetrantennary oligosaccharides (Leger *et al.*, 1989; Fu and van Halbeek, 1992). The most dominant glycoform includes biantennary oligosaccharides located at both asparagine positions, although, changes in physiological conditions can affect the *N*-glycan pattern observed in the host (Montreuil *et al.*, 1997). Very little is known about the *N*-glycan structures of hTf expressed in foreign hosts. Recombinant hTf expressed in baby hamster kidney cells was comprised of numerous glycoforms, however, the detailed *N*-glycan structures were not determined (Mason *et al.*, 1993). In related studies, both human and bovine lactoferrins have been expressed in insect cells and subjected to glycosylation analysis. *N*-linked oligosaccharides obtained from bovine lactoferrin expressed in *Mamestra brassicae* cells were observed to lack any complex or hybrid structures (Lopez *et al.*, 1997). Carbohydrate analysis revealed a small fraction of Gal in oligosaccharides obtained from *N*-glycans of human lactoferrin expressed in *Spodoptera frugiperda* (Sf-9) (Salmon *et al.*, 1997), though the analysis was for monosaccharides only.

One possible reason for the limitation in *N*-glycan processing of glycoproteins in insect cells is a deficiency in the enzymes necessary to produce complex oligosaccharides. In a recent study, coexpression of a recombinant GlcNAcTI in Sf-9 cells resulted in an increase of terminal GlcNAc residues found on the *N*-glycans of fowl plague virus hemagglutinin (Wagner *et al.*, 1996). Additional studies used lectin blot analysis to demonstrate that the expression of GalT in Sf-9 cells led to the incorporation of terminal β-linked Gal residues in the *N*-glycans of baculovirus and heterologous glycoproteins (Jarvis and Finn, 1996; Hollister *et al.*, 1998). To gain greater insight into the oligosaccharide processing pathway of insect cells, an analysis of the *N*-glycans from recombinant hTf was undertaken in the current study. In addition, the oligosaccharides generated following coexpression of GalT were detailed in order to elucidate the structural changes resulting from expression of heterologous oligosaccharide modifying enzymes.

Results

Expression of recombinant hTf in insect cells

The recombinant human transferrin baculovirus, AcNPV-hTf, was harvested 5 days following transfection of Sf-9 cells, plaque purified, and amplified to provide a working virus stock. To verify expression of hTf in insect cells, clarified cell lysates were prepared from T.ni cells infected 3 days with AcNPV-hTf or a negative control baculovirus (AcBB) (Lindsay et al., 1993) and subjected to western blot analysis. The immunoblot revealed a major immunoreactive band of ~78 kDa in cell lysates of cells that were infected with AcNPV-hTf (Figure 1).

T.ni cells were observed to produce higher levels of secreted hTf than Sf-9 cells and were consequently used for subsequent oligosaccharide analysis. Medium from *T.ni* cells infected 3 days with AcNPV-hTf was separated by SDS–PAGE and stained with Coomassie brilliant blue to examine extracellular hTf expression levels. Recombinant hTf is clearly visible in the medium (Figure 2). No band corresponding to hTf was observed in the medium from uninfected cells. The molecular weight (MW) of hTf produced in *T.ni* cells is slightly lower than its native counterpart.

Effects of tunicamycin on hTf produced in T.ni cells

To examine the contribution of N-linked oligosaccharides to the MW of recombinant hTf, T.ni cells were labeled 4 h with ^{35}S-methionine after being infected 48 h with AcNPV-hTf in

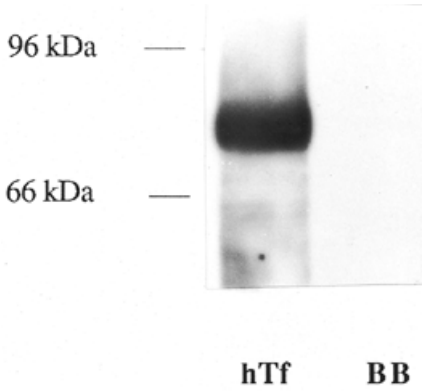


Fig. 1. Western blot of recombinant human transferrin in whole cell lysates of *T.ni* insect cells. Cells were harvested 72 h following infection with AcNPV-hTf (hTf) or a control baculovirus AcBB (BB).

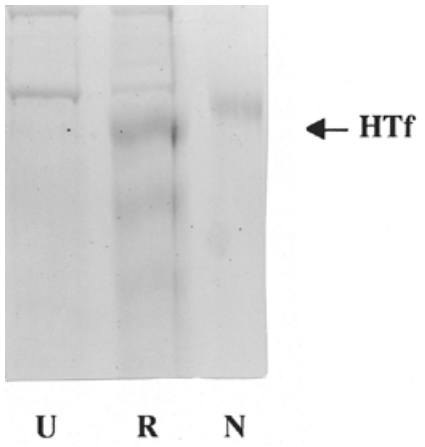


Fig. 2. Coomassie stain of recombinant human transferrin in the medium of *T.ni* insect cells. Medium from uninfected cells (U), medium from cells infected with AcNPV-hTF (R), and purified native human transferrin (N). Cells were harvested 72 h postinfection.

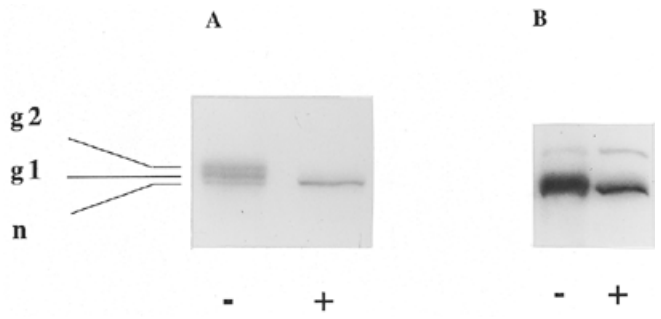


Fig. 3. Autoradiograph of transferrin in whole cell lysates (**A**) and extracellular medium (**B**) following ^{35}S-methionine labeling. At 44 h pi High Five insect cells infected with AcNPV-hTf were placed in methionine deficient medium containing TRAN^{35}S-label in the absence (–) or presence (+) of tunicamycin. Major bands in the whole cell lysate are defined as n, g1, and g2. Transferrin in the medium was concentrated by immunoprecipitation.

the presence or absence of tunicamycin. Following the labeling period, cells were lysed, subjected to SDS–PAGE, dried, and autoradiographed. Secreted hTf was immunoprecipitated from the medium using a polyclonal α-hTf antibody and similarly processed. The autoradiograph depicted in Figure 3 shows that the presence of tunicamycin, an inhibitor of asparagine-linked oligosaccharide processing, results in production of a lower MW form of intracellular and secreted hTf. In the absence of tunicamycin, at least three distinct hTf bands can be seen in the whole cell lysates (Figure 3A). The lower band (n) appears to be non-glycosylated hTf since it is identical in MW to the hTf produced in the presence of tunicamycin. The secreted hTf fraction also appears to contain multiple hTf glycoforms (Figure 3B) which collapse to a lower MW form in the presence of tunicamycin.

Analysis of secreted hTf produced in insect cells

To determine if altering the intracellular level of an enzyme in the oligosaccharide processing pathway can promote elongation of hTf *N*-glycans, a recombinant GalT was overexpressed in insect cells in conjunction with hTf.

Two *T.ni* insect cell cultures were infected with AcNPV-hTf. One of the cultures was also coinfected with the recombinant AcP(+)IE1GalT baculovirus, which contains the gene for GalT under the IE1 promoter. The medium from each of these cell cultures was collected at 66 h pi and hTf was purified on a phenyl Sepharose hydrophobic column. Pooled fractions (Figure 4) containing ≥95% hTf were separated by SDS–PAGE, transferred to a PVDF membrane, and were analyzed for Gal content using HPAEC/PAD following TFA hydrolysis of the hTf bands (Weitzhandler *et al.*, 1993; Fan *et al.*, 1994). Analysis of the monosaccharides indicated that the hTf oligosaccharides from *T.ni* cells coinfected with the GalT recombinant baculovirus contained a measurable amount of Gal while the samples without GalT did not contain significant Gal levels (unpublished observations).

Since the monosaccharide analysis indicated a difference in the Gal levels of the hTf *N*-glycans from cells in the presence or absence of GalT expression, a more detailed comparison of the *N*-glycans was undertaken using HPLC analysis. *N*-glycans were released from recombinant transferrin by glycoamidase A (sweet almond). Based on total carbohydrate analysis, more than 80% of the carbohydrate chains were released by

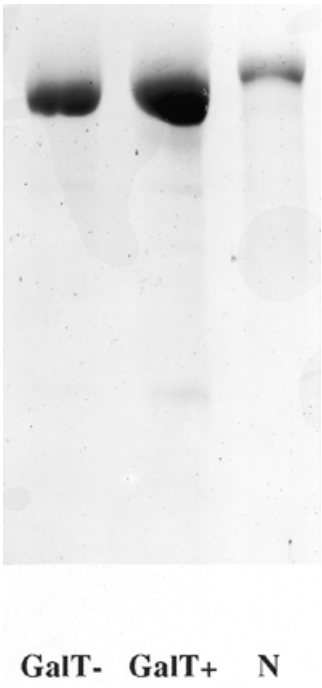


Fig. 4. Coomassie stain of hTf purified from cell culture medium of *T.ni* cells infected with the recombinant hTf baculovirus alone (GalT-) or with both hTf and GalT recombinant baculoviruses (GalT+). Native hTf from human serum (N) is shown to demonstrate the difference in SDS–PAGE mobility between the recombinant and native forms. Approximately 10 μg of the recombinant protein were loaded per lane.

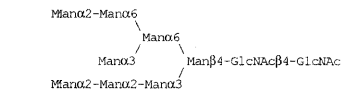
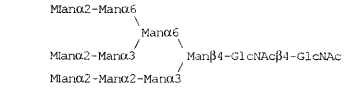
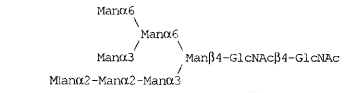
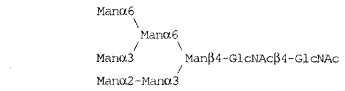
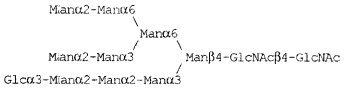
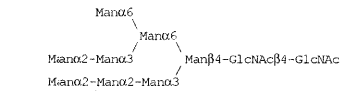
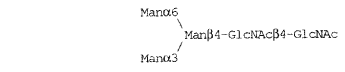
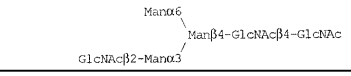
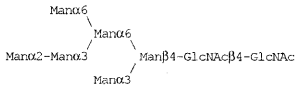
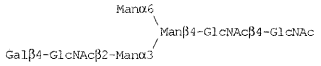
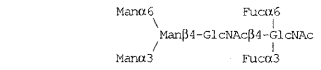
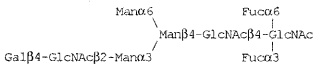
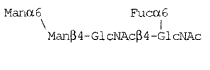
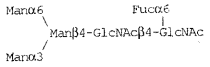
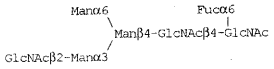
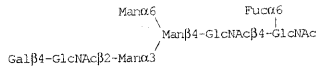
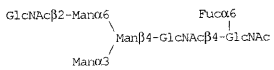
digestion. The reducing ends of the released N-glycans were reductively aminated with 2-aminopyridine. No acidic oligosaccharides were detected on a DEAE-5PW column. The PA-oligosaccharides were then separated by a reversed phase column (Figure 5). Each separated fraction (A through L) was then applied to an amide column. Fraction B was further separated into two fractions B1 and B2 on the amide column while fractions E, F, G, and J were similarly separated into E1 and E2; F1, F2, and F3; G1 and G2; and J1, J2, and J3, respectively (unpublished observations). Three distinct peaks, G2, I, and K, were shown to exist only in the cells coinfected with the GalT baculovirus.

Structural characterization of PA-oligosaccharides

Structural assignment of all neutral *N*-glycans from recombinant transferrin was performed by a 2-D mapping technique as described previously (Takahashi *et al.*, 1995). The coordinates of almost all the *N*-glycans coincided (±5%) with those of known oligosaccharides on the map. PA-oligosaccharides A, B1, B2, C, D, E1, E2, F1, F2, F3, G1, G2, H, J1, J2, J3, K, and L were assigned as code numbers M8.1, M7.2, M9.1, M7.1, M6.1, M9.2, M.8.2, M5.1, 000.1, 100.2, M.6.10, 100.4, 010.1F, 010.0, 010.1, 110.2, 110.4, and 110.1, respectively (Table I). Co-chromatography of each of the sample PA-oligosaccharides with the corresponding reference compound on ODS and amide columns confirmed these assignments.

The structural candidate for glycan E1 was identified as M9.2, which has a Glcα3Manα2Manα2Manα3Manβ4 sequence. Although the presence of glucose is unusual, we confirmed the structure by co-chromatography with the

Table I. Chromatographic properties and the proposed structures of PA-oligosaccharides obtained from recombinant human transferrin secreted from *T.ni* insect cells with and without bovine β(1,4)-galactosyltransferase

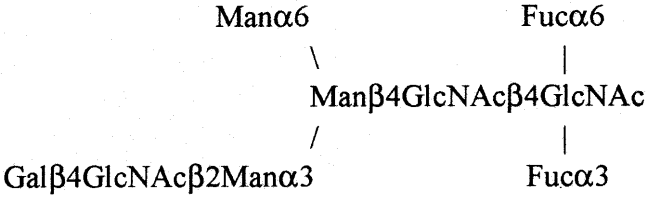
Glycan/code (G.U. ODS, amide)	PA-oligosaccharide structure	Secreted hTf (mol%)	
		-GalT	+GalT
A/M8.1 (4.9,9.0)		3.9	10.1
B1/M7.2 (5.1,8.1)		2.3	5.5
B2/M9.1 (5.2,9.7)		11.6	23.5
C/M7.1 (5.8,8.0)		2.3	5.5
D/M6.1 (6.1,7.1)		4.7	13.4
E1/M9.2 (6.3,10.3)		1.3	3.7
E2/M8.2 (6.4,8.5)		0.3	0.8
F1/M5.1 (7.2,6.2)		4.6	2.4
F2/000.1 (7.4,4.3)		9.0	5.8
F3/100.2 (7.4,4.7)		6.5	3.1
G1/M6.10 (7.9,6.8)		1.1	1.1
G2/100.4 (8.0,5.7)		nd	5.0
H/010.1F (8.5,5.5)		5.9	1.7
I/110.4F (8.9,7.0)		nd	3.3
J1/010.0 (10.1,3.5)		23.4	4.0
J2/010.1 (10.2,4.7)		15.7	6.1
J3/110.2 (10.2,5.1)		3.5	nd
K/110.4 (10.9,6.3)		nd	4.3
L/110.1 (12.7,5.1)		3.9	0.7

reference oligosaccharide obtained from locust lipophorin (Nagao *et al.*, 1987).

Galactose-containing oligosaccharides

N-Glycans G2 (100.4), I (110.4F), and K (110.4), which were distinct to *T.ni* cells overexpressing GalT, were all observed to contain terminal Gal residues. The identification of the structures of glycans G2 and K was not difficult due to the availability of the known references, however, there was no available reference for the structure of glycan I.

After sequential digestion by β-galactosidase and β-*N*-acetylhexosaminidase, the coordinate on the 2-D map of glycan I finally coincided with that of glycan H (Figure 6). Furthermore, the pathway profiles obtained by digestion with β-galactosidase and β-*N*-acetylhexosaminidase from glycan I to H, from glycan K to J2 via J3, and from glycan G2 to F2 via F3 were completely parallel (Figure 6). These results strongly suggest that the Gal-GlcNAc sequence in glycan I is linked to the Manα3Manβ4 antenna, because if the Gal-GlcNAc is linked to the Manα6Manβ4, the glycosidase digestion profile follows a different pathway (Tomiya and Takahashi, 1998). In addition, after prolonged α-fucosidase digestion, glycan I was converted to glycan G2. Therefore, the proposed structure of glycan I is as follows:



The tandem MS spectrum of the parent ion (*m/z* 823.8) gives a fragmentation pattern which is consistent with the structure of glycan I (Figure 7). As shown in Figure 7, daughter ions were due to fragmentation at glycosidic bonds. In particular,

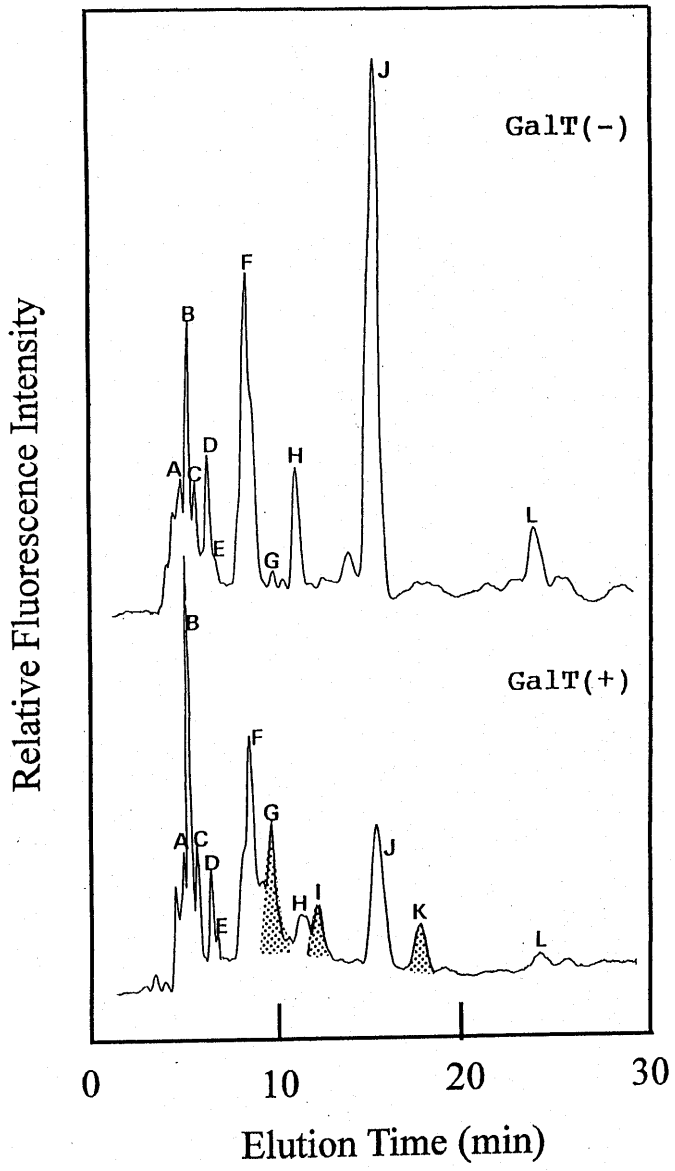


Fig. 5. HPLC elution profiles of PA-oligosaccharides derived from secreted transferrin. Transferrin was purified from the extracellular medium of *T.ni* cells following infection of the cells with [GalT(+)] and without [GalT(-)] the recombinant GalT baculovirus. Following purification, transferrin *N*-glycans were prepared, derivatized, and subjected to separation on the ODS-silica column as described in the text.

the appearance of the ion peak at 1281.6 produced from the original molecule by loss of the $Hex_1HexNAc_1$ portion, suggests the existence of a Gal-GlcNAc sequence in the nonreducing end of the lower antenna. The peak ion 366.2 can be interpreted as the relative molecular mass of Gal-GlcNAc, which complements 1281.6. Subsequently, one Fuc, one mannose, and two Fuc residues are individually lost from the 1281.6 ion and result in the corresponding ions, 1135.5, 1119.5, and 989.4, respectively. By contrast, from the reducing end of glycan I, each ion 300.2, 446.3, and 592.3 is produced and corresponds to the presence of fragments GlcNAc-PA, fucosyl GlcNAc-PA, and difucosyl GlcNAc-PA, respectively.

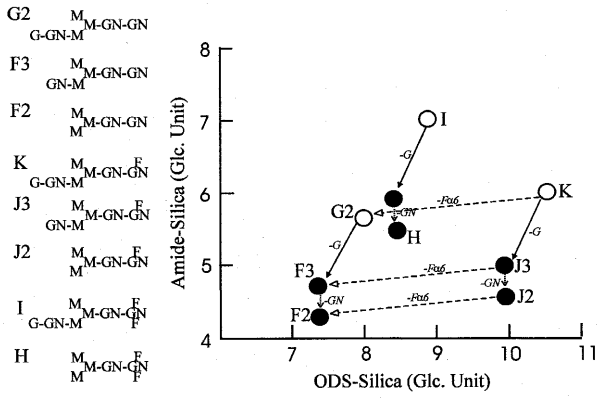


Fig. 6. Identification of glycan I structure using 2D mapping. Structure of glycan I was identified by comparing its coordinates on the 2D map following sequential digestions with β-galactosidase and β-*N*-acetylhexosaminidase with known structures G2 and K. The arrows indicate the change in direction of the coordinates: -G, Gal removal; -GN, GlcNAc removal; -Fα6, α(1,6) fucose removal.

Other small daughter ions are consistent with the loss of each monosaccharide from the glycan I. Thus, ESI-tandem MS data confirmed that glycan I has a Gal-GlcNAc sequence at the nonreducing end and two Fuc residues at the reducing terminal GlcNAc.

Discussion

Western blot and Coomassie stain analyses demonstrated that recombinant hTf was expressed and secreted from insect cells. However, the MW of hTf produced in insect cells is lower than that of the native protein. Native hTf contains two *N*-glycosylation sites that are predominantly occupied by terminally sialylated biantennary complex oligosaccharides (Spik *et al.*, 1975). Metabolic radiolabeling of insect cells infected with the recombinant hTf baculovirus reveals the presence of 3 MW bands representing multiple hTf glycoforms in whole cell lysates. These glycoforms may represent site occupancy variants of hTf where zero, one, and two sites have been glycosylated. Treatment of the insect cells with tunicamycin collapsed these glycoforms to the lowest MW variant, presumably that of nonglycosylated hTf. Other heterologous glycoproteins have been previously found in glycosylated and nonglycosylated forms in insect cells following baculovirus infection (Kawamoto *et al.*, 1994; Tate and Blakely, 1994; Hooker *et al.*, 1999). The downward shift in MW of secreted hTf when treated with tunicamycin suggests that much of the secreted hTf contains oligosaccharides of some type. HTf carbohydrates have been previously shown to have no effect on iron binding, iron transport (Spik *et al.*, 1988), and receptor binding (Mason *et al.*, 1993), but they may be important during the folding and secretory processing.

Analysis of secreted hTf *N*-glycans by three-dimensional HPLC revealed the presence of high mannose, paucimannosidic, and some hybrid oligosaccharide structures. The composition (mol%) of high mannose, paucimannose, and hybrid structures from *T.ni* cells in the absence of GalT was 30.8%, 54.0%, and 13.9%, respectively, whereas the levels were 62.3%, 17.6%,

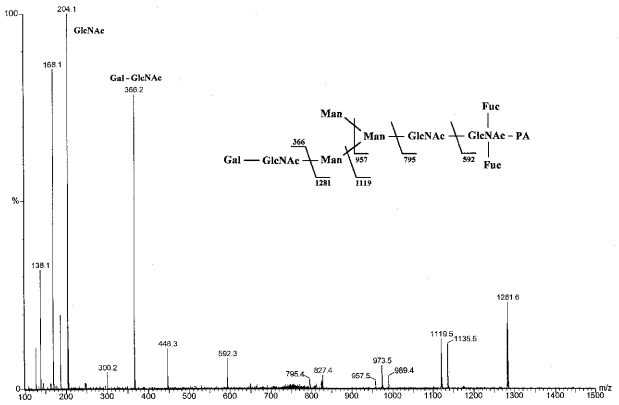


Fig. 7. Electrospray tandem mass spectrum from the parent ion m/z 823.8 $[M+2H]^{2+}$ of glycan I.

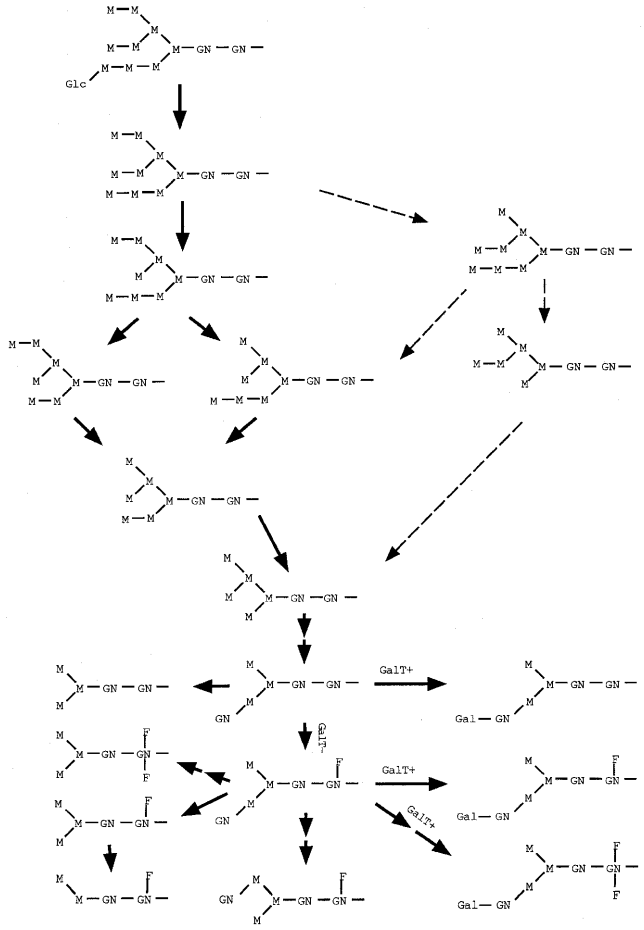


Fig. 8. Proposed *N*-glycan processing pathway in *T.ni* cells. All structures were detected in the medium of cultured *T.ni* cells infected with recombinant transferrin in the presence or absence of overexpressed β(1,4)-galactosyltransferase unless labeled as follows: GalT+, detected only in the medium of cells with β(1,4)-galactosyltransferase overexpression; GalT-, detected only in the medium of cells without β(1,4)-galactosyltransferase overexpression.

and 16.4%, respectively, in the presence of GalT. High mannose structures are commonly found in heterologous glycoproteins from insect cells and may be the result of product secretion or cell lysis. At harvest time, the cell culture infected with both hTF and GalT baculoviruses had a lower viability than the culture infected with the hTF virus only, which was expected from the higher MOI used to infect cells with the two baculoviruses (Kioukia *et al.*, 1995). An increase in lysis of the coinfected cells may result in an increase in the release of intracellular glycoproteins. We have previously observed that intracellular glycoforms include a higher percentage of high mannose structures (Hsu *et al.*, 1997). A higher percentage of lysed cells would result in a release of these partially processed high mannose glycoforms into the medium.

An examination of the high mannose structures provides insights into the processing of oligosaccharides in insect cells. The proposed oligosaccharide processing pathway (Figure 8) appears to follow the same general pathway observed in mammalian cells (Kornfeld and Kornfeld, 1985; Marchal *et al.*, 1999); however, it is possible that alternative pathways may exist in insect cells. The presence of the multiple $Man_8GlcNAc_2$, $Man_7GlcNAc_2$, and $Man_6GlcNAc_2$ isomers suggests that the order of mannose residue cleavage during mannosidase processing is variable. Alternatively, the accumulated high-Man types may have been subjected to degradative α-mannosidase digestions. In addition, one structure, M9.2, is observed which includes an attached glucose. M9.2 is a structure found early in the oligosaccharide processing pathway and is the product of glucose cleavage reactions of the $Glc_3Man_9GlcNAc_2$ core by glucosidases. Alternatively, M9.2 may be a product of re-glucosylation by a previously identified insect glucosyltransferase (Parker *et al.*, 1995) in the cycle of reglucosylation that occurs prior to transport of folded proteins from the ER (Sousa and Parodi, 1995; Wada *et al.*, 1997).

In addition to the high mannose structures were significant levels of paucimannosidic structures and some hybrid structures. Hybrid structures including GlcNAc on both the α(1,3)- and α(1,6)-Man branches were observed in both cultures, confirming the presence of native GlcNAcTI and GlcNAcTII activities in *T.ni* cells. In the absence of GalT expression, however, much of the GlcNAc present on the α(1,3)-Man branch appears to be removed by *N*-acetylglucosaminidase activity which has been found to be present in insect cells (Licari *et al.*, 1993; Altmann *et al.*, 1995). Also observed, in some cases, is the presence of Fuc on the reducing GlcNAc, which, when α(1,3) linked, represents a potentially allergenic glycoform produced by insect cells (Aalberse *et al.*, 1981; Tretter *et al.*, 1993).

Examination of the oligosaccharide structures from hTf produced in cells infected with the GalT baculovirus revealed that transferase overexpression can indeed alter the glycoforms of the expressed transferrin. Three hybrid type oligosaccharides (100.4, 110.4F, 110.4) bearing terminal Gal moieties were identified in hTf from *T.ni* insect cells coinfected with the GalT baculovirus that were not detected in cells that were infected with AcNPV-hTf alone. Interestingly, over 80% of oligosaccharides containing GlcNAc on the α(1,3)-Man arm also include a terminal Gal residue. Sf-9 cells infected with the GalT baculovirus (Wolff *et al.*, unpublished observations) or

stably transformed to express GalT (Hollister *et al.*, unpublished observations) were also observed to produce hybrid *N*-glycans terminating in galactose. In one of these studies, a higher percentage of Gal terminating structures was obtained from a recombinant gp64 fusion protein (Hollister *et al.*, unpublished observations). Differences in the levels of galactosylation may be attributable to a number of factors including the particular glycoprotein, the host cell line, cell lysis effects, and GalT enzyme activity during the infection period (Jarvis *et al.*, 1996).

Of the Gal containing carbohydrate structures, it appears the GalT activity was exclusive to the α(1,3)-Man branch. The absence of Gal residues on the α(1,6)-Man branch may result from low levels of GlcNAc bearing acceptor substrate or the overexpressed mammalian galactosyltransferase may have a high specificity for the α(1,3)-Man arm. When $GlcNAc_2Man_3GlcNAc_2$ was used as an acceptor substrate, several mammalian galactosyltransferases where shown to demonstrate a preference for the α(1,3)-Man arm (Blanken *et al.*, 1984, Paquet *et al.*, 1984; Narasimhan *et al.*, 1985). Alternatively, it is possible that the recombinant GalT is expressed in a cellular compartment prior to the addition of GlcNAc by homologous GlcNAcTII. However, recent evidence suggests that recombinant GalT is localized in the correct organelle (i.e., trans Golgi) of Sf-9 cells (Kawar and Jarvis, unpublished observations).

In addition to enhancing galactosylation, GalT overexpression appeared to inhibit the formation of paucimannosidic products. The percentage of hybrid oligosaccharide structures as a total of hybrid and paucimannosidic structures increased from 20% without GalT to 48% in cells overexpressing GalT. The decrease in the amount of paucimannosidic structures and the concomitant increase in Gal containing hybrid structures in the hTf *N*-glycans from cells overexpressing GalT may be due to competition between GalT and β-*N*-acetylglucosaminidase activities. The transfer of Gal to β(1,2)-GlcNAc of the α1,3-Man arm may prevent cleavage of β(1,2)-GlcNAc by the previously identified insect β-*N*-acetylglucosaminidase.

However, even with GalT overexpression, no complex biantennary structures were observed attached to the recombinant transferrin. In a previous study, approximately 36 mol% of oligosaccharide structures obtained from the secreted fraction of a recombinant IgG produced in *T.ni* cells were of the complex type, some including Gal residues (Hsu *et al.*, 1997). Final *N*-glycan structures may be influenced by the specific polypeptide chain undergoing *N*-glycosylation (Anderson and Goochee , 1994; Pfeiffer *et al.*, 1994) as well as by differences in the cell culture conditions (Anderson and Goochee, 1994). The activity of glycosidases released into the culture medium following cell lysis (Parker *et al.*, 1995) may also alter the *N*-glycan structures of extracellular glycoproteins.

Clearly, the overexpression of GalT enhances the level of oligosaccharide complexity for hTf expressed in *T.ni* cells. Overexpression of GalT may overcome a limitation in constitutive insect cell GalT activity and/or compete with *N*-acetylglucosaminidase activity to alter the *N*-linked oligosaccharide processing pathway. Furthermore, these studies also indicate that several other processing steps, including the addition of GlcNAc on the α(1,6)-Man branch, could possibly be altered using oligosaccharide modifying enzymes to generate a product which is more similar in composition to native transferrin.

Materials and methods

Materials

Trichoplusia ni (*T.ni*, BTI-TN5B1-4, High Five™) and the TA cloning kit were obtained from Invitrogen (Carlsbad, CA). Sf-9 cells and the plasmid containing the gene for human transferrin (TFR27A) were obtained from the American Type Culture Collection (Manassas, VA). Excell 401 and 405 medium was purchased from JRH Biosciences (Lenexa, KS). Oligonucleotides used in the PCR reaction were obtained from Integrated DNA Technologies (Coralville, IA). *Taq* polymerase was purchased from Stratagene (La Jolla, CA). Restriction enzymes, DH5α competent cells, agarose, and 5-bromo-4-chloryl-3-indoyl-β-D-galactopyranoside (X-gal) were obtained from Life Technologies (Gaithersburg, MD). Baculovirus transfer plasmid and linear DNA were obtained from Clonetech (Palo Alto, CA). Qiagen (Valencia, CA) QIAEX kit was used to purify DNA from agarose gels. Hydrochloric acid, ammonium sulfate, and sodium citrate were purchased from J.T.Baker (Phillipsburg, NJ). Coomassie brilliant blue, Tween-20, nitrocellulose membrane, and Bio-Gel P-4 were obtained from Bio-Rad (Hercules, CA). Glycoamidase A (glycopeptidase A, EC 3.5.1.52) from sweet almond (Takahashi, 1977), α-mannosidase, β-galactosidase, and β-*N*-acetylhexosaminidase from jack bean were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). α-L-Fucosidase from bovine kidney was purchased from Boehringer Mannheim (Mannheim, Germany). The pyridylamino (PA) derivatives of isomalto-oligosaccharides (4–20 glucose residues) and of reference oligosaccharides (code nos. 000.1, 010.1, 100.2, 100.4, 110.1, 110.2, 110.4, M5.1, M6.1, M7.1, M7.2, M8.1, M8.2, and M.9.1) were from Seikagaku Kogyo. Code no. 010.0 was obtained by partial α-mannosidase digestion of 010.1. Code no. M6.10 was similarly obtained by partial α-mannosidase digestion of M9.1 (Tomiya *et al.*, 1991). M9.2 was obtained from locust lipophorin (Nagao *et al.*, 1987). Code no. 010.1F was obtained from neuropsin produced in *T.ni* cells (Takahashi *et al.*, unpublished observations). The following materials were obtained from the sources indicated: Dialysis tubing and peroxidase labeled anti-IgG antibody, from Pierce (Rockford, IL); anti-human transferrin antibody, Dako (Carpinteria, CA); Lumiglo, Kirkegaard & Perry (Gaithersburg, MD); Tran^{35}S-label, ICN (Costa Mesa, CA); Amplifier, Amersham Pharmacia Biotech (Piscataway, NJ); PVDF membrane, Millipore (Bedford, MA); Dowex 50W-X8, Dow Chemical Co. (Midland, MI); sodium cyanoborohydride, Aldrich (Milwaukee, WI), 2-aminopyridine, Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Cell culture and virus stock

Suspension cultures of *T.ni* and Sf-9 cells were maintained at 27°C and rotated at 120 r.p.m. in 250 ml shaker flasks containing 35 ml of Excell 405 and Excell 401 serum-free medium, respectively. For protein purification, 500 ml medium in 1 l shaker flasks were maintained as stated above.

The recombinant baculovirus (AcP(+)IE1GalT) encoding the gene for bovine GalT was constructed as previously described (Jarvis and Finn, 1996). The recombinant baculovirus encoding the gene for hTf was constructed as described below.

Baculovirus construction

Recombinant hTf was amplified from plasmid TFR27A by polymerase chain reaction using *Taq* polymerase. The 5′ and 3′ primers were GCG CCC TCG AGA TGA GGC TCG CCG T and CCT ACC TTC TAG ATT TTA AGG TCT ACG GAA, respectively. The 5′ and 3′ primers contain XhoI and BglII restriction sites, respectively, for subsequent cloning into the baculovirus transfer vector. The amplified gene was ligated into a linear cloning vector (PCRII) and transformed into *E.coli* using the TA cloning kit. Following screening for the hTf insert, hTf positive colonies were miniprepped and the plasmid DNA digested with XhoI and BglII restriction enzymes. The digested hTf gene fragment was purified from a 1% agarose TAE gel using a QIAEX DNA extraction kit. The purified and digested gene was ligated into pBacPAK8™, a baculovirus transfer vector which was also digested with XhoI and BglII restriction enzymes and gel purified. The resultant ligation reaction mixture was transformed into *E.coli* DH5α competent cells. *E.coli* colonies grown on LB (w/100 μg/ml ampicillin) agar plates were cultured, miniprepped, and screened for plasmids containing the hTf insert.

Plasmid DNA containing hTf (pBachTf) was transfected with BacPAK6, a linear baculovirus (*Autographa californica* nuclear polyhedrosis virus, AcNPV) genomic DNA fragment, into Sf-9 insect cells. Recombinant baculovirus was identified by selecting clear plaques in low melting point agarose containing X-gal. HTf positive plaques were amplified in Sf-9 suspension cultures to provide working stocks of the recombinant hTf baculovirus (AcNPV-hTf).

Cell infection and harvest

Log phase insect cells were seeded in a 6 well plate at 1×10^6 cells/well (1.5–2 ml). Cells were allowed to attach to the well (20 min at 27°C) and infected with AcNPV-hTf at a multiplicity of infection (MOI) of 5 to ensure synchronous infection. At 72 h postinfection (pi), infected cells were collected with medium in 1.5 ml microfuge tubes and centrifuged 30 s at $16{,}000 \times g$. After removal of the medium, the cell pellets were incubated with 300 μl lysis buffer (1% Triton X-100, 50 mM Tris-HCl pH 8.5, 10 mM EDTA, 150 mM NaCl) and protease inhibitors (0.5 μg/ml antipain, 0.5 μg/ml aprotinin, 15.6 μg/ml benzamidine-HCl, 0.5 μg/ml chymostatin,1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl). Clarified lysates were obtained from crude whole cell lysates by centrifugation for 15 min at $16{,}000 \times g$ at 4°C. The medium from the infected cells was used to evaluate secreted hTf expression levels.

Large scale suspension cultures were infected with AcNPV-hTf alone or with AcNPV-hTf and AcP(+)IE1GalT at a density of 2×10^6 cells/ml. Two hours after infection fresh medium was added to the culture to adjust the cell density to ~1×10^6 cells/ml. At 66 h pi the cultures were centrifuged 20 min at $4000 \times g$ to collect the medium which contains the secreted hTf.

Coomassie stain and Western blot

An equivalent number of cells from whole cell and clarified lysates from infected insect cells were separated by SDS–PAGE (10%) using reducing (60 mM Tris–HCl [pH 6.8], 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) sample buffer. Following electrophoresis, the gel was stained with 0.1% Coomassie brilliant blue stain to visualize protein bands. Secreted hTf was similarly analyzed using an equivalent volume of medium.

For Western blotting, the gel was transferred onto a nitrocellulose membrane following electrophoresis. The membrane was blocked 2 h in a 10% (w/v) dried milk Tris-buffered saline with 0.05% Tween 20 detergent (TBST) solution. Recombinant hTf was detected by probing with a primary polyclonal rabbit anti-human hTf antibody for 1 h in TBST containing 2% dried milk. The primary antibody was followed by a 1 h incubation in TBST with a horse radish peroxidase (HRP) conjugated polyclonal goat anti-rabbit IgG. Protein bands were visualized using LumiGLO™ substrate.

^{35}S-Methionine metabolic labeling

T.ni cells were seeded and infected in a 24-well plate at a density of 2×10^5 cells/well in the presence or absence of 1 μM tunicamycin. At 44 h postinfection the medium was aspirated and the cells were washed once with methionine-deficient Excell 401. The cells were then incubated with 50 μCi/ml of Tran^{35}S-label methionine for 4 h. At the end of the labeling period, the cells were harvested and lysed, and whole cell lysates were subjected to SDS–PAGE as described above. The collected medium was incubated with 25 μl of protein-A Sepharose beads in the presence of 10 μl of polyclonal mouse anti-transferrin and 2 μl of polyclonal rabbit anti-mouse IgG antibodies overnight at 4°C with constant rotation. The beads were then washed twice with 700 μl of cold PBS and boiled 5 min in 40 μl of 2× sample buffer to elute the hTf prior to SDS–PAGE. The radioactive bands on the SDS–PAGE gel were visualized by autoradiography after soaking the gels in Amplifier fluorographic reagent for 20 min and vacuum drying. Kodak BMX x-ray film was used to record the images.

Recombinant hTf purification

Secreted hTf was purified from the insect cell medium using the strategy developed by Ali *et al.* (1996). Following collection, the medium was concentrated 10X using a hollow fiber 5000MWC ultrafiltration membrane (A/G Technology, Needham, MA). The concentrated medium was diluted 50% with a 2.4 M ammonium sulfate/0.8 M sodium citrate buffer (pH 6), centrifuged, and filtered to remove the particulate matter. The sample was loaded on a phenyl Sepharose 6 fast flow column and washed with 1.2 M ammonium sulfate/0.4 M sodium citrate buffer (pH 6)(buffer A), and the proteins were eluted with a 0–100% water gradient in buffer A using a Pharmacia FPLC system (Amersham Pharmacia Biotech. The fractions containing recombinant hTf in high purity (as determined by Coomassie stain) were pooled together, dialyzed against 20 l of water overnight in membrane tubing (7000 MWCO), and lyophilized.

Monosaccharide analysis

The lyophilized hTf samples were resuspended in 50 mM ammonium acetate or phosphate buffered saline. Approximately 400 μg (200 μg/gel) of each hTf sample from *T.ni* cell cultures was subjected to SDS–PAGE (7.5%) and transferred to a PVDF membrane. Following transfer, the gel was stained

with 0.1% Coomassie brilliant blue and visually compared to a nontransferred control to determine the efficiency of hTf transfer to the PVDF membrane. The membrane was stained with 0.1% Coomassie brilliant blue to visualize the protein bands. The protein bands corresponding to hTf were cut from the membrane and placed in pretreated (4 M HCl overnight at 100°C) 1.5 ml vials (2 vials/sample) and rinsed with double distilled water. The membranes were submerged and hydrolyzed in 2 M trifluoroacetic acid (TFA) for 4 h or 4 M HCl for 6 h at 100°C. Following hydrolysis and removal of the membrane, 6 nm 6–0-methyl-D-galactose was added to the samples as a control and the samples were dried using a Speed Vac. The dried samples were dissolved in double distilled water, dried, and dissolved in 250 µl water. The TFA hydrolyates were then passed through a 2 ml bed volume of Dowex 50-X8 resin (prewashed with 2 M HCl and rinsed with water) to remove the charged molecules including amino sugars. The eluent (~6 ml) was dried and dissolved in 250 µl water. Each sample (50 µl) was then passed through a CarboPac PA-1 column (Dionex) and analyzed for monosaccharide composition using High Performance Anion Exchange Chromatography with pulsed amperometric detection (HPAEC/PAD) on a Dionex BioLC (Fan *et al.*, 1994). Monosaccharides were eluted isocratically with 7% NaOH.

Preparation and derivatization of N-glycans from recombinant transferrin

Transferrin produced in insect cells without (9 mg of protein correspond to 240 nmol of oligosaccharides) and with (4 mg of protein correspond to 107 nmol of oligosaccharides) GalT expression was heated at 100°C for 10 min. After digestion of the transferrin glycoprotein with trypsin and chymotrypsin (each 1%, w/w, of the substrate protein), at pH 8.0, the peptide/glycopeptide mixture was treated with glycoamidase A (0.6 mU) in 30 µl of 0.5 M citrate/phosphate buffer at pH 4.0 for 16 h to release the oligosaccharides. The mixture was finally digested with 1% (w/w) of pronase to convert peptide materials to amino acids and small peptides. The supernatant was applied to a column of Bio-Gel P4 (1×40 cm) equilibrated in 10 mM ammonium carbonate. The effluent was monitored using A_{280nm} fluorescence and 1 ml fractions were collected which were then thoroughly dried (Nakagawa *et al.*, 1995). After reductive amination with 2-aminopyridine using sodium cyanoborohydride (Yamamoto *et al.*, 1989), the resultant PA-oligosaccharides were purified by gel filtration on a Sephadex G-15 column (1.0 × 40 cm).

Isolation and characterization of PA-oligosaccharides by three successive HPLC steps

All separations were performed on a LC-10A HPLC system (Shimadzu, Japan). Approximately a 1/200 portion from each of the PA-oligosaccharide mixtures was separated and characterized by HPLC using three-dimensional sugar mapping technique as described previously (Takahashi *et al.*, 1995). PA-oligosaccharides were successively separated on an anion exchange column, TSKgel DEAE-5PW (7.5 × 75 mm; TOSOH, Japan), a reversed-phase column (Shim-pack CLC-ODS 6 × 150 mm, Shimadzu, Japan), and an amide-adsorption column (Amide-80, 4.6 × 250 mm, TOSOH). Elution conditions of the three columns were as described previously (Takahashi *et al.*, 1995; Tomiya *et al.*, 1988). The elution time for each peak was recorded in glucose units (Glc Units) and plotted on the x axis (for the reversed-phase column) and y axis (for the amide-adsorption column). After plotting the x and y coordinates for all PA-oligosaccharides on a two-dimensional (2-D) map, we compared the coordinate of a given sample with those of known PA-oligosaccharides. Each of the sample PA-oligosaccharides was co-chromatographed with a reference PA-oligosaccharide on ODS and amide columns to confirm its identity. The sample and reference PA-oligosaccharides were also digested simultaneously with several exoglycosidases and their coordinates were again compared on the map. PA-oligosaccharides were detected by fluorescence using excitation and emission at 320 and 400 nm, respectively.

Exoglycosidase digestion procedure

Each PA-oligosaccharide (50 pmol) isolated from the successive chromatography was digested with exoglycosidases (β-galactosidase, β-*N*-acetylhexosaminidase, and α-*L*-fucosidase) under conditions described previously (Takahashi and Tomiya, 1992). The elution coordinate of each exoglycosidase-trimmed oligosaccharide was examined on the 2-D map to verify its structural identity.

MALDI-MS analysis

The molecular mass of PA-oligosaccharides were determined by MALDI mass spectrometry using a Voyager Elite time-of-flight mass spectrometer equipped with a delayed-extraction system (PerSeptive Biosystems, Framingham, MA). The samples were ionized by a nitrogen laser at 337 nm. 2,5-Dihydroxybenzoic acid was dissolved in 50% aqueous acetonitrile solution containing 0.1% trifluoroacetic acid at a concentration of 10 mg/ml. The samples were dissolved in distilled water and mixed with the matrix solution at a 1:5 volume ratio. One microliter of aliquot was placed on the sample plate and air dried. The data was analyzed using GRAMS/386 software.

ESI-MS and tandem MS analysis

Electrospray MS and tandem MS data were acquired using a Q-TOF instrument (Micromass, Manchester, UK) and MassLynx data acquisition. This hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer was fitted with a nanoflow electrospray ion source. Samples were dissolved in 50% aqueous methanol solution containing 0.2% formic acid and loaded into a nanoflow tip. A high voltage (1–1.3 kV) was applied to the nanoflow tip of the capillary. The collision energy was increased to an optimum value of 15–30 V using argon collision gas.

Abbreviations

GlcNAcTI, β(1,2)-*N*-acetylglucosaminyltransferase I; II GlcNAcTII, β(1,2)-*N*-acetylglucosaminyltransferase; GlcNAc, *N*-acetylglucosamine; GalT, β(1,4)-galactosyltransferase; Gal, galactose; Fuc, fucose; IgG, immunoglobulin G; *T.ni*, *Trichoplusia ni* insect cell line; Sf-9, *Spodoptera frugiperda* insect cell line; hTf, human serum transferrin; PA, pyridylamino; MOI, multiplicity of infection; pi, postinfection; MW, molecular weight; MS, mass spectrometry.

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