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N-glycan structures of human transferrin produced by *Lymantria dispar* (gypsy moth) cells using the LdMNPV expression system

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_N_-glycan structures of recombinant human serum transferrin (hTf) expressed by *Lymantria dispar* (gypsy moth) 652Y cells were determined. The gene encoding hTf was incorporated into a *Lymantria dispar* nucleopolyhedrovirus (LdMNPV) under the control of the *polyhedrin* promoter. This virus was then used to infect Ld652Y cells, and the recombinant protein was harvested at 120 h postinfection. *N*-glycans were released from the purified recombinant human serum transferrin and derivatized with 2-aminopyridine; the glycan structures were analyzed by a two-dimensional HPLC and MALDI-TOF MS. Structures of 11 glycans (88.8% of total *N*-glycans) were elucidated. The glycan analysis revealed that the most abundant glycans were $Man_{1-3}(\pm Fuc\alpha 6)GlcNAc_2$ (75.5%) and $GlcNAcMan_3(\pm Fuc\alpha 6)GlcNAc_2$ (7.4%). There was only ~6% of high-mannose type glycans identified. Nearly half (49.8%) of the total *N*-glycans contained α(1,6)-fucosylation on the Asn-linked GlcNAc residue. However α(1,3)-fucosylation on the same GlcNAc, often found in *N*-glycans produced by other insects and insect cells, was not detected. Inclusion of fetal bovine serum in culture media had little effect on the *N*-glycan structures of the recombinant human serum transferrin obtained.

Key words: Baculovirus/Gypsy moth/Insect cells/*Lymantria dispar* nucleopolyhedrovirus/*N*-glycan

Introduction

Glycosylation is one of most common posttranslational modifications made to proteins by eukaryotic cells (Jenkins and Curling, 1994). Differences in *N*-glycan structure can sometimes affect the glycoprotein properties, including enzymatic activity, antigenicity, stability, solubility, cellular processing, secretion, and *in vivo* clearance rate (Varki, 1993). The insect cell-baculovirus expression system has been widely used for production of a large number of glycoproteins because of its ability to express high levels of heterologous proteins. This protein expression system primarily uses cell lines established from lepidopteran insects and the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV)–mediated expression vector (Summers and Smith, 1987). Lepidopteran-derived insect cell lines such as Sf9 (from *Spodoptera frugiperda*) and Tn-5B1-4 (from *Trichoplusia ni*) perform many of the post-translational modifications observed in eukaryotic cells, including *N*-glycosylation, and these cells are readily cultured in suspension in commercially available medium. Unfortunately, however, most insect cells examined so far are incapable of synthesizing sialylated complex-type *N*-glycans often found in glycoproteins obtained from mammalian cells.

Earlier studies using dipteran insect cells (Butters and Hughes, 1981; Hsieh and Robbins, 1984) and lepidopteran insect cells with the baculovirus system (Kuroda *et al*., 1990) indicated that insect cells produce mainly high-mannose-type and paucimannosidic-type *N*-glycans. Similar results have been repeatedly published (Altmann *et al*., 1999; März *et al*., 1995). In addition, α(1,3)-fucosylation of Asn-linked GlcNAc is often observed in insect cell–derived glycoproteins, and this modification represents a potential allergen to humans (Fotisch and Vieths, 2001; Tretter *et al*., 1993; Weber *et al*., 1987). The inability of lepidopteran insect cells to synthesize sialylated complex-type *N*-glycans and the presence of α(1,3)-fucosylation have limited the utility of insect cells as host cells for production of pharmaceutical glycoproteins. The limitations of currently used insect cell lines may potentially be overcome by means of genetic manipulation to include the necessary processing enzymes (Ailor *et al*., 2000; Aumiller and Jarvis, 2002; Breitbach and Jarvis, 2001; Hollister *et al*., 1998, 2002; Hollister and Jarvis, 2001; Tomiya *et al*., 2003), or by the use of an alternative insect cell line that may contain mammalian-like *N*-glycan processing capabilities.

As an attempt to survey insect cells potentially better suited for the production of pharmaceutical glycoproteins, a protein expression system based on a gypsy moth–derived cell line and a virus, *Lymantria dispar* multinucleocapsid nucleopolyhedrovirus (LdMNPV) (Yu *et al*., 1992), was tested. LdMNPV was used in the earlier study to express a bacterial β-galactosidase in tissue culture cells derived from gypsy moth (Yu *et al*., 1992). So far, however, the glycosylation pattern of glycoproteins expressed in gypsy moth cells has not been reported. LdMNPV will infect a number of cell lines derived from *L. dispar*, including the cell line Ld652Y, used in the present study. The Ld652Y cell line

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was chosen for the current study because it grows well in suspension in serum-free media at densities and growth rates similar to those of other popular insect cells, such as Sf9 and Tn-5B1-4. In this study, a recombinant human transferrin (hTf) was expressed as a model protein in Ld652Y cells using a recombinant LdMNPV. Structure of most (~90%) of the *N*-glycans released from the recombinant hTf was identified by a combination of matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) and a 2-D mapping technique (Tomiya *et al.*, 1988).

Results

Expression and purification of recombinant hTf from gypsy moth cells

Ld652Y cells were grown in shake-flask suspension cultures up to densities of approximately 7×10^5 cells/ml of medium in the presence or absence of 10% fetal bovine serum (FBS). The cells were then infected at a multiplicity of infection of one tissue culture infectious dose ($TCID_{50}$) per cell with LdMNPV-hTf, which contains the hTf gene. The recombinant hTf was expressed under the control of the *polyhedrin* promoter. At 5 days postinfection, the culture medium was harvested, and the recombinant hTf was isolated by ammonium sulfate fractionation and immunoaffinity chromatography using a Sepharose column onto which anti-hTf antibody was immobilized (Tomiya *et al.*, 2003). This column bound human serum transferrin avidly but not bovine serum transferrin (a potential contaminant from FBS). The purified recombinant hTfs were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Coomassie blue staining (Figure 1). The recombinant hTfs produced by Ld652Y cells, with or without serum in the culture medium, had essentially the same molecular size (~68 kDa) and showed slightly slower mobility than bovine transferrin. The results also indicate bovine transferrin was efficiently removed by the immunoaffinity chromatography.

Structural analysis of N*-glycans of hTf produced by Ld652Y cells*

Detailed glycan analysis was performed with the recombinant hTf obtained under the serum-free culture conditions. *N*-glycans were released from the purified hTf after initial proteolysis using sweet almond glycoamidase A (Takahashi and Nishibe, 1978), which can remove all *N*-glycans including those containing α-Fuc(1-3) substituted on the Asn-linked GlcNAc (Altmann *et al.*, 1995b; Fan and Lee, 1997; Takahashi and Tomiya, 1992; Tretter *et al.*, 1991).

No sialylated glycans were observed when a total mixture of the released glycans was analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The released *N*-glycans were then reductively aminated with 2-aminopyridine, and the resultant pyridylamino (PA) derivatives of glycans were first separated by reversed-phase high-performance liquid chromatography (HPLC) (Tomiya *et al.*, 1988), from which 14 peaks were obtained (Figure 2). Each of these fractions was further separated by normal-phase HPLC using an amide-silica column (Figure 3). After the two chromatographic steps, 19 different PA-glycans were isolated. The eight major PA-glycans (7A, 8A, 9A, 10A, 11A, 12A, 13A, and 14A), accounting for 86.5% of the total glycans, were analyzed by MALDI-TOF MS for confirmation. MS spectra of these major glycans are shown in Figure 4. The elution positions of the major PA-glycans, together with those of several standard PA-glycans

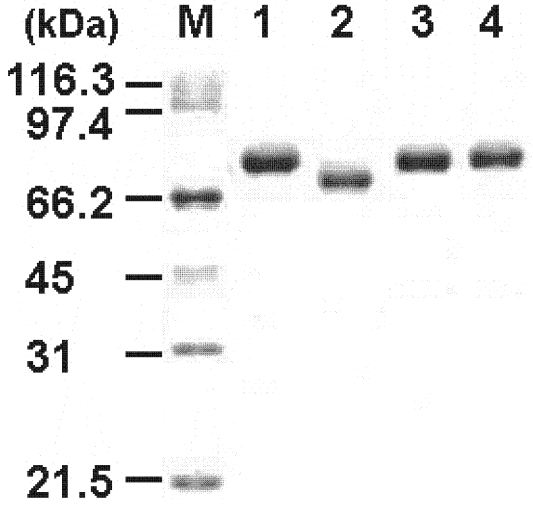


Fig. 1. SDS–PAGE of the purified hTf expressed in Ld652Y cells. The purified proteins were analyzed by SDS–PAGE using 10% acrylamide gel under nonreducing condition. Proteins were stained with Coomassie blue R-250. M, Marker proteins; 1, native human serum transferrin; 2, bovine serum transferrin; 3, recombinant hTf expressed in Ld652Y cells in the serum-free culture medium; 4, recombinant hTf expressed in Ld652Y cells in the culture medium containing 10% FBS.

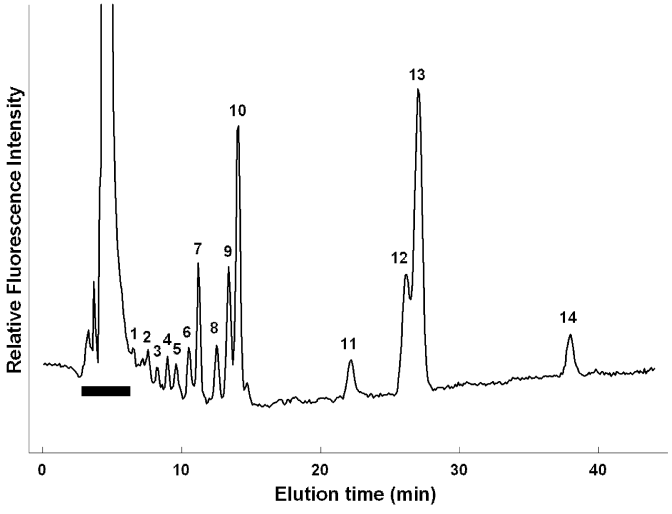


Fig. 2. An elution profile (ODS column) of the PA-glycans from the recombinant hTf expressed in Ld652Y cells in serum-free culture medium. Fourteen different peaks (1–14) were isolated. The peak marked with a bar indicates impurities unrelated to *N*-glycans.

having related structures, are shown on a 2D map in Figure 5.

The molecular mass of PA-glycan 7A ($[M+Na]^+ = 687.95$) corresponds to a glycan of one Man and two GlcNAc residues, suggesting a structure of Manβ(1,4)GlcNAcβ(1,4)-GlcNAc, a linear part of the common *N*-glycan core. Molecular mass of PA-glycans 10A ($[M+Na]^+ = 849.64$) is greater than that of 7A by one Man, and 13A ($[M+Na]^+ = 996.16$), in turn, is greater than 7A by one Fuc. The coordinates of PA-glycan 10A (7.4 glucose units [GU] on the ODS column and 3.3 GU on the amide column) on a 2D map coincided with those of a standard PA-glycan (code no. M2.1), Manα(1,6)Manβ(1,4)GlcNAcβ(1,4)GlcNAc-PA (7.4 GU/3.3 GU) but not with those of a standard PA-glycan (M2.2), Manα(1,3)Manβ(1,4)GlcNAcβ(1,4)GlcNAc-PA (6.3 GU/3.4 GU). The coordinates of PA-glycan 13A (10.4 GU/3.7 GU) coincided with those of a standard PA-glycan (010.1). Furthermore, the coordinates of 13A shifted to those of PA-glycan 10A and a standard PA-glycan (M2.1) after α-L-fucosidase digestion (20 mU, 20 h at 37°C), which removes α(1,6)-linked Fuc but not α(1,3)-linked Fuc from Asn-linked GlcNAc (Tomiya *et al.*, 2003). The change in the coordinates (–3.0 GU on the ODS column and −0.4 GU on the amide column) was also consistent with the unit contribution value of α(1,6)-linked Fuc (3.4 GU/0.4 GU) but not α(1,3)-linked Fuc (–1.8 GU/1.1GU) attached to Asn-linked GlcNAc (Tomiya and Takahashi, 1998). These results suggest that PA-glycans 7A, 10A, and 13A have the structures shown in Table I.

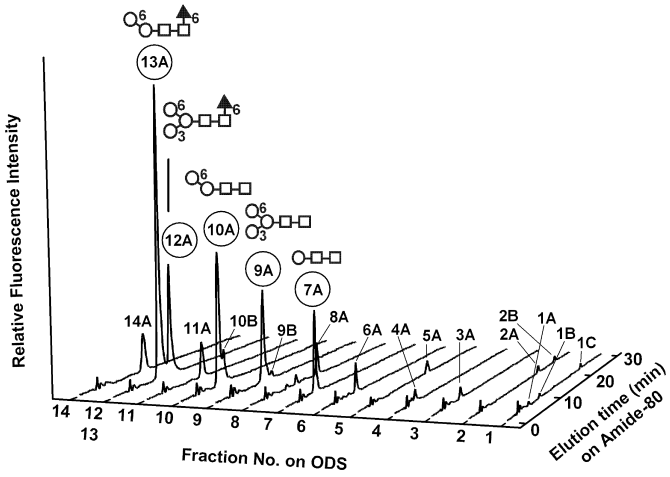


Fig. 3. 2D HPLC chromatogram of PA-glycans derived from the recombinant hTf. The peaks from the ODS column (Figure 2) were subjected to a second separation on a normal phase column (amide-80). Major (>5% of the total glycans) peaks are indicated by circles with numbers, and their structures are shown (squares, GlcNAc; circles, Man; triangles, Fuc).

Molecular masses of PA-glycans 9A ($[M+Na]^+ = 1011.77$) and 12A ($[M+Na]^+ = 1157.98$) indicated that 9A had the common trimannosyl core structure and 12A contained an additional Fuc. The coordinates of PA-glycans 9A on a 2D map coincided with those of a standard PA-glycan (000.1), and those of PA-glycan 12A coincided with those of a standard PA-glycan (010.1), but not with those of a standard PA-glycan (000.1 F) containing Fucα(1,3) instead of Fucα(1,6) (see Scheme 1).

MS data on PA-glycans 11A ($[M+Na]^+ = 1214.97$) indicated that this glycan consisted of the trimannosyl core structure and an additional GlcNAc on either the Manα(1,3)- or the Manα(1,6)-branch. Linkage position of GlcNAc to the trimannosyl core structure in PA-glycan 11A was identified by comparing the coordinates of PA-glycan 11A on a 2D map with those of the standard PA-glycans having GlcNAcβ(1,2) on either the Manα(1,3)-branch (100.2) or the Manα(1,6)-branch (100.1). The coordinates on a 2D map of PA-glycan 11A (9.4 GU, 4.6 GU) was consistent with those of a standard PA-glycan having the trimannosyl core structure having GlcNAcβ(1,2) on the

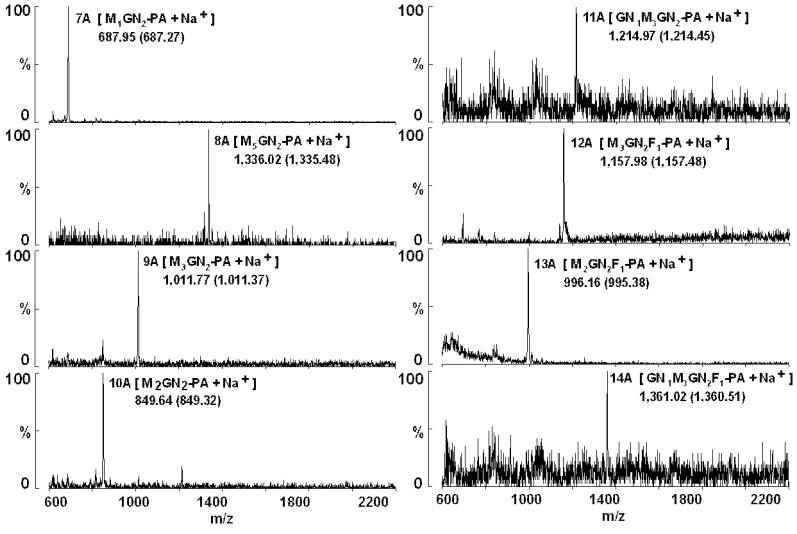


Fig. 4. MALDI-TOF MS spectra of the major PA-glycans from the recombinant hTf expressed in Ld652Y cells. Cells were cultured in serum-free medium. The eight major PA-glycans were isolated by 2D HPLC (Figure 3), and each fraction (~10 pmol) was subjected to MALDI-TOF MS. Peak number and measured mass are shown with the calculated mass (in parentheses).

Manα(1,6)-branch (9.4 GU/4.7 GU) but not with that of the other isomer having GlcNAcβ(1,2) on the Manα(1,3)-branch (7.4 GU/4.7 GU).

The difference between the MS data of PA-glycans 11A and 14A ($[M+Na]^+ = 1361.02$) was equivalent to one Fuc. The coordinates of PA-glycan 14A on a 2-D map (12.8 GU, 5.0GU) coincided with those of the following standard PA-glycan (110.1) within the experimental error (see Scheme 2). Furthermore, the coordinates of PA-glycan 14A on a 2D map shifted to those of PA-glycan 11A (and a standard PA-glycan [100.1]) after α-L-fucosidase digestion, and the magnitude of the change in the coordinates (−3.4 GU/−0.4 GU) was consistent with the unit contribution value of Fuc, which is α(1,6)-linked to GlcNAc adjacent to Asn. These results suggest that PA-glycans 11A and 14A are unfucosylated and α(1,6)-fucosylated monoantennary glycans, respectively, as shown in Table I.

Molecular mass of PA-glycan 8A ($[M+Na]^+ = 1336.02$) corresponds to $Man_5GlcNAc_2$-PA, a high-mannose-type

```
                Manα6
                    \
Code No. 000.1:      Manβ4-GlcNAcβ4-GlcNAc-PA
                    /
                Manα3

                Manα6                Fucα6
                    \                    |
Code No. 010.1:      Manβ4-GlcNAcβ4-GlcNAc-PA
                    /
                Manα3

                Manα6
                    \
Code No. 000.1F:     Manβ4-GlcNAcβ4-GlcNAc-PA
                    /                    |
                Manα3                Fucα3
```

Scheme 1. Structures of the reference PA-glycans 000.1, 010.1, and 000.1F.

```
                GlcNAcβ2Manα6                Fucα6
Code No.                    \                    |
110.1:                       Manβ4GlcNAcβ4GlcNAc-PA
                            /
                        Manα3

                (12.7 GU/ODS, 5.1 GU/amide)
```

Scheme 2. Structure of the reference PA-glycan 110.1.

Table I. Proposed structures of the *N*-glycans found in the recombinant human serum transferrin produced by Ld652Y cells cultured in serum-free medium

Peak (%)	Structure	Peak (%)	Structure	Peak (%)	Structure
7A (6.9)	**Manβ4GlcNAcβ4GlcNAc**	**12A** (11.7)	**Manα6** **Fucα6** \ | **Manβ4GlcNAcβ4GlcNAc** / **Manα3**	5A (1.4)	Manα6 \ Manα6 / \ Manα3 Manβ4GlcNAcβ4GlcNAc / Manα2-Manα3
10A (14.8)	**Manα6** \ **Manβ4GlcNAcβ4GlcNAc**	**11A** (3.6)	**GlcNAcβ2Manα6** \ **Manβ4GlcNAcβ4GlcNAc** / **Manα3**	2A (0.7)	Manα2-Manα6 \ Manα6 / \ Manα3 Manβ4GlcNAcβ4GlcNAc / Manα2-Manα3
13A (34.3)	**Manα6** **Fucα6** \ | **Manβ4GlcNAcβ4GlcNAc**	**14A** (3.8)	**GlcNAcβ2Manα6** **Fucα6** \ | **Manβ4GlcNAcβ4GlcNAc** / **Manα3**	1C (0.2)	Manα2-Manα6 \ Manα6 / \ Manα3 Manβ4GlcNAcβ4GlcNAc / Manα2-Manα2-Manα3
9A (7.8)	**Manα6** \ **Manβ4GlcNAcβ4GlcNAc** / **Manα3**	**8A** (3.6)	**Manα6** \ **Manα6** / \ **Manα3** **Manβ4GlcNAcβ4GlcNAc** / **Manα3**		

Structures of glycans shown in bold were identified by MALDI-TOF MS analysis and 2D mapping technique (Tomiya *et al.*, 1988).
Others were identified by comparison of the coordinates on a 2D map of the glycans with those of authentic standard glycans (Tomiya *et al.*, 1991).

glycan. The branching structure of this glycan was identified by comparing the coordinates of PA-glycan 8A with those of all possible isomers consisting of five Man and two GlcNAc residues (Tomiya *et al*., 1991) and further confirmed by direct cochromatography with a standard high-mannose-type PA-glycan (M5.1).

Presence of α(1,3)-fucosylation in the minor glycans (each ~1% of the total glycans) was also investigated by comparison with the known α(1,3)-fucosylated glycans. Figure 6 shows the elution positions of 11 minor PA-glycans and those of the known glycans having α(1,3)-fucosylated GlcNAc adjacent to Asn, except xylose-containing glycans from plants (Takahashi and Tomiya, 1998; Takahashi *et al*., 1999; Tomiya *et al*., 2003). None of these minor PA-glycans coincided with any of the known PA-glycans containing Fucα(1,3)GlcNAc linked to Asn, suggesting the absence of core α(1,3)-fucosylation.

Coordinates of PA-glycans 5A, 2A, and 1C coincided with those of standard PA-glycans, M6.1, M7.2, and M8.1, respectively. Structural assignment of these glycans was confirmed by comparing their coordinates with those of all possible isomers consisting of two GlcNAc and six, seven, or eight Man residues (Tomiya *et al*., 1991), and by cochromatography with standard PA-glycans M6.1, M7.2, or M8.1. Mass analysis of a total mixture of PA-glycan also indicated the presence of the low levels of $Man_6GlcNAc_2$ ($[M+Na]^+ = 1497.74$), $Man_7GlcNAc_2$ ($[M+Na]^+ = 1663.32$), and $Man_8GlcNAc_2$ ($[M+Na]^+ = 1663.32$).

In summary, the most abundant glycans were Man_{1-3}(±Fucα6)GlcNAc, representing 75.5% of the total *N*-glycans, followed by $GlcNAcMan_3$(±Fucα6)$GlcNAc_2$, representing 7.4% of the total. There was only ~6% of high-mannose-type glycans identified. Nearly half (49.8%) of the total *N*-glycans contained α(1,6)-fucosylation (12A, 13A, and 14A), but α(1,3)-fucosylation on the Asn-linked GlcNAc residue could not be detected within experimental error.

Effects of serum supplementation to the culture medium on the N*-glycosylation of recombinant hTf*

N-glycans of hTf from the Ld652Y cells grown in culture media containing 10% FBS were also analyzed exactly as described for the product from serum-free medium. The *N*-glycan profile on the ODS column showed little difference between these samples, except the total amount of fucosylated glycans (12A, 13A, and 14A) was slightly increased from 49.8% to 55.8% in serum.

Discussion

Earlier studies using dipteran insect cells (Butters and Hughes, 1981; Hsieh and Robbins, 1984) and lepidopteran insect cells with the baculovirus system (Kuroda *et al*., 1990) indicated that insect cells produce mainly high-mannose-type and paucimannosidic-type *N*-glycans. Similar results have been repeatedly shown in subsequent studies (see Altmann *et al*., 1999; März *et al*., 1995). When hTf was expressed in Tn-5B1-4 cells, the distribution of paucimannosidic, high-mannose, and hybrid glycans was 54.0%, 30.8%, and 13.9%, respectively (Ailor *et al*., 2000). Compared with the results of other systems, Ld652Y cells appeared to favor production of high levels of paucimannosidic glycans. The paucimannosidic glycans 7A, 9A, 10A, 12A, and 13A (Table I) represent as much as 76% of the total *N*-glycans. The proportion of high-mannose-type glycans (1C, 2A, 5A, and 8A) was very low (6% of the total). High-mannose-type glycans are commonly found in heterologous glycoproteins from insect cells, especially in intracellular *N*-glycans (Hsu *et al*., 1997). The lysis of cells during the baculovirus infection can lead to the release of proteins carrying such underprocessed *N*-glycans into the medium. In a previous study (Hsu *et al*., 1997), large amounts of high-mannose-type glycans were found intracellularly. This probably was not the case for LdMNPV in this study, because the cell viability was only ~75% at the harvest time, but the total high-mannose-type glycans was only ~6%.

It is worth noting that the cell death following viral infection with LdMNPV is more gradual than that observed using the AcMNPV virus in the previous studies (data not shown). Alternatively, Ld652Y cells may include higher levels of mannosidases, which convert high-mannose-type structures to paucimannosidic structures. Interestingly, 56% of the total *N*-glycans (7A, 10A, and 13A) do not have the Manα1-3GlcNAc branch, and $Man_1GlcNAc_2$ (7A), which has only one Man residue, was as high as 7%. The high proportion of these three *N*-glycans indicates the existence of a highly active α(1,3)-mannosidase in Ld652Y cells.

Ld652Y cells also produced significant amounts of GlcNAc-terminated *N*-glycans (7.4% of the total) as did Tn-5B1-4 cells (Ailor *et al*., 2000). In Tn-5B1-4 cells, the *N*-glycans with GlcNAcβ2Manα3 branch were 10% of the total and those with GlcNAcβ2Manα6 branch, 4% of the total. However, in Ld652Y cells, GlcNAc was found only in the GlcNAcβ2Manα6 branch. It may be that Tn-5B1-4 cells have a higher GlcNAc transferases I activity than Ld652Y, and Ld652Y cells have a higher GlcNAc transferase II activity. Alternatively, the Ld652Y cells may contain a higher level of *N*-acetylglucosaminidase than Tn-5B1-4 cells. An *N*-acetylglucosaminidase that specifically cleaves terminal GlcNAc residues from the GlcNAcβ2Manα3 branch of *N*-glycans has been found in insect cells from *Spodoptera frugiperda*, *Mamestra brassicae*, *Bombyx mori*, and *Trichoplusia ni* (Altman *et al*., 1995a; Kubelka *et al*., 1994; Wagner *et al*., 1996).

Galactosylated *N*-glycans are generally not found in glycoproteins derived from insect cells. Some exceptions are galactosylated *N*-glycans in interferon γ expressed in *Estigmene acrea* cells (Ogonah *et al*., 1996) and in a mouse IgG produced by Tn-5B1-4 cells (Hsu *et al*., 1997). However, no galactosylated *N*-glycan was found when human serum transferrin was expressed in Tn-5B1-4 cells (Ailor *et al*., 2000). This is confirmed by the fact that no detectable change in reverse-phase HPLC was observed (data not shown) after the total mixture of PA-glycans was digested by β-galactosidase.

N-glycans containing core α(1,3)-fucosylation were found in honey bee venom phospholipase A2 and membrane glycoproteins from Sf-21, Mb-0503, Bm-N cells (Kubelka *et al*., 1994). They were also found in a recombinant mouse IgG (Hsu *et al*., 1997) expressed in Tn-5B1-4 cells. On the other

hand, no core α(1,3)-fucosylation was found in the glycans of human interferon ω expressed in Sf9 cells (Voss *et al.*, 1993), interferon γ expressed in Sf9, and *Estigmene acrea* cells (Ogonah *et al.*, 1996). Core α(1,3)-fucosylation was not found in the third eight-cysteine domain of LTBP-1 (Rudd *et al.*, 2000) expressed in Sf9 cells, but it was present in the same protein expressed in Tn-5B1-4 cells (Rudd *et al.*, 2000). α(1,6)-Fucosylation of the Asn-linked GlcNAc is common in mammalian *N*-glycans (Kobata, 1992) and considered innocuous. However, α(1,3)-fucosylation at the same GlcNAc is not found in mammalian glycoproteins and can cause an allergic reaction in humans. Furthermore, the presence of antibodies specific for such a Fuc residue in human blood can possibly affect *in vivo* activity and clearance of biopharmaceutical glycoproteins (Bardor *et al.*, 2003). To have such structures in a glycoprotein makes it less than ideal for therapeutic use (Prenner *et al.*, 1992; Wilson *et al.*, 2001).

The choice of glycoamidase A from sweet almond is important in the current work. It is known that glycoamidase A, but not glycoamidase F (from *Flavobacter*), can release *N*-glycans containing the Fucα(1,3)-GlcNAc-Asn moiety (Altmann *et al.* 1995b; Fan and Lee, 1997; Takahashi and Tomiya, 1992; Tretter *et al.*, 1991). Using the glycoamidase A, we previously found that *N*-glycans in a recombinant hTf expressed in Tn-5B1-4 cells contained as much as 6% of difucosylated trimannosyl core structure (010.1 F) (Ailor *et al.*, 2000), which would have escaped detection if we had used the *Flavobacter* enzyme (see Scheme 3).

Even with the use of glycoamidase A, we could not detect any glycan containing core α(1,3)-fucosylation in the present study. However, the Ld652Y cells may still produce very low levels of core α(1,3)-fucosylated glycans that escaped our detection. At any rate, the present study suggests that the levels of core α(1,3)-fucosylation in Ld652Y cells are negligible or very much lower than that found in Tn-5B1-4 cells. This may be an advantage if gypsy moth–derived cell lines are to be used for glycoprotein production for human use.

Insect cells can grow in serum-containing, serum-free, or even protein-free growth medium. Our results show that inclusion of FBS did not affect *N*-glycosylation patterns in Ld652Y cells except for a small increase in α(1,6)-fucosylated glycans. Elimination of serum from the culture medium will further contribute to lowering the production costs of glycoproteins.

Mammalian cells express sialylated complex-type *N*-glycans on glycoproteins, but Ld652Y cells, like most other insect cell lines, lack the capacity to produce complex-type *N*-glycans containing Gal and sialic acid residues. Other modifications leading to complex-type structures (including tri- and tetraantennary structures), are also not present in Ld652Y cells. To produce more "humanized" glycoproteins in insect cells, such deficiencies must be overcome. These include elimination of core α(1,3)-fucosylation, enhancement of GlcNAc transferases I and II, and β(1,4)-galactosyltransferase activity, introduction of the sialylation module (Altmann *et al.*, 1999; Hollister *et al.*, 2002; Lawrence *et al.*, 2000; Laurence *et al.*, 2001) and suppression of a certain specific β-*N*-acetylglucosaminidase activity (Altman *et al.*, 1995a; Watanabe *et al.*, 2002). More "mammalian-like" *N*-glycans are being produced in insect cells by the introduction of genes of processing-related enzymes (Ailor *et al.*, 2000; Aumiller and Jarvis, 2002; Breitbach and Jarvis, 2001; Hollister *et al.* 1998, 2002; Hollister and Jarvis, 2001; Tomiya *et al.*, 2003). Gypsy moth–derived cell lines may become a preferable target for genetic engineering if the lack of α(1-3)-fucosylation can be profitably utilized.

```
                Manα6              Fucα6
Code No.          \                  |
010.1F:            Manβ4GlcNAcβ4GlcNAc-PA
                  /                  |
               Manα3              Fucα3
```

Scheme 3. Structure of a difucosylated trimannosyl core structure.

Materials and methods

Materials

Excell 420 medium was purchased from JRH Biosicence (Lenexa, KS). FBS and the appropriate cloning kit and restriction enzymes were obtained from Invitrogen (Carlsbad, CA). Glycoamidase A (glycopeptidase A, EC 3.5.1.52) from sweet almond was from Seikagaku America (Falmouth, MA); jack bean β-galactosidase, jack bean β-*N*-acetylhexosaminidase, bovine kidney α-L-fucosidase, 2-aminopyridine, sodium cyanoborohydride, and apo-human transferrin were from Sigma-Aldrich (St. Louis, MO). Dowex 50×2 (H^+) and Sephadex G-15 (medium) were from Dow Chemical (Midland, MI). Shim-pack CLC-ODS column (6×150 mm) was from Shimadzu USA (Columbia, MD). Amide-80 column (4.6×250 mm) was from Tosoh Biosep (Montgomeryville, PA).

Generation of a recombinant hTf-expressing virus strain of LdMNPV

Polyh-htf-pMT/V5-His was constructed to contain the hTf gene (Yang *et al.*, 1984) with accompanied sequence at the 3′ end to generate a V5 tag as well as six histidine residues under the control of the LdMNPV *polyhedrin* promoter (Bischoff and Slavicek, 1996). The following sequence was added to the 3′ end of the hTf gene to generate the V5 epitope and the six his tag: CTC GAG TCT AGA GGG CCC TTC GAA GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC CAT CAC CAT TGA.

To generate a transplacement vector for construction of a recombinant virus, cosmid clone P313 (Riegel *et al.*, 1994) was digested with *Bam*HI and *Hin*dIII, and the 9294-bp fragment from 128,400–137,694 (Kuzio *et al.*, 1999) was cloned into the *Bam*HI and *Hin*dIII sites of pBS sk+ to generate pNP-EGT-9.3. The pNP-EGT-9.3 was digested with *Bst*eII and blunt ended with Klenow. The polyh-htf-pMT/V5-His was digested with *Spe*I and *Pme*I, and the *Spe*I/*Pme*I fragment was isolated and the ends were filled with Klenow. The *Spe*I/*Pme*I fragment from clone polyh-htf-pMT/V5-His was ligated into the

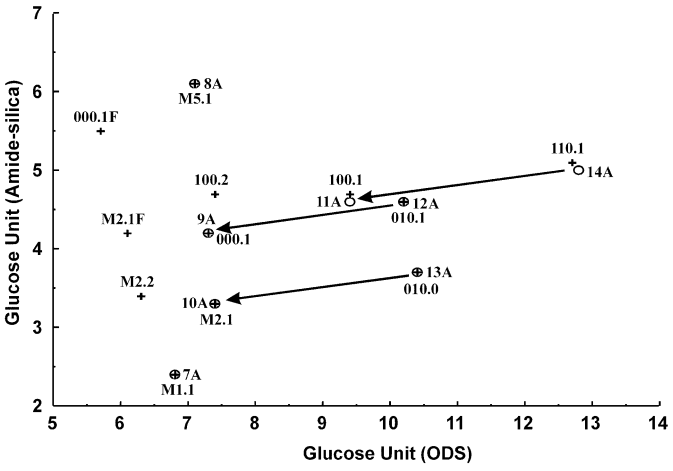
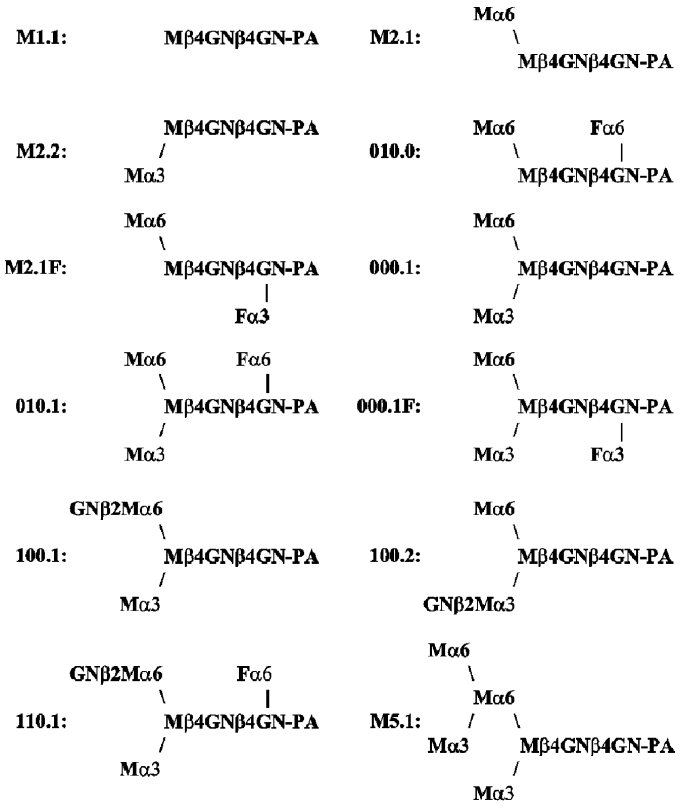


Fig. 5. 2D map aided structural characterization of the major glycans from the recombinant hTf expressed by Ld652Y cells cultured in serum-free medium. The arrows indicate the changes in the coordinates of PA-glycans by α-L-fucosidase digestion. Symbols: circles, major glycans from recombinant hTf; plus signs, reference PA-glycans. Structures of the reference PA-glycans are as follows. M, Man; GN, GlcNAc; F, Fuc.



Scheme 4. Structures of the reference PA-glycans used for analysis of the major glycans shown in Figure 5.

*Bst*eII digested pNP-EGT-9.3 to generate the transplacement vector polyh-htf-his6EGT-. This transplacement vector contains the gene and lacks most of the EGT gene (from 120,641–121,613). Viral strain 122bEGT-LacZ+ was used to generate a recombinant LdMNPV strain expressing the hTf gene (Slavicek *et al.*, 2001). This viral strain had the *LacZ* gene in place of the EGT gene. Viral isolate 122bEGT-LacZ+ genomic DNA and the transplacement vector polyh-htf-his6EGT- were cotransfected into Ld652Y cells as described previously (Bischoff and Slavicek, 1996). Budded virus from the transfection was plaque-purified, and several clear plaques were isolated and plaque-purified again. The purified viral isolates were propagated in Ld652Y cells; genomic DNA was isolated and analyzed by restriction endonuclease digestion (data not shown).

Cell culture and virus infection

Ld652Y cells established from *L. dispar* and LdMNPV were used as a host and virus system. The cells were maintained as described previously (Bischoff and Slavicek, 1996). A suspension culture of Ld652Y cells was maintained at 27°C and rotated at 120 rpm in 250-ml shaker flasks containing 30 ml Excell 420 media. For the recombinant hTf production, 10 100-ml shake flasks, containing serum-free Excell 420 media with or without 10% FBS, were seeded with 7×10^7 Ld652Y cells. The cells were infected with LdMNPV-hTf at 1.0 tissue culture infectious dose ($TCID_{50}$) unit per cell. $TCID_{50}$ was determined as described previously (Slavicek *et al.*, 2001). The cells were incubated at 27°C and rotated at 120 rpm. Five days postinfection, the culture media containing hTf were harvested after removal of cells by centrifugation.

Purification of recombinant hTf from culture medium

The culture supernatant containing hTf (1 L) was concentrated by ultrafiltration, and proteins were precipitated by adding ammonium sulfate (50% final saturation). After removing the precipitate by centrifugation at 8000 rpm for 15 min at 4°C, proteins including hTf were precipitated by adding ammonium sulfate to the supernatant (80% final saturation) and collected by centrifugation. The precipitate was dissolved in buffer A (10 mM Tris–HCl, pH 7.0), and dialyzed against buffer A at 4°C. The hTf-containing sample solution was then applied to a column (ϕ1.6 × 6 cm) of anti-hTf-IgG immobilized Sepharose 4 Fast Flow (Tomiya *et al.*, 2003) equilibrated with buffer A containing 0.5 M NaCl. After unbound proteins were washed off with the same buffer, hTf was eluted with 0.1 M glycine–HCl, pH 2.7, containing 0.5 M NaCl. The eluate was immediately neutralized with 0.5 M Tris–HCl, pH 8.3, dialyzed against water, lyophilized, and used for carbohydrate analyses. The purity of the recombinant hTf preparation was analyzed by SDS–PAGE (10% acrylamide) under nonreducing condition, and proteins were visualized by Coomassie brilliant blue R-250 staining.

Purification and derivatization of N-glycans from recombinant hTf

N-glycans were prepared from the purified recombinant hTf as described previously (Ailor *et al.*, 2000). Briefly, a trypsin-chymotrypsin (each 1%, w/w, of the substrate protein) digest of hTf (5 mg) was treated with glycoamidase A (0.4 mU) in 100 mM sodium citrate-phosphate, pH 5, at 37°C overnight, and the mixture was passed through a

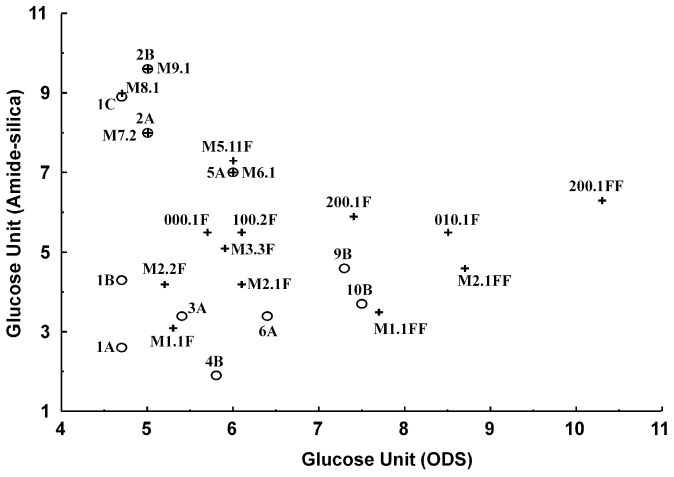
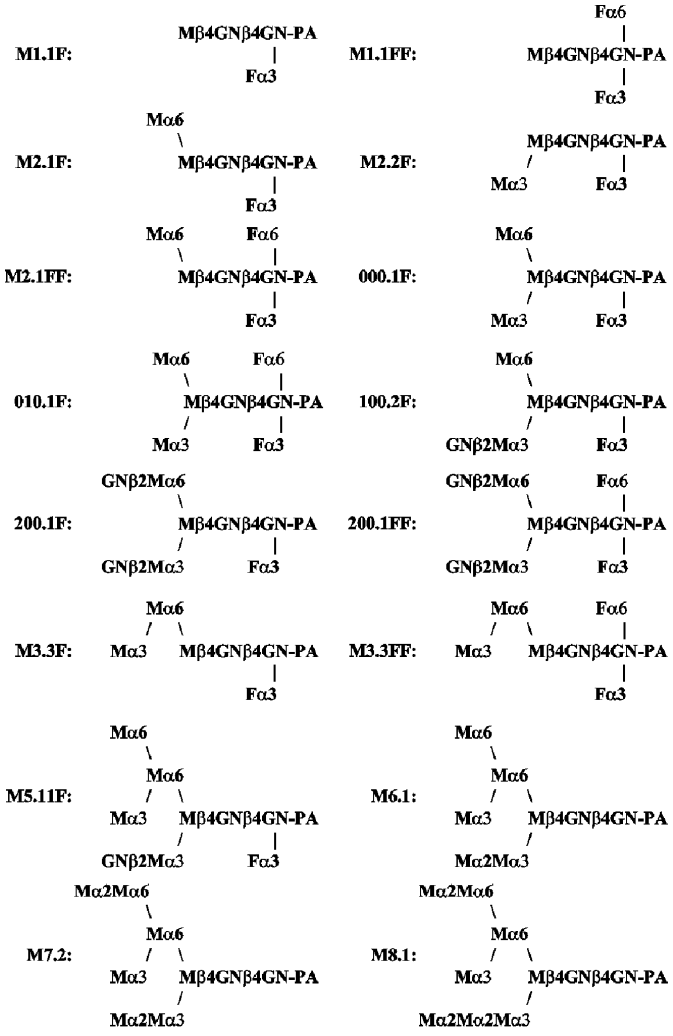


Fig. 6. Comparison of the coordinates on a 2D map of 11 minor PA-glycans with those of reference glycans of high-mannose type and known α(1-3)-fucosylated glycans except xylose-containing glycans from plants (Takahashi, and Tomiya, 1998; Takahashi *et al.*, 1999; Tomiya *et al.*, 2003). Symbols: plus signs, reference PA-glycans; circles, minor glycans from recombinant hTf expressed by Ld652Y cells. Structures of the reference PA-glycans are shown in schemes.

Dowex 50 × 2 (H^+) column (1 ml). The purified glycans in the effluent thus obtained were lyophilized and derivatized by reductive amination with 2-aminopyridine and sodium cyanoborohydride (Nakagawa *et al.*, 1995; Yamamoto *et al.*, 1989), and the PA-derivatized glycans were purified by gel filtration on a Sephadex G-15 column (1.0 × 40 cm) using 10 mM NH_4HCO_3 as eluant.

Isolation and characterization of PA-glycans by two different HPLC steps

The LC-10A HPLC system (Shimadzu USA) was used to analyze the PA-glycans. The PA-glycan mixture was separated and characterized by 2D sugar mapping technique as described previously (Takahashi *et al.*, 1995; Tomiya *et al.*, 1988). PA-derivatized glycans were monitored by fluorescence ($\varepsilon_{ex} = 300$ nm, $\varepsilon_{em} = 360$ nm). The HPLC conditions for analytical chromatography with two columns were the same as described previously (Tomiya *et al.*, 1988). The PA-glycans were successively separated on a reverse-phase column, Shim-pack CLC-ODS (6 × 150 nm), and a normal phase column, Amide-80 (4.6 × 250 mm). The elution time normalized as the elution position of GU, based on elution positions of PA-isomalto-oligosaccharides (DP 4–20). The elution times (in min) of the glycans of interest in ODS and amide-80 column chromatography were converted to GU and plotted on the *x*-axis (ODS column) and *y*-axis (amide-80 column). The resultant 2D map for all PA-glycans was compared with those of known PA-glycans and confirmed by cochromatography with reference glycans. The reference glycans were obtained from human immunoglobulin G, ribonuclease B, and recombinant hTf expressed in *T. ni* cells using the same procedure (Tomiya *et al.*, 2003).



Scheme 5. Structures of the reference PA-glycans used for analysis of the minor glycans shown in Figure 6. M, Man; GN, GlcNac; F, Fuc.

MALDI-TOF MS analysis

The Kompact SEQ MALDI-TOF mass spectrometer (Kratos Analytical, Manchester, UK) was used to analyze PA-glycans in the linear positive-ion mode using 20 mg/ml of 2,5-dihydroxybenzoic acid as reported previously (Papac *et al.*, 1998). The matrix was dissolved in a 1:1 (v/v) mixture of ethanol:10 mM sodium chloride. To crystallize the sample PA-glycans, 0.5 μl of the matrix was placed on a sample plate to which 0.5 μl of the sample (usually 10 pmol) followed by 0.5 μl of matrix were added, and air-dried. A mixture of PA-isomalto-oligosaccharides (DP = 4–20) was used for calibration.

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Abbreviations

AcMNPV, *Autographa californica* multicapsid nucleopolyhedrovirus; FBS, fetal bovine serum; GU, glucose unit; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; HPLC, high-performance liquid chromatography; hTf, human transferrin; LdMNPV, *Lymantria dispar* multicapsid nucleopolyhedrovirus; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MS, mass spectrometry; PA, pyridylamino; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; $TCID_{50}$, tissue culture infectious dose.

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