Production and N-Glycan Analysis of Secreted Human Erythropoietin Glycoprotein in Stably Transfected Drosophila S2 Cells

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Abstract: Schneider 2 (S2) cells from Drosophila melanogaster have been used as a plasmid-based, non-lytic expression system for foreign proteins. Here, a plasmid encoding the human erythropoietin (hEPO) gene fused with a hexahistidine (His6) tag under the control of the Drosophila metallothionein (MT) promoter was stably transfected into Drosophila S2 cells. After copper sulfate induction, transfected S2 cells were found to secrete hEPO with a maximum expression level of 18 mg/L and a secretion efficiency near 98%. The secreted hEPO from Drosophila S2 had an apparent molecular weight of about 23 ~ 27 kDa which was significantly lower than a recombinant hEPO expressed in Chinese hamster ovary (CHO) cells (about 36 kDa). N-glycosidase F digestion almost completely eliminated the difference and resulted in the same molecular weight (~20 kDa) of de-N-glycosylated hEPO proteins. These data suggest that recombinant hEPO from S2 cells was modified with smaller N-glycans. Subsequently, the major N-glycans were identified following glycoamidase A digestion, labeling with 2-amino-pyridine (PA), and two-dimensional high-performance liquid chromatography (HPLC) analysis in concert with exoglycosidase digestion. This analysis of N-glycans revealed that hEPO was modified to include paucimannosidic glycans containing two or three mannose residues with or without core fucose. A similar glycosylation pattern was observed on a recombinant human transferrin expressed in S2 cells. These results provide a detailed analysis of multiple N-glycan structures produced in a Drosophila cell line that will be useful in the subsequent application of these cells for the generation of heterologous glycoproteins. © 2005 Wiley Periodicals, Inc.

Keywords: Drosophila S2 cells; glycoprotein; human erythropoietin; N-glycan structure

INTRODUCTION

Many of the most valuable biopharmaceuticals are secreted recombinant proteins. In addition, secreted protein expression can be advantageous because it facilitates downstream purification processes. Many secreted proteins often require post-translational modifications for their biological activities. During secretion of recombinant proteins via the endoplasmic reticulum (ER) and Golgi apparatus, the proteins must undergo proper folding, assembly, and other post-translational modifications including glycosylation. The glycoprotein, human erythropoietin (hEPO), is a principal growth factor responsible for stimulation of the proliferation and differentiation of responsive bone marrow erythroid precursor cells to more mature erythrocytes (Goldwasser and Kung, 1968). Native hEPO, which consists of 165 amino acids and has a molecular weight of approximately 30 kDa (Jacobs et al., 1985), is synthesized primarily in the adult kidney. Recombinant hEPO expressed in Chinese hamster ovary (CHO) cells has been used to treat anemia induced by impaired erythrocyte production, cancer, and several other pathological conditions (Ludwig et al., 1995).

Native hEPO protein is characterized by three N-linked complex tetra-antennary oligosaccharides (at amino acid residues 24, 38, and 83) and one O-linked oligosaccharide at
residue 126. These carbohydrate chains account for about 40% of the molecular mass (Lai et al., 1986). These glycans, especially the N-glycans, play an important role in the biological and physical activities of the protein by controlling the secretion, biological function, and in vivo circulatory half-life of hEPO (Dordal et al., 1985; Kornfeld and Kornfeld, 1985; Takeuchi and Kobata, 1991; Tsuda et al., 1990). Site-directed mutagenesis revealed that the N-glycans at positions 38 and 83 were associated with secretion of hEPO (Delorme et al., 1992). Comparisons of N-glycans from natural urinary hEPO versus recombinant hEPO (Delorme et al., 1992). Comparisons of N-glycans from natural urinary hEPO versus recombinant hEPO proteins produced in mammalian CHO or baby hamster kidney (BHK) cells (Hokke et al., 1990, 1995; Nimtz et al., 1993; Rahbek-Nielsen et al., 1997; Tsuda et al., 1988) revealed that the N-glycans differed somewhat in their monosaccharide composition and structure, especially regarding the N-acetyllactosamine repeats and sialylation.

There have been many attempts to produce human therapeutic proteins in numerous insect cell lines. The baculovirus expression system has been particularly popular in combination with cell lines such as Spodoptera frugiperta and Trichoplusia ni (Cha et al., 1999a; Joosten and Shuler, 2003; Miller, 1988; Smith et al., 1983). Unfortunately, these systems have had limited commercial use due to a lack of complex glycosylation for many lepidopteran insect cell lines (Jarvis and Finn, 1996; Martin et al., 1988). As compared to their vertebrate counterparts, N-glycans observed on many of these lepidopteran insect cell lines have been characterized by oligomannose-type glycans and paucimannosidic glycans sometimes including a core fucose (Fuc) in the α(1,3) and/or α(1,6) position (Possee, 1997; Tomiya et al., 2003a, 2004; Varki et al., 1999). Previously, hEPO was successfully expressed in insect cells using a baculovirus system (Quelle et al., 1989; Wojchowski et al., 1987). Unfortunately, the baculovirus expression system is lytic in nature and such a system may not be ideal for secretory proteins since the cell membrane can lyse and release intracellular contents and also the infection mechanism may have negative effects on post-translational processing (Ikonomou et al., 2003).

An alternative plasmid-based non-lytic expression system has been developed in the insect cell line, Schneider 2 (S2) cells derived from Drosophila melanogaster (Schneider, 1972). In this system, high copy numbers of recombinant plasmid vectors are inserted into the host cell genome, allowing, after the addition of a chemical inducer, continuous production of the desired foreign protein without cell destruction (Hill et al., 2001; Johansen et al., 1989; Kirkpatrick et al., 1995; Nilsen and Castellino, 1999). Here, we investigated the secretion and detailed N-glycan structures of recombinant hEPO produced in an insect Drosophila S2 cell suspension culture. The recombinant hEPO was a fusion construct including a hexahistidine (His₆) epitope tag that enabled simple purification using immobilized metal affinity chromatography (IMAC). Subsequently, N-glycans were released by glycoamidase A from the purified protein, labeled with 2-aminopyridine (PA), and their structures were analyzed by two-dimensional high-performance liquid chromatography (HPLC) and exoglycosidase digestion in order to elucidate major N-glycan structures obtained from Drosophila S2 cell-expressed recombinant glycoproteins. A second purified protein, recombinant human serum transferrin (hTf), was also analyzed in order to determine consistency in the N-glycan profile. The present study suggests that recombinant glycoproteins expressed in Drosophila S2 cells contain paucimannosidic glycosylation patterns containing one to three mannose residues sometimes including fucose attachments.

MATERIALS AND METHODS

Cell Culture

Stably transfected Drosophila S2 cell lines (Shin et al., 2003) with pMT/BiP/hEPO (Shin and Cha, 2002) that contains the Drosophila metallothionein (MT) promoter that is activated by Cu²⁺ and BiP signal sequence to facilitate secretion were used for hEPO expression. To produce secreted hEPO from the developed S2 cell lines, cells were initially grown to 2 × 10⁶ cells/mL (over 95% viable) in M3 medium (Shields and Sang M3 insect medium; Sigma, St. Louis, MO) with 10% insect medium supplement (IMS; Sigma) and 300 µg/mL hygromycin B (Sigma) using three 100 mm cell culture dishes and then transferred into a 500 mL spinner flask (Wheaton, Millville, NJ). One hundred fifty microliter and 400 mL serum-free M3 media were used for investigation of growth and expression profiles and for purification of recombinant hEPO, respectively. Cells were cultured at 27°C with constant stirring (80 rpm) until a cell density of at least 6 × 10⁶ cells/mL was reached, after which copper sulfate was added (500 µM) to induce expression of the recombinant proteins.

Analytical Methods

Cell number was counted using a hemacytometer (Fisher Scientific, Pittsburgh, PA) and cell viability was determined by trypan blue (Sigma) exclusion using a 0.4% (w/v) solution. Total protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as a protein standard. The quantity of recombinant hEPO was determined using purified recombinant hEPO from CHO cells (kindly provided by Yuhan Corp., Korea) as a calibration standard for Western blot. The cell culture broth was divided into cellular and secreted (conditioned medium) fractions, and the presence of hEPO in each fraction was determined by Western blot analysis. Secretion efficiency of recombinant hEPO was defined as the secreted quantity in the medium divided by the total (secreted and cellular) quantity.

SDS–PAGE and Western Blot Analysis

Samples were mixed with a sample buffer (10% SDS, 10% β-mercaptoethanol, 0.3M Tris-HCl (pH 6.8), 0.05%
bromophenol blue, and 50% glycerol), boiled for 5 min, and resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), followed by silver staining (Bio-Rad) or electrophoretic transfer to a Hybond™-PVDF membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) for Western blot. After blocking for 1 h in Tris Buffered Saline (TBS; 20 mM Tris-HCl, pH 7.5 and 500 mM NaCl) containing 5% non-fat dry milk, the membrane was incubated in an antibody solution (1% non-fat dry milk in TBS with 0.05% Tween-20 (TTBS) containing rabbit anti-hEPO polyclonal antibodies (1:1,000 v/v) (R&D Systems Inc., Minneapolis, MN), and probed with a goat anti-rabbit monoclonal antibody conjugated with alkaline phosphatase (1:1,000 v/v) (Sigma). After successive washing with TTBS and TBS, FAST Red TR/Naphthol AS-MX (4-Chloro-2-methylbenzene diazonium/3-hydroxy-2-naphthoic acid 2, 4-dimethylanilide phosphate; Sigma) was added for detection, and the reaction was quenched with distilled water. The developed membrane was scanned, and a digitized image was analyzed by Gel-pro Analyzer software (Media Cybernetics, Silver Spring, MD).

**Affinity Purification**

Three days after the induction of His_{6}-tagged hEPO expression, the culture medium (400 mL) was harvested, and clarified by centrifugation at 5,000 rpm for 5 min. The medium was concentrated by ultrafiltration using a 10,000 molecular weight cut-off membrane (Amicon stirred cell, Model 8200; Millipore, Bedford, MA). Copper sulfate, the inducer of protein expression, was removed from the concentrated medium by dialyzing twice against 50 mM NaH_{2}PO_{4}—bromophenol blue, and 50% glycerol), boiled for 5 min, and resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), followed by silver staining to determine purification purity and yield.

**De-N-Glycosylation of Purified Recombinant hEPO**

The samples (each contained 1 µg protein) were heated at 100°C for 5 min in the presence of 1% SDS, and then digested by N-glycosidase F (2 mU) (Roche Molecular Biochemicals, Mannheim, Germany) at 37°C for 16 h in the presence of 2% Triton X-100. The de-N-glycosylated proteins were analyzed by 12.5% SDS–PAGE with silver staining.

**Analysis of N-Glycans**

The purified recombinant hEPO was dissolved in distilled water, and heat denatured, and digested overnight with trypsin (1%, w/w) in 200 mM Tris-HCl, 100 mM MgCl_{2} buffer (pH 8.0) at 37°C. After heating the sample in a boiling water for 5 min, glycopeptides were digested overnight with glycoamidase A (also called glycopeptidase A) (Seikagaku America, East Falmouth, MA) at pH 5. Released oligosaccharides were partially purified by passing the samples through a Dowex 50 (H\(^{+}\)) column (1 mL), lyophilized, and derivatized with PA as previously described (Kondo et al., 1990). The PA-derivatized N-glycans were purified by gel filtration on a Sephadex G-15 column (1.0 x 40 cm; Amersham Biosciences, Uppsala, Sweden) using 10 mM NH_{4}HCO_{3} as an eluant. The PA-glycans were analyzed with an LC-10Avp HPLC system (Shimadzu Scientific Instruments, Columbia, MD). The sample PA-N-glycans were separated by normal-phase HPLC using an Amide-80 column (2 x 250 mm; Tosoh Biosep, Montgomeryville, PA) (first dimension). Elution was performed by a linear gradient of acetonitrile (50%–80%, v/v) in 10 mM ammonium formate, pH 7.0, and monitored by fluorescence (λ_{ex} = 300, λ_{em} = 360 nm), and major peaks were collected. Each fraction was dried up, and dissolved in water, and subjected to reversed-phase HPLC using a Shim-Pack VP-ODS column (2 x 150 mm; Shimadzu Scientific Instruments) (second dimension). Elution was performed by a linear gradient of 1-butanol (0.1%–0.25%, v/v) in 10 mM sodium phosphate, pH 4.3), and monitored by fluorescence (λ_{ex} = 315, λ_{em} = 385 nm). Each column was calibrated with a mixture of PA-isomalto-oligosaccharides prior to the analyses of sample PA-glycans (Tomiya et al., 1988).

**RESULTS**

**Secreted Production and Purification of Recombinant hEPO by Drosophila S2 Cells**

Stably transfected Drosophila S2 cells were cultured in 150 mL serum-free medium in a 500-mL spinner flask for expression of secreted recombinant hEPO. Prior to induction, the S2 cells were healthy, with over 95% viability (Fig. 1). The Drosophila MT promoter was employed to induce recombinant hEPO expression by treatment of S2 cells (6 x 10^{6} cells/mL) with copper sulfate. After induction, the cells stopped growing and showed a rapid decrease in viability, likely due to toxic nature of copper sulfate (Guecheva et al., 2001; Kishimoto et al., 1992; Radyuk et al., 2003) and/or the metabolic burden of foreign protein expression.

We quantified the secreted and cellular-expressed recombinant hEPO using Western blot analysis (Fig. 2A), from which we established secretion and cellular expression.
profiles for hEPO (Fig. 2B). While the cellular hEPO concentrations were minimal throughout the culture period, the quantity of secreted hEPO increased continuously with culture time, reaching a maximum of ~18 mg/L around 7 days after induction. This secretion level was substantially higher than that seen for hEPO in the mammalian CHO cell system (about 1.4 mg/L) in one previous study (Davis et al., 1987). However, production of other human proteins in the S2 cell system (2.3 mg/L for human interleukin-2 (hIL-2) (Shin et al., 2003) and 40.8 mg/L for hTF (Lim et al., 2004)) have shown that secretion levels in S2 cells can vary depending on the foreign protein being expressed. Secretion efficiency, defined as the secreted quantity in the medium divided by the total quantity, was found to be consistent at around 97%–98% (Fig. 2C). These results indicate that the stably transfected S2 cell system had relatively efficient secretion of the synthesized hEPO, consistent with previous reports on production of S2-derived recombinant hTF (Lim et al., 2004). Interestingly, two prominent protein bands were observed inside the cells in the Western blot of Figure 2 expressed by S2 cells to suggest the intracellular accumulation of variants of hEPO. Since the recombinant hEPO was fused with a His6 tag, we employed IMAC for purification and analyzed purification using silver-stained SDS–PAGE (Fig. 3). About 2.3 mg of the purified recombinant hEPO was obtained from 400 mL of the culture with a yield of 33% and a purity of 96%.

Figure 1. Cell growth and viability of a recombinant hEPO-expressing S2 cells in a total volume of 150 mL culture. Protein expression was induced with 500 μM copper sulfate on day 2. Each measured value represents the average of two independent cultures.

Figure 2. A: Western blot analysis of secreted and cellular hEPO from S2 cells; (B), concentration of hEPO in the culture supernatant and cellular fraction; (C), secretion efficiency from transfected S2 cells. Twelve percent SDS–polyacrylamide gels and anti-hEPO antibody were used for the analysis. 1×, sample without concentration; 50×, 50-fold concentrated sample. Each measured value represents the average of two independent culture experiments.

Size Comparison of Recombinant hEPO From S2 Cells and CHO Cells

We compared the hEPO expressed and purified from S2 cells with recombinant hEPO obtained from CHO cells (Fig. 4, lanes 1 and 3). In S2 cells, we detected a relatively broad band, possibly comprising up to three bands, of expressed proteins with a molecular weight of ~25 kDa (ranging from 23 to 27 kDa) (Fig. 4, lane 3). This apparent molecular weight was significantly lower than that of CHO cell-derived hEPO.

Figure 3. SDS–PAGE analyses of proteins in each step of purification. Lane M, marker proteins; lane 1, post-ultrafiltration; lane 2, post-dialysis; lane 3, post-IMAC purification; lane 4, post the second dialysis. Proteins were visualized by silver staining.
In order to obtain further structural information on the N-glycosylation patterns of recombinant hEPO from S2 cells, we subjected the N-glycans from purified hEPO to two-dimensional HPLC analysis (Tomiya et al., 1988; Tomiya and Takahashi, 1998). N-glycans released from the hEPO by glycoamidase A (from sweet almond) digestion were purified and labeled with PA. The resultant pyridylamino (PA) derivatives of the glycans were separated by normal-phase HPLC using an amide-silica column. Quantity of PA-glycans in the peaks was estimated based on their peak areas. As shown in Figure 5, four major peaks (peaks 1, 2, 3, and 4), accounting for 80% of the total N-glycans, were resolved by the normal-phase HPLC. The elution position of peak 1, 2, 3, and 4 coincided with those of authentic PA-glycan standards b, c, d, and e listed in the legend of Figure 5. The recombinant hEPO expressed in S2 cells did not contain significant levels of any complex-type N-glycans (such as GlcNAc-terminated or Gal-terminated biantennary glycan) and oligomannose-type N-glycans. The major PA-glycan fractions were then subjected to reversed-phase HPLC. The results from the two columns generated a two-dimensional glycan map (Fig. 6A). Peaks 1 and 3 appeared to contain primarily single species of glycan, respectively while peaks 2 and 4 were resolved into two peaks, respectively, on the second octa-decyl silica (ODS)-column. In addition to coincidence with the authentic

Figure 4. SDS–PAGE analyses of intact and de-N-glycosylated hEPO from CHO cells and S2 cells. Purified hEPO from CHO and S2 cells were treated with N-glycanase F (2 mU at 37 °C for 16 h). The intact and N-glycosidase F treated samples were subjected to SDS–PAGE (12% acrylamide gel). Lane M, marker proteins; lane 1, purified hEPO (intact) from CHO cells; lane 2, N-glycosidase F digested hEPO from CHO cells; lane 3, purified hEPO (intact) from S2 cells; lane 4, N-glycosidase F digested hEPO from S2 cells.

Figure 5. A: Normal-phase HPLC elution profile of PA-glycans derived from a recombinant EPO expressed in Drosophila S2 cells. N-glycans were prepared from purified hEPO, derivatized, and separated on an Amide-80 column as described in Materials and Methods. Arrows indicate the elution positions of reference PA-glycans. Peaks 1, 2, 3, and 4 were coincidental with the reference PA-glycans b, c, d, and e, respectively. B: Reference PA-glycans. Closed square, GlcNAc; circle filled with gray, Man; triangle filled with gray, Fuc; open circle, Gal.

Structure of Major N-Glycans in a S2 Cell-Expressed Recombinant hEPO

In order to obtain further structural information on the N-glycosylation patterns of recombinant hEPO from S2 cells,
standard PA-glycans, elution position of peaks 1 and 3 coincided with the standard PA-glycans \( b \) and \( d \), respectively, on the ODS-column to confirm their identities as \( \text{Man}_{a}(1,6)\text{Man}_{b}(1,4)\text{GlcNAc}_b(1,4)\text{GlcNAc}-PA \) (peak 1 in Table I). PA-glycans 2a, 2b, 4a, and 4b were subjected to a mild \( \alpha \)-fucosidase that could remove core \( \alpha(1,6) \)-linked Fuc but not core \( \alpha(1,3) \)-linked Fuc (Tomiya et al., 2003b), and then the enzyme digests were analyzed both by normal-phase and reverse-phase HPLC (Fig. 7A). Elution position of each peak expressed in terms of time was converted into a value expressed in glucose unit (GU) and plotted on a 2D-map (Fig. 7B). The unit contributions of relevant sugar residues to the elution position (expressed in Glc units) of an \( N \)-glycan have been previously documented (Tomiya and Takahashi, 1998). The coordinates of PA-glycans \( 2b \) and \( 4b \) on the 2D-map coincided with those of authentic standard PA-glycans \( c \) and \( e \) listed in the legend of Figure 5. Furthermore, their coordinates shifted to those of PA-glycans 1 (and standard \( b \)) and 3 (and standard \( d \)), respectively (Fig. 7B) following fucosidase digestion to confirm that PA-glycans \( 2b \) and \( 4b \) have core \( \alpha(1,6) \)-linked Fuc on PA-glycans 1 and 3, respectively, and their structures were identified as shown in Table I. By fucosidase digestion, the coordinates of PA-glycans 2a and 4a on the 2D-map shifted in parallel to the changes observed with PA-glycans 2b and 4b indicating PA-glycans 2a and 4a also have \( \alpha(1,6) \)-linked Fuc. However, the exact structures of peak 2a and 4a that represent about 2% and 11% of the total \( N \)-glycans, respectively, could not be identified at this time as their elution positions did not correspond with known standards in our hands. In total, all specified \( N \)-glycans from recombinant hEPO expressed in \textit{Drosophila} S2 cells consist of paucimannosidic structures. The most abundant glycan (peak 4b), representing 39% of the total \( N \)-glycans, was \( \text{Man}_a(1,3)[\text{Man}_a(1,6)\text{Man}_b(1,4)\text{GlcNAc}_b(1,4)\text{GlcNAc}-PA \) (peak 1 in Table I) and another 11% of the \( N \)-glycans (peaks 1 and 2b) included \( \text{Man}_a(1,6)[\text{Man}_b(1,4)\text{GlcNAc}_b(1,4)\text{Fuc}_a(1,6)\text{GlcNAc} \) paucimannosidic structures. Together these

Table I. Structures of major \( N \)-glycans attached to recombinant hEPO and hTf expressed in \textit{Drosophila} S2 cells.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Structure</th>
<th>hEPO (relative amount, %)</th>
<th>hTf (relative amount, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Man(_a)6 \ / \ Man(_b)4GlcNAc(_b)4GlcNAc-PA</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>2b</td>
<td>Man(_a)6 \ / \ Fuc(_a)6 \ / \ Man(_b)4GlcNAc(_b)4GlcNAc-PA</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>Man(_a)6 \ / \ Man(_b)4GlcNAc(_b)4GlcNAc-PA \ / \ Man(_a)3</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>4b</td>
<td>Man(_a)6 \ / \ Fuc(_a)6 \ / \ Man(_b)4GlcNAc(_b)4GlcNAc-PA \ / \ Man(_a)3</td>
<td>39</td>
<td>48</td>
</tr>
</tbody>
</table>
identified N-glycans account for a majority (~71%) of the hEPO N-glycans obtained from Drosophila S2 cells.

In order to evaluate the consistency of N-glycans for recombinant proteins obtained from S2 cells, a second recombinant protein, hTf, expressed and purified from S2 cells (Lim et al., 2004) was subjected to a similar 2D HPLC analysis. The same four major peaks were identified eluting from the amide-silica column for the hTf sample, although the percentages of each differed somewhat. Separation of the four major peaks on the second ODS column resulted in the identification of the same principal N-glycans listed in Table I. The principal hTf N-glycan (peak 4b in Fig. 6B), accounting for 48% of the total, was again a paucimannosidic glycan with trimannosyl core and a core α(1,6)-linked fucose. The non-fucosylated trimannosyl core was also present in significant amounts (~22%). Inclusion of the other specified dimannosyl paucimannosic structures (11% of total N-glycans) led to the identification of 81% of the transferrin N-glycans, all of which consisted of paucimannosidic structures with or without additional Fuc residues.

DISCUSSION

This study represents the first detailed effort to identify the range of N-glycan structures attached to recombinant glycoproteins expressed in Drosophila S2 cells, a widely used insect cell line for glycoprotein expression. Here, we successfully produced secreted recombinant His$_6$-tagged hEPO in high yield (up to 18 mg/L), and purified it by a simple protocol. A purified hEPO had an apparent molecular weight of about 23 ~ 27 kDa in Drosophila S2 cells while CHO cell-derived hEPO had a molecular weight of about 36 kDa. N-glycosidase F treated hEPO proteins from both cell lines showed comparable molecular weights of ~20 kDa, indicating that the secreted hEPO from S2 cells was modified with smaller N-glycans. Glycan analyses of the S2-derived hEPO revealed that the most prominent N-glycan (peak 4b in Fig. 6B) contained three mannoses with Fuc and accounted for 39% of the total N-glycans. A 2D HPLC analyses for the glycan profile of the purified recombinant hTf from S2 cells also showed a similar profile (Fig. 6B). Together, these N-glycan analyses indicate that recombinant glycoproteins expressed in S2 cells are modified by mainly paucimannosidic glycans containing two to three mannoses with or without Fuc. The recombinant hEPO expressed in S2 cells does not contain significant levels of any complex-type N-glycans (such as GlcNAc-terminated or Gal-terminated biantennary glycan) and oligomannosetype N-glycans. The glycan structures reported here clearly indicates that S2 cells do not have capability of synthesizing complex-type N-glycans, instead they produce paucimannosidic glycans. These profiles of the N-glycan structure are similar to those of glycoproteins expressed in other insect cell lines derived from lepidopteran insects such as S. frugiperda and T. ni (Ailor et al., 2000; Davidson et al., 1990; Hsu et al., 1997; Marchal et al., 1999). Earlier studies on endogenous Drosophila glycoproteins revealed oligomannosidic (four ~ nine mannoses) and mono- or di-fucosylated paucimannosidic N-glycans consisting of two ~ three Man residues (Fabini et al., 2001; Williams et al., 1991). We found that N-glycans attached to S2 cell-expressed human glycoproteins include only paucimannosidic structures but do include a number of different forms as compared to the single N-glycan structure (Man$_3$GlcNAc$_2$Fuc) reported previously for human urokinase-type plasminogen activator receptor (Gardsvoll et al., 2004).

Shown in Figure 8 is a proposed N-glycan processing pathway in Drosophila S2 cells. Like in mammalian cells.
and other insect cells, N-glycosylation of a protein in S2 cells is initiated by transfer of a Glc3Man3GlcNAc2 precursor glycan to Asn residue in a Asn-X-Thr/Ser sequon on a nascent polypeptide (Marshall, 1974). The three terminal Glc residues and four Man residues are successively removed by α-glucosidase I, II, ER α-mannosidase (Kerscher et al., 1995), and Golgi α-mannosidase I to produce Man3GlcNAc2 (for recent reviews, see Betenbaugh et al., 2004; Tomiya et al., 2003a). Thereafter, a β(1,2)-linked GlcNAc residue is added to Man3(1,3)Manβ(1,4)-branch by Drosophila β-N-acetylglucosaminyltransferase I (Sarkar and Schachter, 2001) which produces GlcNAc-Man3GlcNAc2. We found as much as 60% of the total N-glycans in the recombinant hEPO were modified with core α(1,6)-Fuc as identified forms (47%) and unidentified forms (13%). In mammalian cells, core α(1,6)-fucosylation first takes place on GlcNAcMan3GlcNAc2 (Longmore and Schachter, 1982; Wilson et al., 1976), therefore, we speculate that core fucosylation in S2 cells also first occurs at this point and possibly later on GlcNAcMan3GlcNAc2, as well, by recently identified Drosophila core α(1,6)-fucosyltransferase (Roos et al., 2002). Removal of two terminal mannose residues on GlcNAcMan3(±Fucα(1,6))GlcNAc2 by α-mannosidase II (Foster et al., 1995; Rabouille et al., 1999) generates GlcNAcMan3(±Fucα(1,6))GlcNAc2. Since no significant levels of GlcNAc-containing glycans were detected in the recombinant hEPO in the present study, it is suggested that S2 cells contain β-N-acetylglucosaminidase activity which efficiently removes β(1,2)-linked GlcNAc in GlcNAcMan3(±Fucα(1,6))GlcNAc2 and generates Man3(±Fucα(1,6))GlcNAc2. The activity of β-N-acetylglucosaminidase was described in another Drosophila melanogaster cell line, Kc (Sommer and Spindler, 1991). A similar enzyme activity was also observed in some other lepidopteran cell lines (Altman et al., 1995; Licari et al., 1993), and in Caenorhabditis elegans (Zhang et al., 2003).

The difference in N-glycosylation patterns between Drosophila and mammalian cells could represent a critical limitation in commercialization of insect cell lines as recombinant protein expression systems for human therapeutic proteins such as hEPO. However, the results in this study and the proposed N-glycan processing pathway in Drosophila S2 cells (Fig. 8) provide clues to overcome this limitation. Indeed, recent efforts in engineering glycosylation pathways in baculovirus/lepidopteran insect cell systems has demonstrated the potential of humanizing N-glycan patterns by expressing several mammalian glycosyltransferases (Ailor et al., 2000; Breitbach and Jarvis, 2001; Hollister et al., 1998, 2002; Hollister and Jarvis, 2001; Jarvis et al., 2001; Seo et al., 2001; Tomiya et al., 2003b), sialic acid pathway enzymes (Lawrence et al., 2000, 2001; Viswanathan et al., 2001, and both glycosyltransferases and sialic acid pathway enzymes (Aumiller et al., 2003). Furthermore, SDS–PAGE analyses of the secreted hEPO (Fig. 4) and Western blot analysis of the intracellular fractions (Fig. 2) indicated that the recombinant protein from S2 cells included variants that may not be fully glycosylated at all three N-glycosylation sites. This underglycosylation may represent insufficient availability of dolicholphosphate-linked oligosaccharide precursor for glycosylation when a glycoprotein is expressed in extremely high level. Future efforts may be needed to address this limitation in processing as well as the lack of complex N-linked oligosaccharides for the effective use of Drosophila S2 cells in the production of human-type glycoproteins.

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**Figure 8.** A proposed N-glycan processing pathway in Drosophila S2 cells. Glc I, α-glucosidase I; Glcase II, α-glucosidase II; ER Manase, ER α-mannosidase; Golgi Manase I, Golgi α-mannosidase I; GlcNAcT I, β(1,2)-N-acetylglucosaminyltransferase I; FucT C6, core α(1,6)-fucosyltransferase (FucT 8); Manase II, α-mannosidase II; GlcNAcase, β-N-acetylglucosaminidase; Manase, α-mannosidase. Closed square, GlcNAc; circle filled with gray, Man; triangle filled with gray, Fuc; closed circle, Glc.
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