J. Microbiol. Biotechnol. (2008), **18**(2), 383–391

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Expression and Characterization of Human *N*-Acetylglucosaminyltransferases and α2,3-Sialyltransferase in Insect Cells for *In Vitro* Glycosylation of Recombinant Erythropoietin

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Received: August 23, 2007 / Accepted: November 4, 2007

The glycans linked to the insect cell-derived glycoproteins are known to differ from those expressed in mammalian cells, partly because of the low level or lack of glycosyltransferase activities. GnT II, GnT IV, GnT V, and ST3Gal IV, which play important roles in the synthesis of tetraantennary-type complex glycan structures in mammalian cells, were overexpressed in *Trichoplusia ni* cells by using a baculovirus expression vector. The glycosyltransferases, expressed as a fusion form with the IgG-binding domain, were secreted into the culture media and purified using IgG sepharose resin. The enzyme assay, performed using a pyridylaminated-sugar chain as an acceptor, indicated that the purified glycosyltransferases retained their enzyme activities. Human erythropoietin expressed in *T. ni* cells (rhEPO) was subjected to *in vitro* glycosylation by using recombinant glycosyltransferases and was converted into complex-type glycan with terminal sialic acid. The presence of *N*-acetylglucosamine, galactose, and sialic acid on the rhEPO moiety was detected by a lectin blot analysis, and the addition of galactose and sialic acid to rhEPO was confirmed by autoradiography using UDP-^{14}C-Gal and CMP-^{14}C-Sia as donors. The *in vitro* glycosylated rhEPO was injected into mice, and the number of reticulocytes among the red blood cells was counted using FACS. A significant increase in the number of reticulocytes was not observed in the mice injected with *in vitro* glycosylated rhEPO as compared with those injected with rhEPO.

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Keywords: Glycosyltransferase, baculovirus, glycoprotein, sialic acid, erythropoietin

The carbohydrate moieties of a glycoprotein play important roles in determining its structure and are involved in various biological functions including cell-cell recognition, differentiation, and proliferation [34]. In mammalian cells, the *N*-glycosylation pathway is initiated by the addition of $Glu_3Man_9GlcNAc_2$ to the Asn residue of a nascent polypeptide in the endoplasmic reticulum (ER) lumen at the Asn-X-Ser/Thr sequence, where X can be any amino acid except Pro. After 3 glucose and 4 mannose molecules are trimmed by glycosidases in the ER and Golgi, the sugar chain is further modified by glycosyltransferases in a stepwise manner to yield a complex type of sugar chain in the Golgi [20].

The baculovirus-insect cell expression system has been widely used to produce recombinant proteins for research and industrial purposes [25]. The major advantages of this expression system are that it can produce large amounts of recombinant proteins and undergoes post-translational modification in a eukaryotic environment [15, 25]. The glycoproteins expressed in insect cells possess *N*-glycans with core structures similar to those produced in the mammalian system, but insect cells fail to glycosylate proteins in precisely the same manner as mammalian cells do [2, 14, 15].

Lepidopteran insect cells generally appear to have extremely low levels of *N*-acetylglucosaminyltransferase II (GnT II), *N*-acetylglucosaminyltransferase IV (GnT IV), *N*-acetylglucosaminyltransferase V (GnT V), and galactosyltranferase activities, and no detectable sialyltransferase activity [2, 14, 26]. Furthermore, some insect cells possess *N*-acetylglucosaminidase that removes the terminal GlcNAc

residue from $GlcNAcMan_3GlcNAc_2$-N-Asn and eliminates the intermediate required for complex *N*-glycan production [14]. Consequently, the major *N*-glycan produced by insect cells is the paucimannose structure $Man_3GlcNAc_2$(±Fuc)-N-Asn.

Glycosyltransferases are generally type II transmembrane proteins consisting of a short *N*-terminal cytoplasmic tail, transmembrane domain, protease-sensitive stem region, and C-terminal catalytic domain [29]. Glycosyltransferases were successfully expressed in insect cells as fusion proteins with an IgM signal peptide and IgG-binding domain. The expressed proteins were secreted into the culture medium, cleaved at the same site as that observed in mammalian cells, and easily purified from the culture medium by using column chromatography. Most importantly, their biochemical activity was retained [17, 22].

Erythropoietin (EPO) is a glycoprotein that stimulates the proliferation and differentiation of erythroid precursor cells to more mature erythrocytes [19]. Secreted EPO contains approximately 40% carbohydrate and has an approximate molecular mass of 35 kDa. The carbohydrate content has been shown to affect protein stability and solubility, and the terminal sialic acid plays an important role in the long half-life of the protein during circulation [21, 30].

In this study, a large amount of soluble human GnT II, GnT IV, GnT V, and Gal β1,4-GlcNAcα2,3-sialyltransferase (ST3Gal IV) were expressed in insect cells by using a transfer vector equipped with an IgM signal peptide and IgG-binding domain [17]. The purified glycosyltransferases were biochemically active and subsequently applied for *in vitro* glycosylation of human EPO produced in insect cells, in an attempt to transform the paucimannose type of oligosaccharide into the complex type, mimicking that produced in mammalian cells.

Materials and Methods

Cells and Viruses

Spodoptera frugiperda (*Sf*9, Invitrogen) cells, used for the transfection and expression of recombinant human glycosyltransferases (GnT II, GnT IV, GnT V, and ST3Gal IV), were cultured at 27°C in Grace's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS, Hyclone), 50 μg/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Inc.) [16, 18]. *Trichoplusia ni* (*T. ni*, BTI-TN5B1-4, High Five, Invitrogen) cells, used for the mass production of recombinant GnT II, GnT IV, GnT V, and ST3Gal IV, were cultured at 27°C in HyQ SFX insect medium (Hyclone). *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant viruses were propagated in *Sf*9 cells. For the expression of recombinant GnT II, GnT IV, GnT V, and ST3Gal IV, a monolayer of cells was infected with a recombinant baculovirus at 27°C for 1 h with gentle rocking and incubated at 27°C [5].

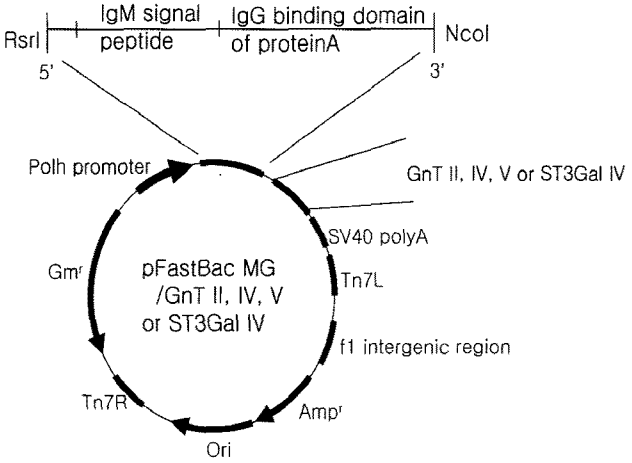

Fig. 1. Physical map of the transfer vector carrying cDNA encoding glycosyltransferase.
cDNAs encoding human GnT II, GnT IV, GnT V, or ST3Gal IV lacking the transmembrane domains were inserted into pFastBacMG, a transfer vector modified from pFastBac to induce fusion with the mouse-derived IgM signal peptide and protein A-derived IgG-binding domain [17].

Construction of Transfer Vectors

cDNAs encoding human GnT II, GnT IV, GnT V, and ST3Gal IV, whose transmembrane domains were removed to enhance their solubility and secretion from the cells, were amplified by PCR using the primers listed in Table 1. After digestion with restriction enzymes, the PCR products were cloned into the corresponding enzyme sites of pFastBacMGa [17]. The resulting recombinant transfer vectors were named pFastBacMG-GnT II, pFastBacMG-GnT IV, pFastBacMG-GnT V, and pFastBacMG-ST3Gal IV (Fig. 1).

Table 1. Primers used for cloning of cDNAs encoding glycosyltransferases.

Glycosyltransferase	Primer	Orientation	Sequence[a] (5'-3')	Restriction site
GnT II	NYGnT II-F	Sense	CCgaattcATGAGGTTCCGCATCTAC	EcoRI
	NYGnT II-R	Antisense	CCaagcttTCACTGCAGTCTTCTATA	HindIII
GnT IV	NYGnT IV-F	Sense	TAgtcgacATATGAGGCTCCGCAATGGA	SalI
	NYGnT IV-R	Antisense	GGaagcttAGATGATCAGTTGGTGGC	HindIII
GnT V	NYGnT V-F	Sense	ATgtcgacTAATGGCTCTCTTCACTCCG	SalI
	NYGnT V-R	Antisense	TActcgagCTATAGGCAGTCTTTGCA	XhoI
ST3Gal IV	ST3Gal IV-F2	Sense	GGgaattcGGGAAGACAGGTACATCGAGCT	EcoRI
	ST3Gal IV-R	Antisense	AAAaagcttCAGAAGGACGTGAGGTTC	HindIII

[a]Lowercase letters indicate restriction enzyme sites.

Construction of Recombinant Baculoviruses

Recombinant baculoviruses were constructed as described previously [17]. In order to induce transposition between the recombinant transfer vector and AcNPV DNA, pFastBacMG-GnT II, pFastBacMG-GnT IV, pFastBacMG-GnT V, and pFastBacMG-ST3Gal IV were transformed into *E. coli* DH10Bac (Invitrogen) harboring the AcMPV bacmid. The transformed *E. coli* was incubated for 24 h on Luria-Bertani (LB) plates containing 50 μg/ml kanamycin, 7 μg/ml gentamycin, 10 μg/ml tetracycline, 100 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), and 40 μg/ml isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant bacmid DNA extracted from white colonies was transfected into *Sf*9 cells by using Cellfectin (Invitrogen), and the transfected cells were cultured at 27°C for 72 h. The culture medium was collected and centrifuged at 500 ×*g* for 5 min to remove cells and large debris.

Purification of Recombinant Human Glycosyltransferases

For the expression of recombinant GnT II, GnT IV, GnT V, and ST3Gal IV, 2×10^7 High Five cells were seeded in a 150-mm tissue culture dish (Nunc). The cells, infected with the recombinant virus, were grown at 27°C. At 72 h post-infection, the culture medium was collected and clarified by centrifugation for 10 min at 500 ×*g*. Concentrated equilibrium buffer (50 mM sodium phosphate and 750 mM NaCl; pH 8.0) was added to the supernatant to adjust the final concentration at 10 mM sodium phosphate and 150 mM NaCl (pH 8.0), and the mixture was filtered using a 0.45-μm membrane (Millipore). IgG-Sepharose 6 Fast Flow (Amersham-Pharmacia Biotech) was washed with distilled water to remove ethanol, equilibrated with equilibration buffer (10 mM sodium phosphate and 150 mM NaCl; pH 8.0), and then packed in a column. The equilibrated culture medium was passed through the IgG-Sepharose column. The column was washed with equilibrium buffer and eluted using elution buffer (100 mM glycine and 500 mM NaCl; pH 2.7). The elution fractions were immediately collected in a tube with 2 M Tris-Cl (pH 8.8). The amount of purified protein was estimated using a BCA protein assay kit (Pierce) with bovine serum albumin (BSA) as a standard.

Immunoblot Analysis

Proteins separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were electrotransferred to a nitrocellulose membrane (Schleicher & Schuell) by using a semi-dry transfer blotter (Owl) at 200 mA for 1.5 h. The membrane was blocked for 1.5 h at room temperature (RT) in a blocking buffer (TBS containing 0.1% Tween-20 and 4% skim milk) and was incubated at RT for 1 h with 1:2,000 diluted rabbit antisera in a washing buffer (TBS containing 0.1% Tween-20 [TTBS]). It was then washed three times with washing buffer (10 min for each wash) and incubated at RT for 1 h with 1:5,000 diluted alkaline phosphatase-conjugated goat anti-rabbit IgG antibody in the washing buffer. After washing as described previously, the membrane was developed in alkaline phosphatase buffer (100 mM Tris-Cl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$) by adding *p*-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) [23].

Enzyme Assay

The reaction for GnT II was performed at 37°C for 6 h with 25 mM cacodylate buffer (pH 7.4), 25 mM NaCl, 40 mM $MnCl_2$, 10 mM UDP-GlcNAc (Sigma) as a donor, 25 μM $Man_2ManGlcNAc_2$-PA (PA016, Takara) as an acceptor, and 0.26 μg of purified GnT II in a final volume of 20 μl. The reaction for GnT IV was performed at 37°C for 6 h with 25 mM cacodylate buffer (pH 7.4), 25 mM NaCl, 5 mM $MnCl_2$, 10 mM UDP-GlcNAc, 25 μM $(GlcNAcMan)_2ManGlcNAc_2$-PA (PA012, Takara) as an acceptor, and 0.7 μg of purified GnT IV in a final volume of 20 μl. The reaction for GnT V was performed at 37°C for 6 h with 25 mM cacodylate buffer (pH 7.4), 25 mM NaCl, 2 mM $MnCl_2$, 10 mM UDP-GlcNAc, 25 μM $(GlcNAcMan)_3ManGlcNAc_2$-PA (PA013, Takara) as an acceptor, and 0.9 μg of purified GnT V in a final volume of 20 μl. The reaction for ST3Gal IV was performed at 37°C for 6 h with 25 mM cacodylate buffer (pH 7.4), 25 mM NaCl, 2 mM $MnCl_2$, 2.5 mM CMP-NeuAc (Sigma), 25 μM $(GalGlcNAcMan)_2ManGlcNAc_2$-PA (PA001, Takara) as an acceptor, and 1.6 μg of purified ST3Gal IV in a final volume of 20 μl. Alternatively, 65 mM sodium cacodylate buffer (pH 6.0) or 50 mM morpholino-ethylsulfonic acid (MES) buffer (pH 6.8) was adopted for the ST3Gal IV reaction. After the reactions were stopped by chilling on ice, the products were separated by high-performance liquid chromatography (HPLC) (600 series, Waters) using a PALPAK TypeR column (Takara) with the following conditions: a linear gradient of 0.05–0.2% *n*-butanol in 100 mM triethylamine and 100 mM acetic acid (pH 4.0) for 40 min at a flow rate of 1 ml/min or 1.2 ml/min. Monitoring was conducted with a scanning fluorescence spectrophotometer (Waters 474, Waters) with excitation at 320 nm and emission at 400 nm.

***In Vitro* Galactosylation and Sialylation of rhEPO**

For *in vitro* galactosylation, the reaction mixture comprising 25 mM cacodylate buffer (pH 7.4), 25 mM NaCl, 8 mM $MnCl_2$, 3 mM UDP-Gal, 2.4 μg recombinant human EPO expressed in insect cells (rhEPO), and 0.17 μg β-1,4-galactosyltransferase (GalT I) in a final volume of 50 μl was incubated at 37°C for 12 h. Subsequent *in vitro* sialylation was performed by either of 2 methods: (i) The galactosylation reaction mixture was passed through CENTRI-SPIN (Princeton Separations) containing 65 mM cacodylate buffer (pH 6.0), 65 mM NaCl, and 3% Triton X-100; 1 μl of 50 mM CMP-NeuAc and 0.4 μg of α2,6 sialyltransferase (ST6Gal I) were added to the reaction mixture and incubated at 37°C for 12 h. (ii) Alternatively, 7 μl of 210 mM sodium cacodylate buffer (pH 5.7), 1 μl of 50 mM CMP-NeuAc, and 0.4 μg of ST6Gal I were added to the galactosylation reaction mixture and incubated at 37°C for 12 h. In another method, *in vitro* galactosylation and sialylation were performed with 50 mM MES buffer (pH 6.8), 8 mM $MnCl_2$, 3 mM UDP-Gal, 1 mM CMP-NeuAc, 2.4 μg rhEPO, 0.17 μg GalT I, and 0.4 μg ST6Gal I incubated at 37°C for 12 h. For autoradiography, 0.04 μCi UDP-^{14}C-Gal (NEN) and 0.04 μCi CMP-^{14}C-NeuAc (NEN) were added to the reaction mixture as sugar donors, and the blotted samples were exposed to IP-Plate (Fujifilm) for 72 h at RT. The exposed IP-Plate was read using a BAS1500 IP Reader (Fujifilm).

***In Vitro* Glycosylation of rhEPO**

For the *in vitro* glycosylation of rhEPO for use in a bioassay, the reaction mixture comprising 25 mM cacodylate buffer (pH 7.4), 25 mM NaCl, 10 mM $MnCl_2$, 6 mM UDP-GlcNAc, 6 mM UDP-Gal, 2 mM CMP-NeuAc, 5 μg rhEPO, 1.04 μg GnT II, 0.72 μg GnT IV, 1.60 μg GnT V, 0.34 μg GalT I, and 1.6 μg ST3Gal IV in a final volume of 50 μl was incubated at 37°C for 12 h. For α2,6 sialylation, 7 μl of 210 mM sodium cacodylate buffer (pH 5.7), 2 μl

of 50 mM CMP-NeuAc, and 0.8 μg of ST6Gal I were added to the reaction mixture and incubated at 37°C for an additional 12 h.

Lectin Blot

The reaction mixtures containing *in vitro* glycosylated rhEPO were subjected to SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell) by using a semi-dry transfer blotter (Owl) at 200 mA for 1.5 h. The membrane was blocked by soaking it in the blocking buffer (TBS containing 0.5% Tween-20) for 1.5 h at RT. Next, peroxidase-conjugated lectin, *N*-acetylglucosamine-binding wheat germ agglutinin (WGA, EYlab), galactose-binding *Ricinus communis* agglutinin (RCA, Sigma), α2,6-linked sialic acid-binding *Sambucus nigra* agglutinin I (SNA I, EYlab), or α2,3-linked sialic acid-binding *Maackia amurensis* agglutinin (MAA, EYlab, U.S.A.) was added to the membrane in TTBS and incubated overnight at 4°C. The membrane was washed three times (10 min each wash) with TTBS. The blots were visualized using a SuperSignal West Pico chemiluminescent substrate kit (Pierce).

Bioactivity of rhEPO

Five μg each of rhEPO and *in vitro* glycosylated rhEPO and 0.32 μg of Chinese hamster ovary (CHO) cell-derived EPO in phosphate buffered saline (PBS) (pH 7.4) containing 0.1% BSA (Pierce) were subcutaneously injected into 6-week-old female B6D2F1 (Slc:BDF1) mice. On the fourth day, blood was collected from the sacrificed animals in a tube containing EDTA-Na. One ml of Retic-COUNT reagent (Becton Dickinson) was added to 5 μl of well-mixed whole blood and vortexed gently. The sample was incubated at RT for 30 min in the dark. The reticulocytes were counted by fluorescence-activated cell sorting (FACS; FACSCalibur, Becton Dickinson).

Results and Discussion

Expression and Purification of *N*-Acetylglucosaminyltransferases and α2,3-Sialyltransferase

Glycosyltransferases possess a type II membrane protein topology with a short *N*-terminal cytoplasmic tail, hydrophobic membrane-anchor domain, proteolytically sensitive stem region, and large C-terminal active domain [29]. In order to express glycosyltransferases in insect cells, cDNAs encoding human GnT II, GnT IV, GnT V, and ST3Gal IV were inserted into pFastBacMG to yield pFastMG-GnT II, pFastMG-GnT IV, pFastMG-GnT V, and pFastMG-ST3Gal IV, respectively (Fig. 1). The transmembrane domains of the human glycosyltransferases were removed to prevent possible interference with the secretion of recombinant proteins from the cells. pFastBacMG is a transfer vector designed to produce a protein fused with the mouse-derived IgM signal peptide for efficient secretion and the protein A-derived IgG-binding domain for convenient purification [17]. The recombinant baculoviruses rvGnT II, rvGnT IV, rvGnT V, and rvST3Gal IV were recovered from *Sf9* cells transfected with the bacmid DNAs extracted from *E. coli* DH10Bac that was previously transformed with the transfer vectors.

T. ni cells were then infected with the recombinant baculoviruses, and the proteins in the culture medium were collected at various time intervals. The proteins were separated by 12% SDS-PAGE (Fig. 2, panels A, and C) and subjected to immunoblot analysis with rabbit antisera (Fig. 2, panels B and D). At 36 h post-infection, rabbit antisera-reactive bands appeared in the culture medium of cells infected with rvST3Gal IV (Fig. 2, panels C and D), but no corresponding band was detected in the medium of AcMNPV-infected cells (Fig. 2, panels A and B). Similar results were observed for the culture media of cells infected with rvGnT II, rvGnT IV, and rvGnT V (data not shown). The observed molecular masses of the recombinant GnT II, GnT IV, GnT V, and ST3Gal IV expressed in the insect cells were 52 kDa, 66 kDa, 89 kDa, and 44 kDa, respectively. The molecular masses were matched with the calculated values obtained from the amino acid composition of each glycosyltransferase deduced from the corresponding cDNA sequences.

In order to obtain large amounts of recombinant GnT II, GnT IV, GnT V, and ST3Gal IV, the culture media of the recombinant virus-infected *T. ni* cells were harvested at 72 h post-infection and loaded in a column packed with IgG-cross-linked Sepharose resin to exploit the IgG-binding domain for easy purification. Eluates from the rvST3Gal IV-infected cell culture medium column were separated by 12% SDS-PAGE (Fig. 2, panel E) and subjected to immunoblot analysis (Fig. 2, panel F). A single protein band (expected molecular mass shown in panels E and F of Fig. 2) indicated that a large amount of pure recombinant ST3Gal IV was obtained by one-step column chromatography. Similar results were also acquired for the culture medium of cells infected with rvGnT II, rvGnT IV, and rvGnT V (data not shown).

All the expressed glycosyltransferases were well secreted from the cell and could be efficiently purified by immunoaffinity column chromatography; this suggested that the mouse-derived IgM signal peptide and the IgG-binding domain of protein A fused at the *N*-terminus of each glycosyltransferase and could efficiently facilitate secretion and purification, respectively, as observed in a previous study [17]. Approximately 263 μg of GnT II, 186 μg of GnT IV, 168 μg of GnT V, and 403 μg of ST3Gal IV were harvested from 200 ml of cell culture medium.

Enzyme Activities of Recombinant Glycosyltransferases

In order to examine whether the recombinant glycosyltransferases expressed in insect cells retained their biochemical activity, an enzyme assay was carried out with pyridylamino (PA)-labeled oligosaccharide as an acceptor. Reaction mixtures comprised $Man_2ManGlcNAc_2$-PA (PA016, Takara) as an acceptor and UDP-GlcNAc as a sugar donor for GnT II, $(GlcNAcMan)_2ManGlcNAc_2$-PA (PA012, Takara)

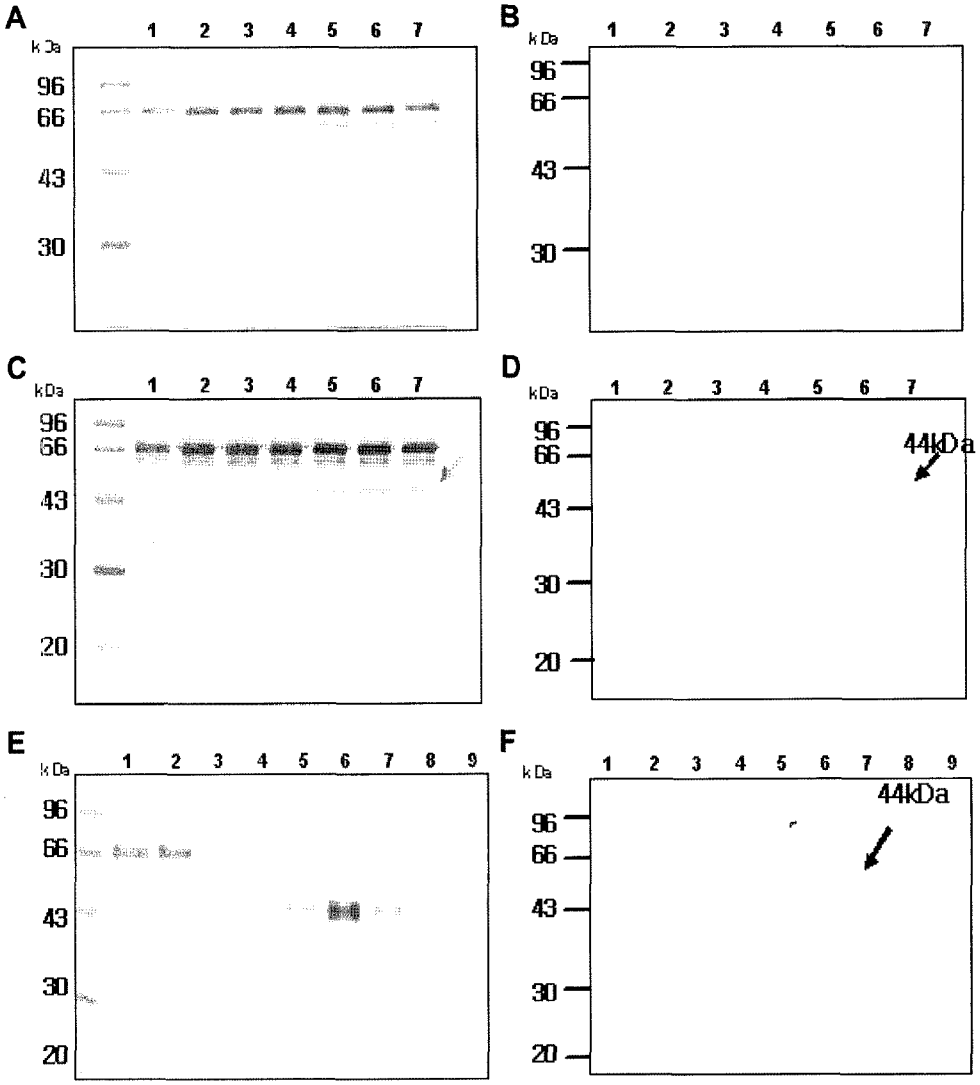

Fig. 2. Expression and purification of recombinant ST3Gal IV in *T. ni* cells.
AcMNPV- or recombinant virus-infected cell culture media were separated by 12% SDS-PAGE. The gels were stained with Coomassie blue (panels A, C, and E) and subjected to immunoblot analysis with rabbit antisera (panels B, D, and F). **A** and **B**: AcMNPV-infected cell media; **C** and **D**: rvST3Gal IV-infected cell media. Lane M: standard molecular mass marker; lane 1: cell culture medium collected at 0 h post-infection; lane 2: cell culture medium collected at 12 h post-infection; lane 3: cell culture medium collected at 24 h post-infection; lane 4: cell culture medium collected at 36 h post-infection; lane 5: cell culture medium collected at 48 h post-infection; lane 6: cell culture medium collected at 60 h post-infection; lane 7: cell culture medium collected at 72 h post-infection. Recombinant ST3Gal IV was purified by affinity chromatography using IgG-tagged Sepharose resin (**E** and **F**). Lane M: standard molecular mass marker; lane 1: recombinant virus-infected medium; lane 2: flow-through of the affinity column; lanes 3–9: eluates from the affinity column.

as an acceptor and UDP-GlcNAc as a sugar donor for GnT IV, $(GlcNAcMan)_3ManGlcNAc_2$-PA (PA013, Takara) as an acceptor and UDP-GlcNAc as a sugar donor for GnT V, and $(GalGlcNAcMan)_2ManGlcNAc_2$-PA (PA001, Takara) as an acceptor and CMP-NeuAc as a sugar donor for ST3Gal IV. The reaction products were separated on a PALPAK TypeR column.

A new peak appeared in the reaction product of GnT II at 16 min retention time in addition to a PA016 peak at 12 min retention time (panel A, Fig. 3), suggesting that GnT II transferred GlcNAc from UDP-GlcNAc to PA016. A relatively low peak level at 16 min retention time indicated that the reaction catalyzed by GnT II was not efficient. The relatively low enzyme activity of GnT II as compared with that of the other recombinant glycosyltransferases expressed and analyzed in this study could most probably be attributed to the acceptor molecules used (PA016: $(Man)_2ManGlcNAc_2$-PA); higher enzyme activity was observed in the reaction in which $(GlcNAc\beta1,2Man)(Man)ManGlcNAc_2$ was used as an acceptor [33].

A peak at 17 min retention time, the equivalent position of $(GlcNAcMan)_3ManGlcNAc_2$-PA (PA013), in addition to a PA012 peak at 12 min retention time, appeared in the reaction product of GnT IV (panel B, Fig. 3). A new

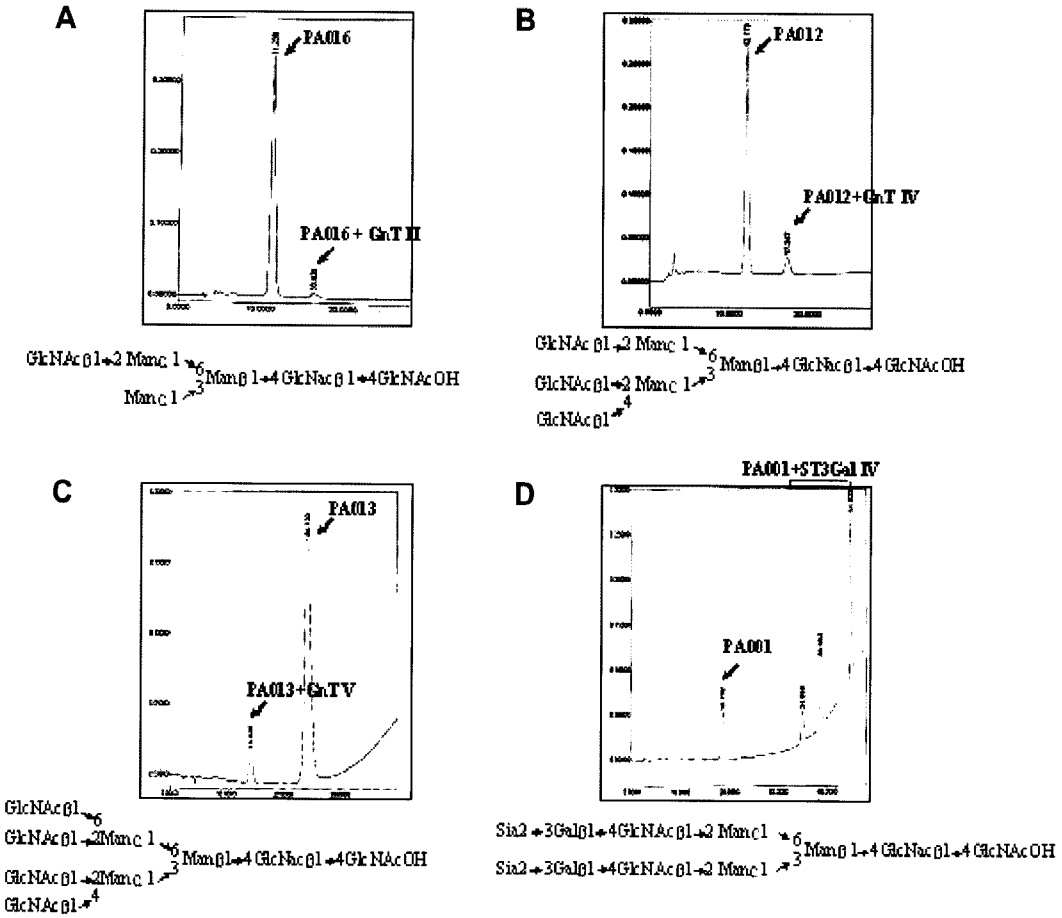

Fig. 3. Analysis of GnT II, GnT IV, GnT V, and ST3Gal IV activities.
The reaction mixtures comprising the enzymes (GnT II, GnT IV, GnT V, or ST3Gal IV), acceptors (PA016, PA012, PA013, or PA001), and donors (UDP-GlcNAc or CMP-NeuAc) were incubated, and the resulting products were fractionated by HPLC on a PALPAK TypeR column. **A**. Structure and elution profile of PA016 after the reaction of GnT II; **B**. Structure and elution profile of PA012 after the reaction of GnT IV; **C**. Structure and elution profile of PA013 after the reaction of GnT V; **D**. Structure and elution profile of PA001 after the reaction of ST3Gal IV.

peak at 14 min retention time, the equivalent position of $(GlcNAcMan)_4ManGlcNAc_2$-PA (PA014), in addition to a PA013 peak at 24 min retention time, appeared in the reaction product of GnT V (panel C, Fig. 3). These results indicated that GlcNAcs were attached to PA012 and PA013 in the reaction mediated by GnT IV and GnT V.

Finally, 3 new peaks at 34 min, 38 min, and 44 min retention times in addition to a PA001 peak at 18 min retention time appeared in the reaction product of ST3Gal IV (panel D, Fig. 3). Since PA001 has 2 terminal galactose residues that could be independently linked to sialic acid, the 3 different reaction products could possibly be synthesized owing to its reaction with ST3Gal IV, and they appeared in peaks at 34 min, 38 min, and 44 min retention times. Moreover, ST3Gal IV exhibited higher activities in 25 mM sodium cacodylate buffer (pH 7.4) than in 65 mM sodium cacodylate buffer (pH 6.0) or MES buffer (pH 6.8) (data not shown).

Taken together, the above results indicated that glycosyltransferases, expressed in insect cells as *N*-terminus fusion proteins with IgG-binding domains, can be produced in large quantities as biochemically active forms from limited culture volumes, and they can be purified relatively simply. These recombinant glycosyltransferases should be useful for the modification of glycans expressed as the paucimannose or oligomannose type to the complex type by *in vitro* glycosylation.

In Vitro Glycosylation of EPO Expressed in Insect Cells

Although insect cells can provide a eukaryotic protein-modification environment, including glycosylation, most recombinant glycoproteins produced in the insect cell system are not sialylated [13, 24]. The ability of glycosyltransferases to attach sugar residues at the terminal position of glycoprotein *in vitro* was exploited using recombinant human EPO (rhEPO) expressed in insect cells. The rhEPO fused with the IgG-binding domain at the *N*-terminus was expressed in *T. ni* cells by using baculovirus as a vector and was purified by immunoaffinity column (data not shown). EPO stimulates the viability, proliferation, and differentiation of erythroid precursor cells to more mature erythrocytes [7, 19, 30]. The terminal sialic acid plays an

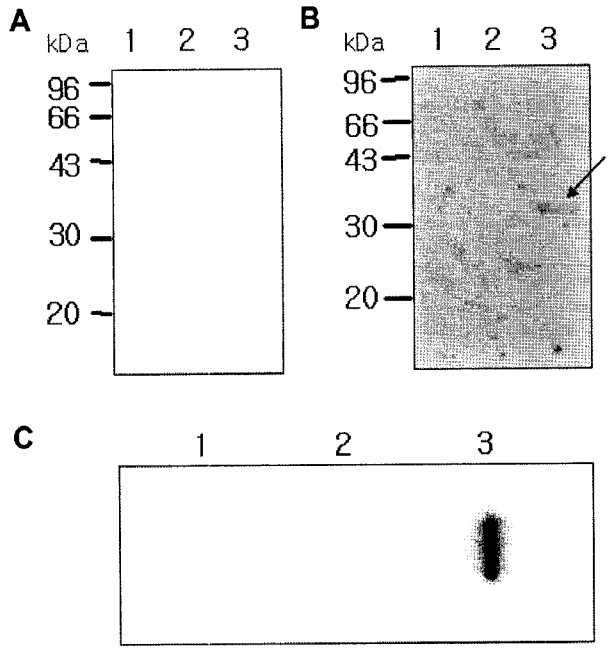

Fig. 4. *In vitro* galactosylation of rhEPO expressed in insect cells.

Recombinant EPOs treated with GalT I were separated by 12% SDS-PAGE. The gel was stained with Coomassie blue (**A**), transferred to a nitrocellulose membrane, and probed with RCA-I (**B**). Lane 1: GalT I and UDP-Gal; lane 2: rhEPO treated with UDP-Gal; lane 3: rhEPO treated with UDP-Gal and GalT I. Alternatively, slot-blot analysis was carried out with the reaction products by using UDP-^{14}C-Gal as a galactose donor (**C**). Lane 1: GalT I and UDP-^{14}C-Gal; lane 2: rhEPO treated with UDP-^{14}C-Gal; lane 3: rhEPO treated with UDP-^{14}C-Gal and GalT I.

important role in pharmacokinetics and extends the half-life of EPO within the blood circulation system [6, 31].

Since galactosyltransferase activity is low and sialyltransferase activity is absent in lepidopteran insect cells, sialic acid is absent, and a few galactose molecules are found in most recombinant glycoproteins expressed in insect cells [10, 11]. rhEPO was treated with recombinant GalT I expressed in insect cells with UDP-Gal as a galactose donor; the reaction mixture was separated by 12% SDS-PAGE, and lectin blot analysis was performed with RCA-I. A positive band was observed in the sample treated with GalT I (Fig. 4, lane 3 of panel B) but not in the reaction mixtures lacking GalT I or rhEPO (Fig. 4, lanes 1 and 2 of panel B). The addition of galactose to rhEPO was confirmed in the slot-blot analysis performed using UDP-^{14}C-Gal (Fig. 4, lane 3 of panel C). No band was detected in the reaction mixtures lacking GalT I or rhEPO (Fig. 4, lanes 1 and 2 of panel C).

GalT I-treated rhEPO was further sialylated with recombinant ST6Gal I and CMP-Sia as a sialic acid donor. The reaction mixture was separated by 12% SDS-PAGE and subjected to lectin blot analysis with SNA-I. A positive band was detected with the galactosylated rhEPO treated with ST6Gal I (Fig. 5, lanes 5, 6, and 7 of panel B) but not in the sample lacking ST6Gal I or galactosylated rhEPO (Fig. 5, lanes 1–4 of panel B). The addition of sialic acid was also confirmed in the reaction with CMP-^{14}C-Sia (Fig. 5, slots 5, 6, and 7 of panel C). The appearance of additional bands with molecular mass of 43 kDa, the equivalent position of recombinant GalT I (Fig. 5, slot 2 of panel C), implied that GalT I in the reaction mixture could also be galactosylated and sialylated since GalT I, a glycoprotein [8, 27], was produced in insect cells.

The *N*-linked saccharides of recombinant EPO expressed in CHO cells comprise tetraantennary saccharides [30], whereas the recombinant EPO expressed in insect cells has a paucimannose or high mannose structure because of the absence or low levels of *N*-acetylglucosaminyltransferase, galactosyltransferase, and sialyltransferase activities [1, 3, 6, 9, 32]. Glycoproteins possessing oligosaccharides with galactose or mannose residues at the terminal position are rapidly cleared from circulation after binding to galactose or mannose receptors present in the liver [28], resulting in the reduction of their serum half-life. Consequently, the necessity to develop a system to convert a glycoprotein with terminal galactose or mannose to one with terminal sialic acid is constantly increasing.

In order to transform the probable paucimannose form of rhEPO into a highly sialylated complex form that is expressed in mammalian cells, *in vitro* glycosylation was conducted using recombinant glycosyltransferases expressed in insect cells. *In vitro* glycosylation using GnT II, GnT IV, and GnT V followed by galactosylation and sialylation using GalT I, ST6Gal I, and ST3Gal IV was attempted. The addition of *N*-acetylglucosamine, galactose, and sialic acid to rhEPO was confirmed by WGA, RCA-I, and SNA-I lectin blot analysis, respectively (data not shown). These results indicate that *N*-acetylglucosamine, galactose, and sialic acid can be attached to glycoproteins expressed in insect cells *in vitro* by the consecutive addition of GnT II, GnT IV, GnT V, GalT I, and ST6Gal I into the reaction mixture.

Bioassay of *In Vitro* Glycosylated EPO in Mice

In order to examine whether the terminal sialic acid added to the recombinant EPO expressed in insect cells *in vitro* can enhance the biological activity of the EPO, female B6D2F1 (Slc:BDF1) mice were injected with rhEPO. Four days later, blood samples were collected from the mice, and the number of red blood cells was counted. As shown in Table 1, the percentage of reticulocytes among 50,000 red blood cells was markedly increased in CHO cell-derived EPO-injected mice (42.1%) than in rhEPO-injected mice (12.7%), and the latter value was in turn slightly higher than that in PBS-injected mice (10.0%). No significant enhancement of reticulocyte formation was observed in the *in vitro* glycosylated rhEPO-injected mice (12.6%) as compared with the rhEPO-treated mice (12.7%) (Table 2).

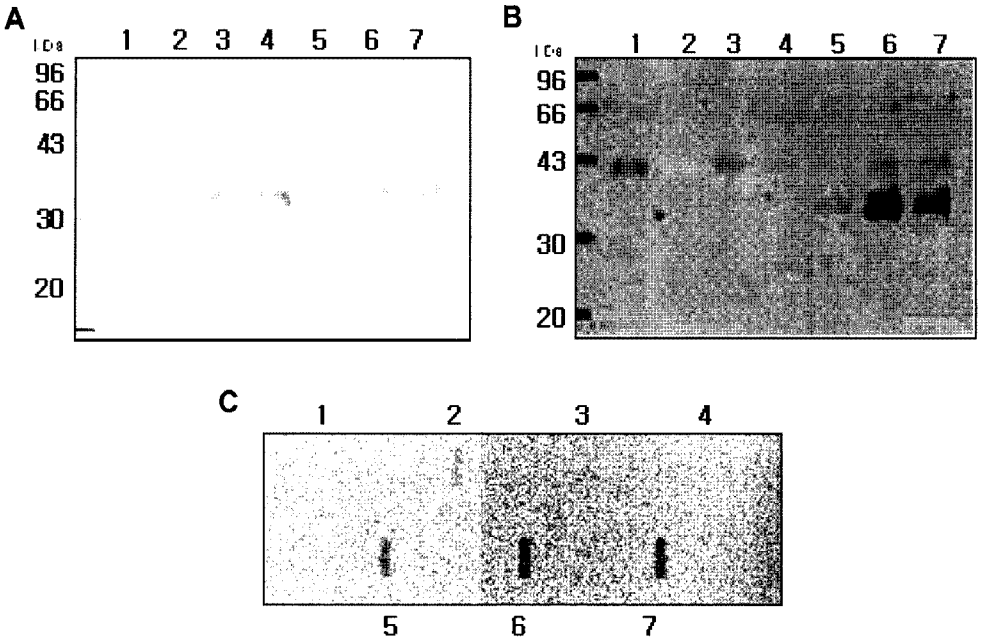

Fig. 5. *In vitro* sialylation of rhEPO expressed in insect cells.
Recombinant EPOs treated with GalT I and ST6Gal I were separated by 12% SDS-PAGE. The gel was stained with Coomassie blue (**A**), transferred to nitrocellulose membrane, and probed with SNA-I (**B**). Alternatively, slot-blot analysis was carried out with the reaction products by using CMP-^{14}C-Sia as a sialic acid donor (**C**). Lane 1: rhEPO; lane 2: GalT I and ST6Gal I; lane 3: GalT I-treated rhEPO; lane 4: ST6Gal I-treated rhEPO; lane 5: GalT I-treated rhEPO subjected to ST6Gal I after buffer adjustment using a spin column; lane 6: GalT I-treated rhEPO subjected to ST6Gal I after buffer adjustment to 210 mM sodium cacodylate (pH 5.7); lane 7: rhEPO treated with GalT I and ST6Gal I in 50 mM MES buffer (pH 6.8).

Although the addition of *N*-acetylglucosamine, galactose, and sialic acid to rhEPO was confirmed by lectin blot analysis (data not shown), it was expected that only a limited number of sialic acid residues could be attached to the glycan because of the complicated multistep reaction condition. Besides, the long reaction time (24 h) at 37°C would possibly degrade rhEPO extensively. To improve its biological activity significantly, it is therefore required to establish an efficient reaction condition for *in vitro* glycosylation of rhEPO. Reducing the entire reaction time and/or modifying the multistepped reaction process into a single-stepped reaction should prevent the degradation of rhEPO and enhance the reaction efficiency as well. Alternatively, the construction of glycosyltransferase gene-transduced insect cell lines that can express humanized glycoproteins would eventually resolve the current obstacles for the production of complex glycans with highly sialylated forms in insect cells [4, 9, 10, 12, 14, 15, 26].

Acknowledgments

This work was supported by a Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST) (R01-2006-000-10635-0) and by a Sogang University Grant (20041036).

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Table 2. Biological activity of EPO in mice.

Sample	No. of mice	% of reticulocytes[a] (mean±SD)
PBS	6	10.0±0.9
rhEPO	10	12.7±0.8
rhEPO+GnTs+GalT I+ST3Gal IV+ST6Gal I	10	12.6±0.5
CHO cell-derived EPO	10	42.1±0.5

[a]Blood samples were collected at day 4 after injection. Reticulocytes and mature erythrocytes were measured by counting 50,000 cells in 5 μl of whole blood.

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