Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Biotechnology 102 (2003) 61–71
JOURNAL OF Biotechnology
www.elsevier.com/locate/jbiotec

Improvement of glycosylation in insect cells with mammalian glycosyltransferases

Geen-Dong Chang [b,1], Chen-Ju Chen [a], Chih-Yin Lin [a], Hsei-Chorn Chen [a], Hungwen Chen [a,b,*]

[a] *Institute of Biological Chemistry, Academia Sinica, Nankang, Taipei 115, Taiwan*
[b] *Graduate Institute of Biochemical Sciences, National Taiwan University, Taipei 106, Taiwan*

Received 22 July 2002; received in revised form 23 October 2002; accepted 8 November 2002

Abstract

The *N*-glycans of recombinant glycoproteins expressed in insect cells mainly contain high mannose or tri-mannose structures, which are truncated forms of the sialylated *N*-glycans found in mammalian cells. Because asialylated glycoproteins have a shorter half-life in blood circulation, we investigated if sialylated therapeutic glycoprotein can be produced from insect cells by enhancing the *N*-glycosylation machinery of the cells. We co-expressed in two insect cell lines, Sf9 and Ea4, the human α1-antitrypsin (hα1AT) protein with a series of key glycosyltransferases, including GlcNAc transferase II (GnT2), β1,4-galactosyltransferase (β14GT), and α2,6-sialyltransferase (α26ST) by a single recombinant baculovirus. We demonstrated that the enhancement of *N*-glycosylation is cell type-dependent and is more efficient in Ea4 than Sf9 cells. Glycan analysis indicated that sialylated hα1AT proteins were produced in Ea4 insect cells expressing the above-mentioned exogenous glycosyltransferases. Therefore, our expression strategy may simplify the production of humanized therapeutic glycoproteins by improving the *N*-glycosylation pathway in specific insect cells, with an ensemble of exogenous glycosyltransferases in a single recombinant baculovirus.

Keywords: Human α1-antitrypsin; Glycosylation; Glycosyltransferase; Insect cell

1. Introduction

The baculovirus-insect cell system is a popular and efficient eukaryotic expression system, in which overexpressed recombinant proteins are correctly folded and post-translationally modified through phosphorylation and glycosylation. However, a major limitation regarding the production of therapeutic glycoproteins in the baculovirus-insect cell system is the lack of complex-type *N*-glycans, containing terminal sialic acid residues. Asialoglycoproteins, in blood circulation, usually have a shorter half-life due to the scavenging effect of the asialoglycoprotein receptor on hepatocytes. Therefore, the efficacy of therapeutic glycopro-

* Corresponding author. Tel.: +886-2-2785-5696x6090; fax: +886-2-2788-9759.
E-mail address: hwchen@gate.sinica.edu.tw (H. Chen).
[1] This author, C.-J. Chen and C.-Y. Lin all contributed equally to this work.

0168-1656/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0168-1656(02)00364-4

teins will be severely reduced upon incomplete glycosylation.

In the mammalian *N*-glycosylation pathway, the pre-assembled $Glc_3Man_9GlcNAc_2$ oligosaccharides are first transferred onto nascent polypeptide chains within the endoplasmic reticulum. Subsequently, three glucose residues on the precursor oligosaccharide are trimmed off by glucosidases I and II and then one or two mannose residues are further trimmed off by α1,2-mannosidase. As glycoproteins enter the Golgi complex, mannosidases IA, IB, and perhaps, other class I α-mannosidases remove the other two or three α1,2-linked mannose residues. The resulting $Man_5GlcNAc_2$ oligosaccharide is extended with a GlcNAc residue by the GlcNAc transferase I (GnT1) to produce the $GlcNAcMan_5GlcNAc_2$ oligosaccharide from which two more mannose residues are further removed by the Golgi-specific α-mannosidase II. The resulting $GlcNAcMan_3GlcNAc_2$ oligosaccharide can then be converted to a sialylated complex-type *N*-glycan by a series of glycoltransferases, including GlcNAc transferase II (GnT2), galactosyltransferase (GT), and sialyltransferase (ST).

In contrast, most *N*-glycans of natural or recombinant glycoproteins expressed in lepidopteran insect cells contain high mannose, nonfucosylated or α1,6-fucosylated tri-mannose structures (Altmann et al., 1999; Kubelka et al., 1994). It is speculated that insect cells may lack or possess lower levels of enzymes essential for the elongation reactions leading to complex-type *N*-glycans. Nevertheless, several insect counterparts of the mammalian enzymes in the *N*-glycosylation pathway have been characterized. For instance, the insect α1,2-mannosidase and α-mannosidase II have been isolated (Jarvis et al., 1997; Kawar et al., 1997) and the enzyme activities of GnT1 and 2 have been detected in four lepidopteran cell lines, including Sf9 (Altmann et al., 1993). In insect cells, after transfer of GlcNAc onto the $Man_5GlcNAc_2$ oligosaccharide by GnT1, an α-mannosidase II and a β-*N*-acetylglucosaminidase in the Golgi complex are involved in the removal of the mannose and GlcNAc residues to yield the trimannosyl core structure (Licari et al., 1993; Wagner et al., 1996a). Recently, an *Estigmena acrea*-derived Ea4 cell line was established and had a more extensive glycosylation potential such that human interferon γ (INF-γ), when expressed in Ea4 cells, contained terminal GlcNAc residues (Ogonah et al., 1996). The more extensive glycosylation potential in Ea4 cells was attributed to the lack of β-*N*-acetylglucosaminidase activity (Wagner et al., 1996a).

Since the carbohydrate moieties of glycoproteins affect the biological activity, stability, immunogenicity, and pharmacokinetics of the glycoproteins, several approaches have been taken to improve *N*-glycosylation in insect cells. One approach is to express the target glycoprotein together with exogenous glycosyltransferases in the host cells. For instance, coinfection of Sf9 cells with a hemagglutinin (HA) recombinant baculovirus and a human GnT1 recombinant baculovirus increases the level of HA carrying terminal GlcNAc residues (Wagner et al., 1996b). Alternatively, expression of the bovine β14GT driven by the immediately early gene *ie1* promoter and hr5 enhancer has been used to modify the *N*-glycosylation pathway in Sf9 cells, producing more extensively processed *N*-glycans (Jarvis and Finn, 1996). Another approach is to establish genetically transformed cell lines that stably express exogenous glycosyltransferases (Jarvis et al., 1998). For instance, a β14GT-expressing Sf9 cell line, Sfβ4GalT, when infected with a human tissue plasminogen activator (tPA) recombinant baculovirus, produces β1,4-galactosylated tPA (Hollister et al., 1998).

Recently, several studies have further demonstrated the production of terminally sialylated glycoproteins in insect cells with the aid of heterologous β14GT and α26ST. In one study, baculoviral expression vectors were designed to coexpress β14GT and α26ST in insect cells under the control of the *ie1* promoter and hr5 enhancer such that the major structural glycoprotein, gp64, of the derived recombinant baculovirus was sialylated in insect cells (Jarvis et al., 2001). In other studies, stable insect cell lines expressing β14GT and α26ST were established and were also able to produce sialylated gp64 after infection with baculovirus (Hollister and Jarvis, 2001; Breitbach and Jarvis, 2001). Due to temporal and spacial trim-

ming and elongation enzymes being involved in the insect *N*-glycosylation pathway, these studies indicated that the insect *N*-glycosylation pathway may be directed to terminal sialylation by supplementation of the relevant elongation glycosyltransferases.

In our production of sialylated therapeutic glycoproteins, we avoided labor-intensive efforts in establishing new insect cell lines and used instead a quadruple expression vector (Belyaev and Roy, 1993) to construct an expression cassette for simultaneous expression in insect cells of the human α1-antitrypsin (hα1AT) and three key glycosyltransferases GnT2, β14GT, and α26ST from the mammalian biosynthetic pathway for complex-type *N*-glycans. Theoretically, these enzymes should be sufficient to produce biantennary sialylated *N*-glycans in the Ea4 cell line, because in this cell line the activity of GnT1 is not counteracted by β-*N*-acetylglucosaminidase (Wagner et al., 1996a). In this report, we demonstrate that production of sialylated hα1AT glycoproteins can be achieved by simultaneous expression of the three glycosyltransferases and hα1AT in Ea4 cells, but not in Sf9 cells, by a single recombinant baculovirus. Our results potentially provide a new tool for the production of humanized therapeutic glycoproteins in insect cells.

2. Materials and methods

2.1. Insect cells and cell culture

Sf9 and Ea4 cells were maintained in serum free medium, Sf-900II SFM (GIBCO BRL, Rockville, MD) supplemented with 0.125 μg ml^{-1} of amphotericin B, 50 μg ml^{-1} of streptomycin, and 50 U ml^{-1} of penicillin as suspension cultures at 28 °C. The Sf9 cells were maintained at densities between 0.5 and 3.0×10^6 cells ml^{-1}. The Ea4 cells were maintained at densities between 0.2 and 2.0×10^6 cells ml^{-1}.

2.2. Construction of recombinant baculoviruses

The pAcAB4 transfer vector (Pharmingen, San Diego, CA), which contains two *p10* and two *polyhedrin* promoters, was used for transfer constructs in this study. For simplicity, we denoted the duplicated promoters as left and right promoters. The hα1AT cDNA was inserted into the left *p10* promoter. The resulting construct was named pAT. The pATGTGnT226ST construct was generated by inserting human GnT2, β14GT, and α26ST cDNAs downstream of the left *polyhedrin*, right *p10*, and right *polyhedrin* promoters in the pAT plasmid, respectively (Fig. 1A).

Generation of the recombinant baculovirus was performed by co-transfecting the transfer construct DNA and the *Bsu*36I-digested *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) DNA into Sf9 cells. Recombinant viruses were isolated by plaque assay and amplified in Sf9 cells. Positive clones were identified by PCR screening of the genomic DNA prepared from Sf9 cells infected with candidate baculovirus clones. The positive clones, BacAT and BacATgng26, derived from pAT and pATGTGnT226ST plasmids, respectively, were further plaque-purified twice, amplified, and used in this study.

Primer sequences used in PCR screens are as follows: For BacAT, the hα1AT: GAGGCCATACCCATGTC and GATTTCGCTCTAACATACCACC. For BacATgng26, the hα1AT: ATTCCGGAGGCTCAGAT and TAAGCTGGCAGACCTTC; the GnT2: GTGCCCAAATTGAGTCA and GATTTCGCTCTAACATACCACC; the β14GT: AGAGGCATGTCTATATCTCGCCCAA and ATAACAGCCATTGTAATGAGACGCA; the α26ST: CAGGTGTGGAACAAGGA and AGTTCATCAGGCGAATGG.

2.3. RT-PCR

Sf9 cells in 60-mm culture plate were infected with BacATgng26 for 72 h. Total RNA was prepared with a commercial kit (Qiagen, Valencia, CA) and treated with DNase I to remove contaminated genomic DNA. First-strand cDNA was prepared from 5 μg of total RNA with random hexadeoxynucleotides, according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ). Primer sequences for PCR

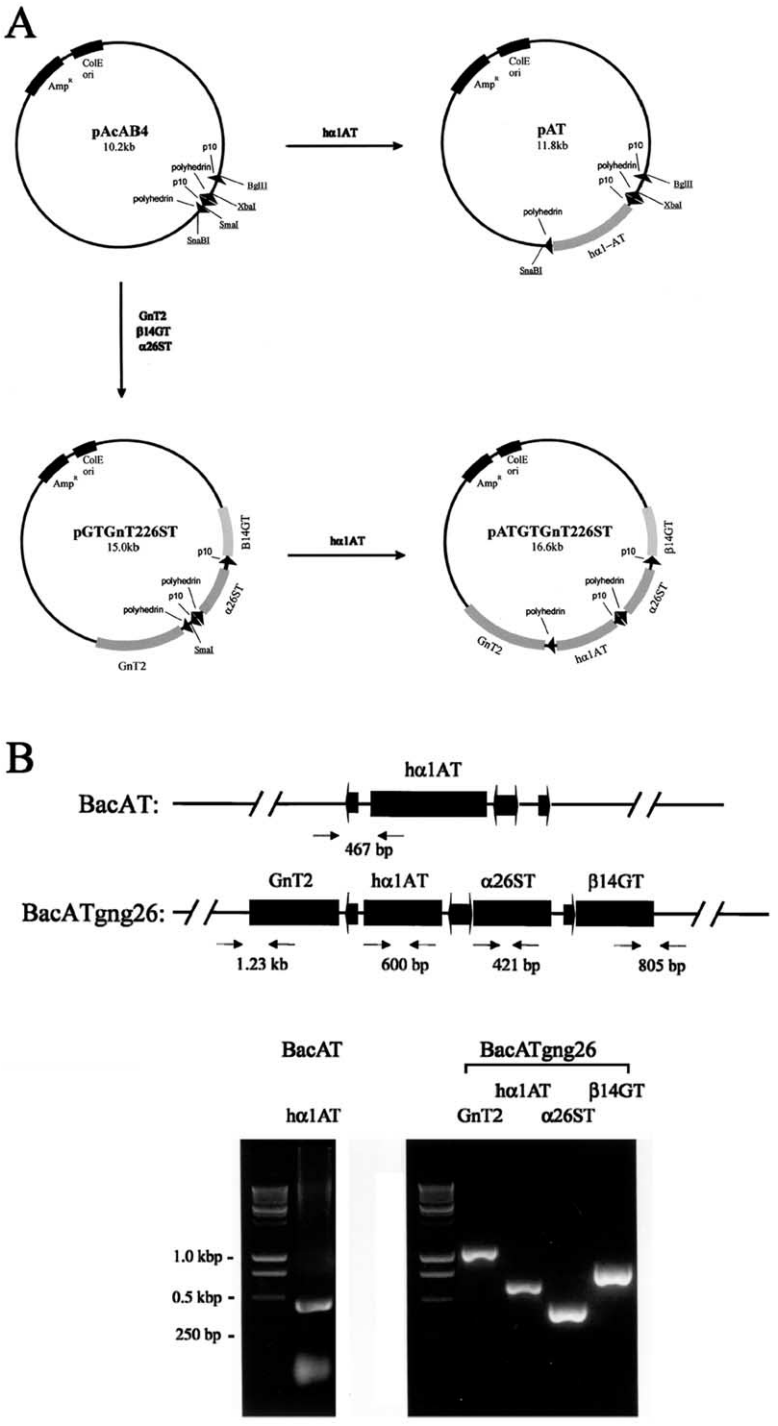

Fig. 1. (A) Construction of pAT and pATGTGnT226ST. Human cDNAs encoding α1AT, GnT2, β14GT, and α26ST were stepwise cloned into the parental transfer vector, pAcAB4 as described in Section 2. The triangles denote the very late gene *p10* or *polyhedrin* promoter and its transcriptional direction. (B) PCR analysis of positive recombinant baculoviruses. The position of designated primers for PCR is denoted with arrows. The numbers beneath the primer pairs denote the predicted size of amplified fragments. BacAT and BacATgng26 are recombinant baculovirus strains derived from pAT and pATGTGnT226ST, respectively. Genomic DNA of Sf9 cells infected with BacAT and BacATgng26 was prepared at 72 h post-infection and used for PCR analysis. Sizes of DNA markers are listed on the left.

are GnT2: GACCCTGAGGAATGTAGATA and CTACCTTGTCAGCCATGCCA; β14GT: AGAGGCATGTCTATATCTCGCCCAA and CTAGCTCGGTGTCCCGATGT. The primer pairs for hα1AT and α26ST are the same as described above.

2.4. Glycosyltransferase activity assay

Sf9 cells in 25-cm^2 culture flasks were infected with BacAT and BacATgng26, respectively. Cell extracts were prepared 72 h post-infection. For β14GT, cells were lysed with 0.5 ml of assay buffer (50 mM cacodylate, pH 7.4, 54 mM NaCl, 10 mM $MnCl_2$, 0.4% Triton-X100) and incubated on ice for 10 min. The lysate was clarified for 15 min at the top speed in a microcentrifuge. One hundred micrograms of the supernatant protein were incubated with 0.5 μCi ml^{-1} UDP-[^{14}C]galactose, 2 mM ATP, and 360 μM ovalbumin at 30 °C for 1 h. The reactions were stopped with 1 ml of cold 5% (w/v) phosphotungstic acid and spotted onto glass fiber filters. The filters were washed once with cold 5% phosphotungstic acid and then with cold 100% ethanol twice. The filters were dried and measured with a liquid scintillation cocktail in a radioactivity counter.

For α26ST, cells were lysed with 0.5 ml of assay buffer (0.1 M Tris-maleate, pH 7.3 and 0.4% Triton-X100) and incubated on ice for 10 min and clarified as described above. One hundred micrograms of the supernatant protein were incubated with 0.3 μCi ml^{-1} CMP-[^{14}C]sialic acid and 0.5 mM asialofetuin at 37 °C for 30 min. The reactions were stopped with 9 mM $HgCl_2$ and 1 ml of cold 10% (w/v) trichloroacetic acid (TCA) and spotted onto glass fiber filters. The filters were washed once with cold 10% TCA, twice with cold 5% TCA, and twice with cold 100% ethanol. The filters were measured as described above.

2.5. Immunoprecipitation

Antisera against hα1AT were prepared in rabbits with pure hα1AT (Calbiochem-Novabiochem, San Diego, CA). Sf9 and Ea4 cells on 30-mm culture plates were infected with BacAT and BacATgng26 at a multiplicity of infection (MOI) of 5. At 24 h post-infection, the infected cells were labeled with ^{35}S-methionine (100 μCi ml^{-1}) in methionine-deficient Sf-900II SFM. At 48 h post-infection, the media were collected and supplemented with RIPA (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholate) and incubated with the rabbit polyclonal antibody against hα1AT and protein A-agarose beads at 4 °C for 2 h. The immunoprecipitates were washed three times with RIPA and analyzed by SDS–PAGE and fluorography.

2.6. Expression and purification of hα1AT

Recombinant hα1AT proteins were prepared by infecting 5×10^8 Ea4 cells at mid-exponential growth phase with BacATgng26. The culture media were collected and centrifuged at 72 h post-infection. The clarified supernatant was applied to a trypsin–agarose column (Sigma–Aldrich, St. Louis, MO) equilibrated with PBS. After extensive washing with PBS, hα1AT proteins were eluted with the SDS–PAGE sample buffer. For comparison, trypsin inhibitors were purified by the same procedure from 50 ml of fetal calf serum (FCS).

2.7. Analysis of hα1AT glycosylation

For endoglycosidase digestion, the immunoprecipitated ^{35}S-hα1AT proteins were dissolved in the denaturation buffer (0.5% SDS and 1% β-mercaptoethanol) and boiled for 10 min. The denatured proteins were incubated with 100 IUB milliunits of endoglycosidase H in the reaction buffer (50 mM sodium citrate, pH 5.5) at 37 °C overnight. Alternatively, the denatured proteins were incubated with 8 IUB milliunits of peptide:*N*-glycosidase F in the reaction buffer (50 mM sodium phosphate, pH 7.5) supplemented with 1% NP-40 at 37 °C overnight. The reaction mixtures were analyzed by SDS–PAGE and fluorography.

For lectin blotting analysis, affinity-purified hα1AT proteins were resolved by SDS–PAGE, transferred onto Immobilon PVDF membrane (Millipore, Bedford, CA), and analyzed by the DIG glycan differentiation kit (Roche Molecular Biochemicals, Mannheim, Germany) according to

the manufacturer's instruction. In brief, the membrane was blocked in Tris-buffered saline (TBS: 50 mM Tris, pH 7.5, 150 mM NaCl) containing 0.5% Tween 20 overnight at 4 °C. The membrane was cut into strips and incubated with digoxigenylated lectins in TBS containing 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ for 1 h at room temperature. The digoxigenylated lectins used in this study were GNA, recognizing terminal mannose, SNA, recognizing sialic acid linked α(2–6) to galactose, PNA, recognizing the core disaccharide galactose linked β(1–3) to GalNAc, and DSA, recognizing galactose linked β(1–4) to GlcNAc (Jarvis and Finn, 1995). After extensive washing, the membranes were incubated with an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody. Signals were developed by a standard color reaction. Specificities of lectins were verified by using different glycoproteins provided in the aforementioned kit as positive and negative controls. In this study, fetuin, carboxy-peptidase Y (CPY), asialofetuin, and transferrin, were used as positive controls for PNA, GNA, DSA, and SNA, respectively. CPY, fetuin, transferrin, and asialofetuin were used as negative controls for PNA, GNA, DSA and SNA, respectively.

3. Results

3.1. Generation of recombinant baculovirus harboring GnT2, β14GT, α26ST and hα1AT cDNAs

In order to simplify the production of sialylated glycoproteins from insect cells, we used a single recombinant baculovirus to co-express a target protein with a series of key glycosyltransferases from the mammalian *N*-glycosylation pathway. We used the quadruple expression vector pAcAB4, which allows simultaneous expression of four foreign genes during the very late phase of the baculovirus infection cycle, as the transfer vector to generate recombinant baculoviruses. As shown in Fig. 1A, cDNAs encoding hα1AT, GnT2, β14GT, and α26ST were inserted downstream of the very late gene *p10* and *polyhedrin* promoters in the pAcAB4 plasmid. The resulting construct was named as pATGTGnT226ST. As a control, we constructed the pAT plasmid, which harbors only the hα1AT cDNA in the pAcAB4 plasmid (Fig. 1A). To verify that the cDNAs had integrated into the baculoviral genome, genomic DNA, extracted from Sf9 cells infected with the recombinant baculovirus clones, was PCR amplified with specific primers. As shown in Fig. 1B, positive clones BacAT and BacATgng26 were generated from the pAT and pATGTGnT226ST transfer constructs, respectively.

3.2. Simultaneous expression of GnT2, β14GT, α26ST and hα1AT

To investigate whether the inserted foreign cDNAs were expressed from the recombinant baculovirus, BacATgng26, the mRNA levels of hα1AT, GnT2, β14GT, and α26ST in the very late phase of the infection cycle (72 h post-infection) were examined by RT-PCR. As shown in Fig. 2A, specific bands for each gene were observed. Similarly, the mRNA level of hα1AT from the BacAT-infected Sf9 cells was also detected (data not shown). These results indicate that both recombinant baculoviruses are functional at the transcriptional level.

To examine the enzyme activities of the expressed glycosyltransferase, cell extracts were prepared from BacAT- and BacATgng26-infected Sf9 cells at 72 h post-infection. The cell extracts were incubated with UDP-[^{14}C]galactose and ovalbumin for the β14GT assay or CMP-[^{14}C]sialic acid and asialofetuin for the α26ST assay. Significant β14GT and α26ST activities were observed in the BacATgng26-infected Sf9 cells, but not in the BacAT-infected cells (Fig. 2B). Taken together, these data indicated that the BacATgng26 recombinant baculovirus can simultaneously express hα1AT, GnT2, β14GT and α26ST transcripts and enzyme activities for β14GT and α26ST were demonstrated.

3.3. Differential enhancement of *N*-glycosylation in Sf9 and Ea4 cells

The effect of the three exogenous glycosyltransferases on the *N*-glycosylation of hα1AT in insect

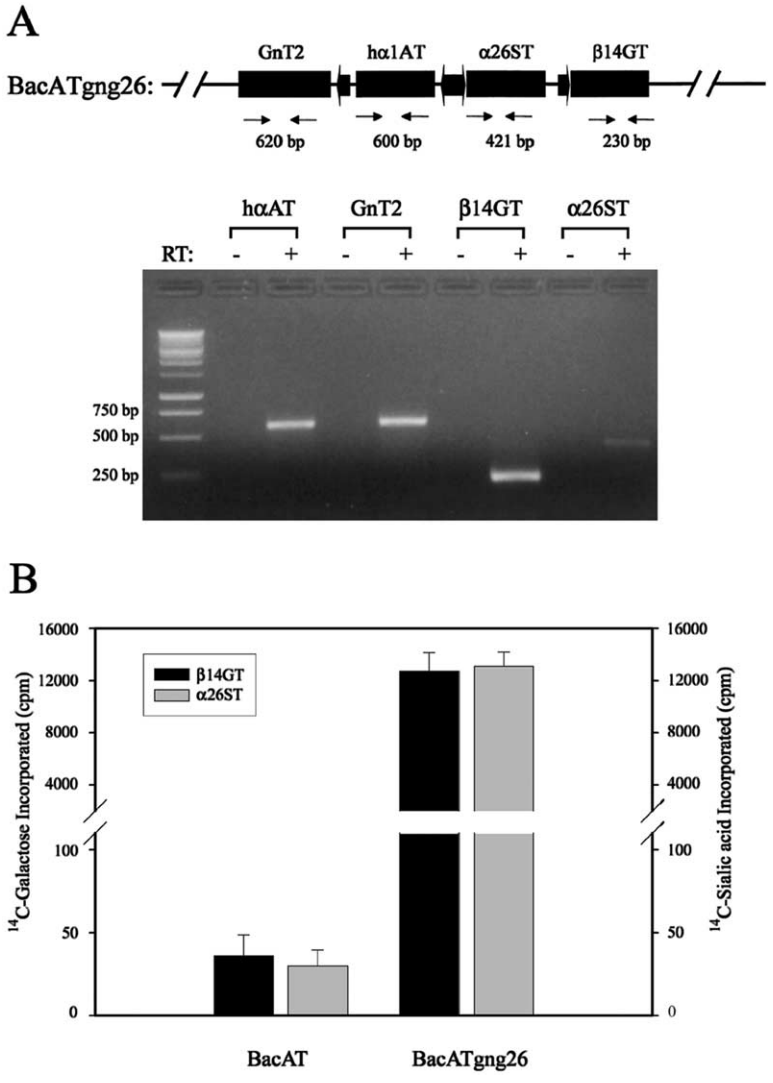

Fig. 2. (A) Simultaneous expression of human cDNAs encoding α1AT, GnT2, β14GT, and α26ST in insect cells. Total RNA was extracted from BacATgng26-infected Sf9 cells at 72 h post-infection. First-strand cDNA was prepared from 5 μg of total RNA with reverse transcriptase (RT). A mock control was prepared in the absence of RT. PCR was performed with specific primers and templates derived from first-strand cDNA or the mock control. Note that the designated primers (arrows) for glycosyltransferases are different from those used in Fig. 1B. The predicted sizes of amplified DNA fragments are listed beneath the primers. Sizes of DNA markers are listed on the left. (B) Glycosyltransferase assay. Sf9 cells were infected with BacAT and BacATgng26, respectively. Seventy-two hours after infection, cell extracts from infected cells were incubated with UDP-[^{14}C]galactose and ovalbumin for the β14GT assay or CMP-[^{14}C]sialic acid and asialofetuin for the α26ST assay. Enzyme activity was measured as the transference of the radioactive sugar residue (in cpm) to the acceptor protein in the presence of 100 μg of cell extracts. Mean values and S.D. obtained from three independent experiments are plotted. The black bar indicates the β14GT activity. The grey bar indicates α26ST activity.

cells was now investigated. Sf9 and Ea4 cells were infected with BacAT and BacATgng26 and the secreted hα1AT proteins analyzed. To avoid the leakage of intracellular hα1AT from dead and infected cells at a very late phase of the infection cycle, we characterized the secreted hα1AT at 48 h post-infection. The BacAT- and BacATgng26-infected Sf9 and Ea4 cells were pulse-labeled with

^{35}S-methionine from 24 to 48 h post-infection. The secreted hα1AT proteins were immunoprecipitated and analyzed by SDS–PAGE and fluorography. Compared with the BacAT-infected Sf9 cells, a minor band of higher molecular mass was seen in the BacAT-infected Ea4 cells (Fig. 3, the arrow in lane 9 and lane 12). This observation is in agreement with a previous finding (Ogonah et al., 1996) that Ea4 has a better glycosylation efficiency. Highly glycosylated hα1AT proteins were the major species in BacATgng26-infected Ea4 and Sf9 cells (Fig. 3, lanes 3 and 6), therefore these data indicated that extensive glycosylation was attained in the presence of the exogenous glycosyltransferases.

The precipitated hα1AT samples were now treated with endo H and PNGase F, in order to characterize the *N*-glycans on hα1AT proteins. As shown in Fig. 3, PNGase F completely removed the *N*-glycans in the four samples (lanes 2, 5, 8 and 11). The hα1AT proteins, from Sf9 cells infected with BacAT and from Ea4 cells infected with BacAT or BacATgng26, were resistant to endo H (lanes 7, 10 and 1). However, endo H resistance is not an exclusive indication of complex glycosylation because some oligomannose structures are endo H-resistant (Ogonah et al., 1996). In contrast, the endo H digestion revealed four bands of hα1AT molecules from the BacATgng26-infected Sf9 cells (lane 4). Therefore, different glycoforms of hα1AT proteins were produced from the BacATgng26-infected Sf9 cells. These data suggest that the exogenous glycosyltransferases enhance *N*-glycosylation in Ea4 and Sf9 cells. However, this enhancement is more efficient in Ea4 cells.

3.4. Glycan analysis of hα1AT from the BacATgng26-infected Ea4 cells

We next investigate the glycosylation profile of hα1AT proteins from the BacAT- and BacATgng26-infected Ea4 cells at 72 h post-infection. To avoid possible antibody affinity bias for particular glycoforms, an affinity purification method based on the enzyme and substrate interaction was used. The secreted hα1AT proteins in the culture media were affinity purified using a trypsin–agarose matrix. Approximately 95% pure hα1AT proteins were obtained (data not shown). To characterize the glycan composition, the hα1AT proteins were analyzed by overlay assays with different lectins. For comparison, positive- and negative-control glycoproteins, commercially purchased pure hα1AT proteins, and trypsin inhibitors, purified from fetal calf serum, were also included in this study. As shown in Fig. 4A, Western analysis with a polyclonal hα1AT antibody detected three major hα1AT bands from the BacATgng26-infected Ea4 cells and a major band for the commercial hα1AT and fetal calf α1AT proteins (lanes 1–3). The positive-control glycoproteins for each lectin probe were specifically detected (lanes 7, 12, 17 and 22), whereas the negative-control glycoproteins were not (lanes 8, 13, 18 and 23). The PNA and GNA lectins did not react with the commercial hα1AT, trypsin inhibitors from FCS, and purified hα1AT from the BacATgng26-infected Ea4 cells (lanes 4–6 for PNA and lanes 9–11 for GNA). Detection with the DSA and SNA lectins indicated that the commercial hα1AT, trypsin inhibitors from FCS, and the upper hα1AT band from the BacATgng26-infected Ea4 cells contained galactose and sialic acid residues in its *N*-glycans (lanes 14–16 for DSA and lanes 19–21 for SNA). Multiple bands were detected by DSA and SNA for the trypsin inhibitors purified from FCS (lanes 15 and 20) because FCS contains different trypsin inhibitors. Similar studies on the BacAT-derived hα1AT did not reveal positive signals for DSA and SNA lectins (Fig. 4B, lanes 6 and 11). Taken together, these results suggested that sialylated hα1AT proteins were synthesized after co-expression with exogenous GnT2, β14GT, and α26ST in Ea4 cells.

4. Discussion

In the present study, we demonstrated that simultaneous expression of GnT2, GT and ST resulted in the production of sialylated hα1AT proteins in Ea4 cells. Several lines of evidence support this observation. Firstly, the expression of these cDNAs was detected at the mRNA level by RT-PCR. Secondly, functional expression of gly-

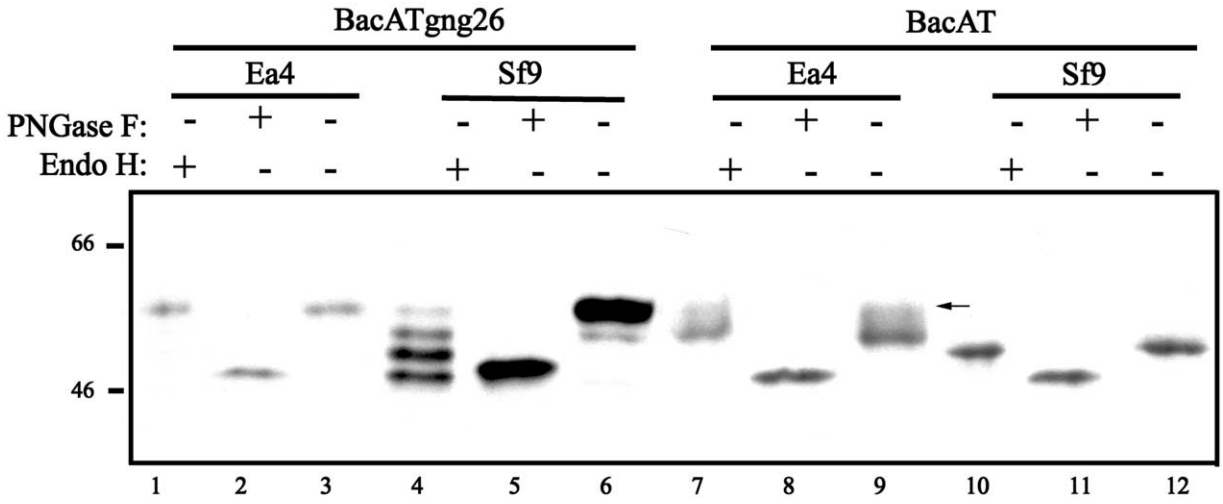

Fig. 3. Differential enhancement of the *N*-glycosylation pathways in Sf9 and Ea4 cells. Sf9 and Ea4 cells (1×10^6 cells) in 30-mm culture plates were infected with BacAT and BacATgng26 at an MOI of 5. Radio-labeling of hα1AT with ^{35}S-methionine and immunoprecipitation of secreted hα1AT proteins are described in Section 2. The precipitates were treated with 100 IUB milliunits of endoglycosidase H (Endo H) and 8 IUB milliunits of peptide:*N*-glycosidase F (PNGase F), respectively, overnight at 37 °C. The reaction mixtures were analyzed by SDS–PAGE and visualized by fluorography. The arrow in lane 9 indicates the hα1AT proteins of higher molecular mass in the BacAT-infected Ea4 cells. Numbers on the left indicate the molecular weight of protein markers.

cosyltransferase cDNAs was verified by enzymatic assays for β14GT and α26ST. Thirdly, overlay assays with lectin probes revealed the presence of sialic acid residues on the expressed hα1AT proteins from the BacATgng26-infected Ea4 cells. GnT2 activity in the BacATgng26-infected cells was not assayed in this study due to a shortage of enzyme substrate. However, functional expression of GnT2 has been demonstrated in insect cells infected by a recombinant baculovirus harboring the human GnT2 cDNA (Tan et al., 1995). Given that β14GT and α26ST were active in the enzyme assays, it is likely that GnT2 was also functional in the BacATgng26-infected insect cells. We speculate that the expressed hα1AT proteins from Ea4 cells co-expressing the three glycosyltransferases may contain sialylated complex-type *N*-glycans. However, further studies are required to identify the precise structures of the expressed sialylated hα1AT proteins.

Interestingly, from the BacATgng26-infected Ea4 cells a major band of hα1AT protein was detected by immunoprecipitation, whereas, at least three bands of hα1AT protein were detected by affinity purification with trypsin agarose. This discrepancy may be due to several possibilities. Firstly, the expression time course in the immunoprecipitation study was 48 h, whereas, in the affinity purification study it was 72 h. In the latter, premature hα1AT proteins, released from dead cells, may contribute to the lower protein bands. Secondly, the antibody used in the immunoprecipitation study may have a differential affinity for the different glycoforms of hα1AT protein. Thirdly, a high secretion rate of hα1AT protein may impede the glycosylation of hα1AT proteins. Therefore, the lower bands of hα1AT protein affinity-purified with trypsin agarose may be attributed to underglycosylated or unglycosylated hα1AT proteins.

Recently, cell lines genetically-engineered to express the relevant elongation glycosyltransferases in the glycosylation pathway have been established. CHO cell lines expressing GT and ST have been established (Weikert et al., 1999). Although highly sialylated glycoproteins have

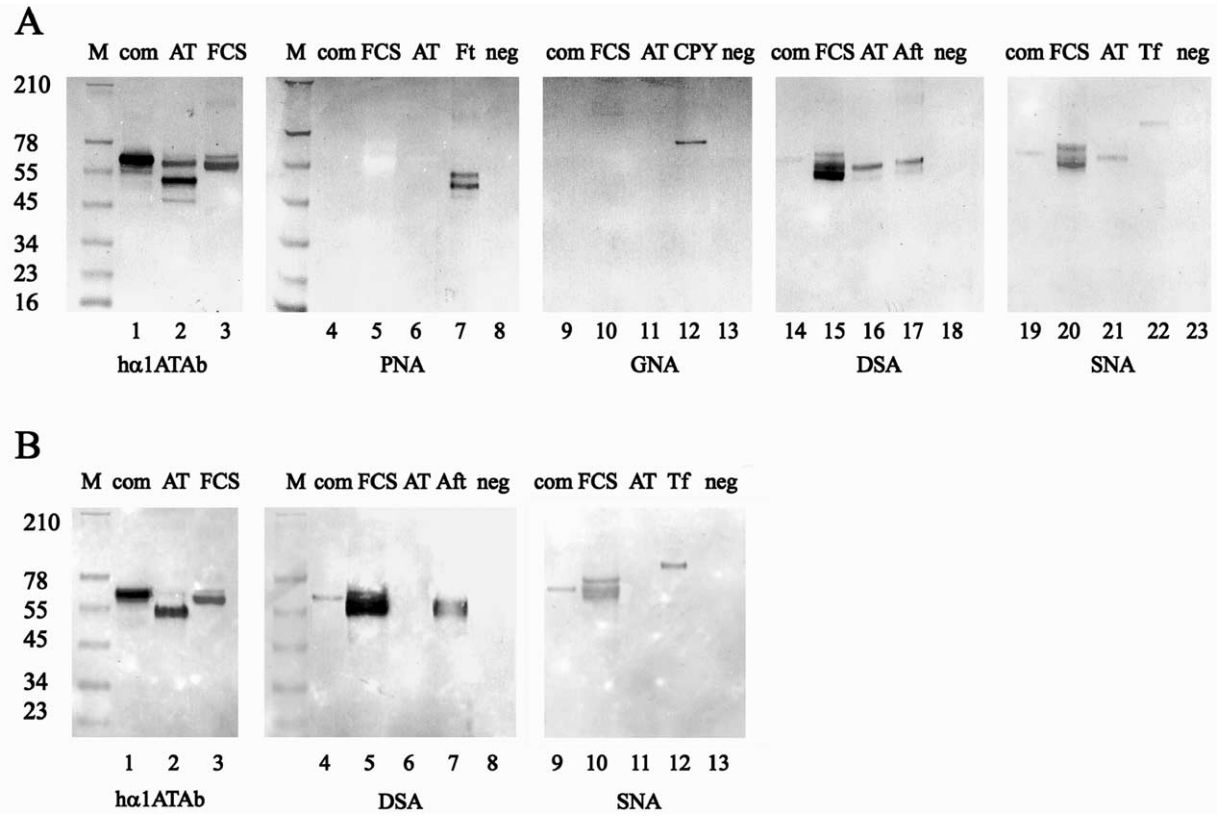

Fig. 4. Lectin blotting analysis. The secreted hα1AT proteins (AT) in the culture media from (A) BacATgng26- or (B) BacAT-infected Ea4 cells were affinity purified by trypsin-agarose chromatography. Trypsin inhibitors from fetal calf serum (FCS) and a commercially pure hα1AT protein (com) were also included. Western analysis with a rabbit polyclonal antibody was performed to localize the α1AT protein (lanes 1–3). For lectin blotting analysis, 0.1 μg of each protein sample was analyzed. A positive control glycoprotein was included for each lectin probe. Ft: fetuin, CPY: carboxypeptidase Y, Aft: asialofetuin, Tf: transferrin, neg: negative control as described in Section 2. Note that FCS contains many different trypsin inhibitors, therefore, many signals were revealed by DSA and SNA lectins (A: lanes 15 and 20; B: lanes 5 and 10).

been produced from these cells, as yet a portion of the secreted glyoproteins is still undersialylated. The authors speculated that this phenomenon might be due to the pools of selected CHO cell clones used in the study. Alternatively, stable Sf9 and Tn-5B1-4 insect cell lines expressing mammalian GT and ST have been established (Hollister and Jarvis, 2001; Breitbach and Jarvis, 2001). When baculovirus gp64 was expressed in both stable lines and the Sf9 class I α-mannosidase was expressed in the stable Sf9 line, terminally sialylated target proteins were detected. However, the antibiotics-selected Tn-5B1-4 insect cell line was not as genetically stable as the Sf9 line (Breitbach and Jarvis, 2001). It is not clear if these lines can be easily maintained and are genetically stable for scaled-up expression.

The direction of the insect *N*-glycosylation pathway to terminal sialylation, in our expression system, was achieved by concurrently expressing the target protein with key glycosyltransferases in insect cells through use of a single recombinant baculovirus. Our results suggest that terminal sialylation can be easily achieved without cell selection and cloning. However, we also faced the difficulty that not all hα1AT proteins were sialylated in our expression system. Future studies on our expression system are warranted to im-

prove the sialylation efficiency in order to produce more humanized glycoproteins for therapeutic purposes.

Acknowledgements

We thank Dr. M. Brantly for the gift of hα1AT cDNA, Dr. E.G. Berger for the gift of human β14GT cDNA, Dr. H. Schachter for the gift of human GnT2 cDNA and Dr. Hsou-min Li for critically reading this manuscript. This work was supported by grants (to H.C.) from the National Science Council (Grant 88-CPC-E-001-001) and Academia Sinica of Taiwan.

References

Altmann, F., Kornfeld, G., Dalik, T., Staudacher, E., Glossl, J., 1993. Processing of asparagine-linked oligosaccharides in insect cells. *N*-Acetylglucosaminyltransferase I and II activities in cultured lepidopteran cells. Glycobiology 3, 619–625.

Altmann, F., Staudacher, E., Wilson, I.B., Marz, L., 1999. Insect cells as hosts for the expression of recombinant glycoproteins. Glycoconj. J. 16, 109–123.

Belyaev, A.S., Roy, P., 1993. Development of baculovirus triple and quadruple expression vectors: co-expression of three or four bluetongue virus proteins and the synthesis of bluetongue virus-like particles in insect cells. Nucleic Acids Res. 21, 1219–1223.

Breitbach, K., Jarvis, D.L., 2001. Improved glycosylation of a foreign protein by Tn-5B1-4 cells engineered to express mammalian glycosyltransferases. Biotechnol. Prog. 17, 822–827.

Hollister, J.R., Jarvis, D.L., 2001. Engineering lepidopteran insect cells for sialoglycoprotein production by genetic transformation with mammalian beta 1,4-galactosyltransferase and alpha 2,6-sialyltransferase genes. Glycobiology 11, 1–9.

Hollister, J.R., Shaper, J.H., Jarvis, D.L., 1998. Stable expression of mammalian beta 1,4-galactosyltransferase extends the *N*-glycosylation pathway in insect cells. Glycobiology 8, 473–480.

Jarvis, D.L., Finn, E.E., 1995. Biochemical analysis of the *N*-glycosylation pathway in baculovirus-infected lepidopteran insect cells. Virology 212, 500–511.

Jarvis, D.L., Finn, E.E., 1996. Modifying the insect cell *N*-glycosylation pathway with immediate early baculovirus expression vectors. Nat. Biotechnol. 14, 1288–1292.

Jarvis, D.L., Bohlmeyer, D.A., Liao, Y.F., Lomax, K.K., Merkle, R.K., Weinkauf, C., Moremen, K.W., 1997. Isolation and characterization of a class II alpha-mannosidase cDNA from lepidopteran insect cells. Glycobiology 7, 113–127.

Jarvis, D.L., Kawar, Z.S., Hollister, J.R., 1998. Engineering *N*-glycosylation pathways in the baculovirus-insect cell system. Curr. Opin. Biotechnol. 9, 528–533.

Jarvis, D.L., Howe, D., Aumiller, J.J., 2001. Novel baculovirus expression vectors that provide sialylation of recombinant glycoproteins in lepidopteran insect cells. J. Virol. 75, 6223–6227.

Kawar, Z., Herscovics, A., Jarvis, D.L., 1997. Isolation and characterization of an alpha 1,2-mannosidase cDNA from the lepidopteran insect cell line Sf9. Glycobiology 7, 433–443.

Kubelka, V., Altmann, F., Kornfeld, G., Marz, L., 1994. Structures of the N-linked oligosaccharides of the membrane glycoproteins from three lepidopteran cell lines (Sf-21, IZD-Mb-0503, Bm-N). Arch. Biochem. Biophys. 308, 148–157.

Licari, P., Jarvis, D.L., Bailey, J.E., 1993. Insect cell hosts for baculovirus expression vectors contain endogenous exoglycosidase activity. Biotechnol. Prog. 9, 146–152.

Ogonah, O.W., Freedman, R.B., Jenkins, N., Patel, K., Rooney, B.C., 1996. Isolation and characterization of an insect cell line able to perform complex N-linked glycosylation on recombinant proteins. Biotechnology 14, 197–202.

Tan, J., D'Agostaro, A.F., Bendiak, B., Reck, F., Sarkar, M., Squire, J.A., Leong, P., Schachter, H., 1995. The human UDP-*N*-acetylglucosamine: alpha-6-D-mannoside-beta-1,2-*N*-acetylglucosaminyltransferase II gene (MGAT2). Cloning of genomic DNA, localization to chromosome 14q21, expression in insect cells and purification of the recombinant protein. Eur. J. Biochem. 231, 317–328.

Wagner, R., Geyer, H., Geyer, R., Klenk, H.D., 1996a. *N*-Acetyl-beta-glucosaminidase accounts for differences in glycosylation of influenza virus hemagglutinin expressed in insect cells from a baculovirus vector. J. Virol. 70, 4103–4109.

Wagner, R., Liedtke, S., Kretzschmar, E., Geyer, H., Geyer, R., Klenk, H.D., 1996b. Elongation of the *N*-glycans of fowl plague virus hemagglutinin expressed in *Spodoptera frugiperda* (Sf9) cells by coexpression of human beta 1,2-*N*-acetylglucosaminyltransferase I. Glycobiology 6, 165–175.

Weikert, S., Papac, D., Briggs, J., Briggs, J., Cowfer, D., Tom, S., Gawlitzek, M., Lofgren, J., Mehta, S., Chisholm, V., Modi, N., Eppler, S., Carroll, K., Chamow, S., Peers, D., Berman, P., Krummen, L., 1999. Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins. Nat. Biotechnol. 17, 1116–1121.