Glycobiology vol. 6 no. 2 pp. 165–175, 1996

Elongation of the *N*-glycans of fowl plague virus hemagglutinin expressed in *Spodoptera frugiperda* (Sf9) cells by coexpression of human β1,2-*N*-acetylglucosaminyltransferase I

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***Spodoptera frugiperda* (Sf9)-cells differ markedly in their protein glycosylation capacities from vertebrate cells in that they are not able to generate complex type oligosaccharide side chains. In order to improve the oligosaccharide processing properties of these cells we have used baculovirus vectors for expression of human (β1,2-*N*-acetylglucosaminyltransferase I (hGNT-I), the enzyme catalysing the crucial step in the pathway leading to complex type *N*-glycans in vertebrate cells. One vector (Bac/GNT) was designed to express unmodified GNT-I protein, the second vector (Bac/tagGNT) to express GNT-I protein with a tag epitope fused to its N-terminus. In Sf9-cells infected with Bac/tagGNT-virus a protein of about 50 kDa representing hGNT-I was detected with an antiserum directed against the tag epitope. HGNT-I activity was increased at least threefold in lysates of infected cells when *N*-acetylglucosamine (GlcNAc)-free ovalbumine was used as substrate. To monitor hGNT-I activity in intact Sf9-cells, the glycosylation of coexpressed fowl plague virus hemagglutinin (HA) was investigated employing a galactosylation assay and chromatographic analysis of isolated HA *N*-glycans. Coexpression of hGNT-I resulted in an at least fourfold increase of HA carrying terminal GlcNAc-residues. The only structure detectable in this fraction was $GlcNAcMan_3GlcNAc_2$. These results show that hGNT-I is functionally active in Sf9-cells and that the *N*-glycans of proteins expressed in the baculovirus/insect cell system are elongated by coexpression of glycosyltransferases of vertebrate origin. Complete complex type oligosaccharide side chains were not observed when hGNT-I was overexpressed, thus supporting the concept that Sf9-cells do not contain glycosyltransferases acting after hGNT-I.**

Key words: β1,2-*N*-acetylglucosaminyltransferase I/baculovirus vectors/expression of recombinant proteins/*N*-glycosylation in Sf9-cells

Introduction

Detailed insight has been obtained in the biosynthesis, structure, and functions of protein bound carbohydrate side chains. There is now substantial evidence that they play a crucial role in maintaining of structure and stability of glycoproteins, in targeting of proteins to different cellular compartments, and in mediating cell adhesion and tissue organisation (for reviews, see Hubbard and Ivatt, 1981; Olden *et al.*, 1982; Elbein, 1987; Rademacher *et al.*, 1988; Kobata, 1992; Lis and Sharon, 1993; Varki, 1993). Oligosaccharides have also been shown to influence the antigenicity of proteins by modulating polypeptide epitopes (Klenk, 1990; Munk *et al.*, 1992).

Compared to the wealth of information available on the nature of glycans of vertebrate origin our knowledge of carbohydrates from invertebrate sources, in particular insects, is fragmentary. Only recently, with the development of the baculovirus expression system for the production of recombinant proteins (for reviews, see Kang, 1988; Luckow and Summers, 1988; Miller, 1988; O'Reilly *et al.*, 1994), the use of lepidopteran cell lines has stimulated the interest in the glycosylation capacity of insect cells. Many posttranslational modifications occur in a way closely related to vertebrate systems, thus often generating biologically active recombinant proteins from insect cells. However, for most of the glycoproteins expressed so far by use of baculovirus vectors, the oligosaccharide side chains have been shown to differ remarkably from their vertebrate counterparts. Apart from human plasminogen which was reported to carry complex type carbohydrates when produced in two insect cell lines (Davidson *et al.*, 1990, 1991a), all other recombinant glycoproteins expressed in such systems had only high-mannose type and short truncated *N*-linked carbohydrates (Jarvis and Summers, 1989; Kuroda *et al.*, 1990; Chen *et al.*, 1991; Wathen *et al.*, 1991; Noteborn *et al.*, 1992; Veit *et al.*, 1993; Voss *et al.*, 1993; Yeh *et al.*, 1993; Hogeland and Deinzer, 1994; Ponimaskin *et al.*, 1994). This also holds true for the endogenous glycoproteins of insect cells which were similarly shown to lack complex type oligosaccharide side chains (Butters and Hughes, 1981; Hsieh and Robbins, 1984; Williams *et al.*, 1991; Staudacher *et al.*, 1992a). Very recently, relatively low levels of β1,2-*N*-acetylglucosaminyltransferase-I (GNT-I) activity have been detected in four insect cell lines using synthetic oligosaccharides as substrates (Altmann *et al.*, 1993; Velardo *et al.*, 1993). The corresponding *N*-glycans with terminal *N*-acetylglucosaminyl (GlcNAc)-residues, however, have been found only as minor constituents of the carbohydrate side chains of endogenous membrane glycoproteins prepared from *Mamestra brassicae*, *Bombyx mori*, and *Spodoptera frugiperda* insect cell lines (Kubelka *et al.*, 1994), of honeybee venom phospholipase A_2 (Kubelka *et al.*, 1993) and of apolipophorin III from migratory locust (Hård *et al.*, 1993). These data gave rise to the now widely accepted notion that insect cells only possess an incomplete subset of enzymes involved in glycosylation reactions as compared to that of vertebrate cells.

The biosynthesis of *N*-linked oligosaccharides has been studied in great detail in vertebrate cells (Kornfeld and

Kornfeld, 1985; Schachter, 1986). In mammalian cells, GNT-I catalyses an essential, committed step in the conversion of oligomannosidic to complex- or hybrid-type *N*-glycans, which is the transfer of a GlcNAc-residue from UDP-GlcNAc to the mannose-residue linked (α1,3) to the (β1,4)-linked core mannose-residue of $Man_5GlcNAc_2$-Asn. Thereby, the oligosaccharide becomes substrate for further processing enzymes. GNT-I is a type II transmembrane protein resident in the medial Golgi apparatus of vertebrate cells and represents a typical member of the vast family of glycosyltransferases (for review, see Paulson and Colley, 1989). The human GNT-I gene has been cloned and sequenced. It encodes a protein of 445 amino acids with a calculated molecular mass of 50,886 Da (Kumar *et al.*, 1990). No *N*-glycosylation site has been detected in the predicted hGNT-I protein sequence. A soluble form of rabbit GNT-I (Sarkar, 1994) and α1,3galactosyltransferase (Joziasse *et al.*, 1990) have been purified from Sf9-cells after expression from recombinant baculoviruses.

The hemagglutinin (HA) of influenza A viruses is a prototype of a class I transmembrane glycoprotein whose structure has been elucidated thoroughly (Wilson *et al.*, 1981; Wiley and Skehel, 1987). The HA used in this study was derived from fowl plague virus (influenza virus A/FPV/Rostock/34[H7N1]). When HA is transported to the cell surface, it undergoes *N*-glycosylation, trimerisation, and cleavage into the two fragments HA1 and HA2. Seven consensus sequences for *N*-linked glycosylation have been found in the protein, all of them being used for the attachment of oligosaccharide side chains (Keil *et al.*, 1984). The HA molecule has been expressed in *Spodoptera frugiperda* (Sf9)-cells (Kuroda *et al.*, 1986) and in larvae of *Heliothis virescens* (Kuroda *et al.*, 1989) and has been shown to undergo essentially the same posttranslational modifications as observed in vertebrate cells. However, trimerisation and cleavage reactions are significantly retarded and less efficient in insect cells (Kuroda *et al.*, 1991). The glycosylation status of HA produced in Sf9-cells has also been examined and compared to that from chicken embryo cells (Kuroda *et al.*, 1990). Only high mannose type structures and short truncated trimannosyl structures were detected on HA from Sf9-cells, whereby the trimannosyl structures replaced the complex type oligosaccharides found on HA produced in chicken embryo cells.

Since many structural and functional properties of a glycoprotein depend on glycosylation, it is desirable to furnish a recombinant protein with its authentic glycans. We have therefore made an attempt to modulate the oligosaccharide pattern of recombinant proteins expressed in the baculovirus system by supplying the insect cell's glycosylation machinery with an enzyme crucial for the generation of complex type glycans. To this end, we have expressed human GNT-I in Sf9-cells. Human GNT-I synthesized in insect cells showed activity both in an *in vitro* assay and towards the oligosaccharides of HA that was expressed simultaneously in Sf9-cells.

Results

Expression of human GNT-I in insect cells

Two recombinant baculoviruses were constructed to express the human *N*-acetylglucosaminyltransferase I (hGNT-I) in *Spodoptera frugiperda* (Sf9)-cells. The first virus, named Bac/GNT, was designed for the expression of a non modified authentic human GNT-protein. The second recombinant baculovirus, Bac/tagGNT, was engineered to express a hGNT-I molecule containing a N-terminally fused highly immunogenic epitope from a H3 serotype HA of influenza virus in order to confer an antigenic tag to the protein (Kolodziej and Young, 1991) (for details, see Materials and methods).

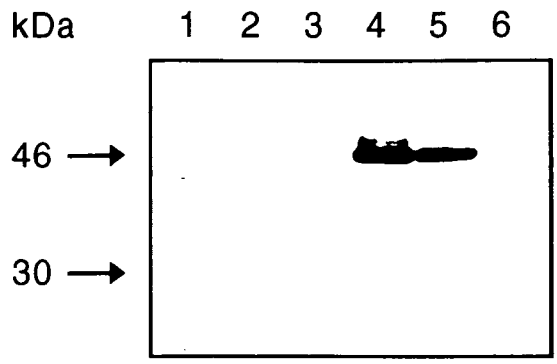

Fig. 1. Detection of tagged GNT-I protein in insect cells by immunoblotting. Proteins from lysates of Sf9-cells infected with different baculoviruses for 48 h were separated by SDS–PAGE and then blotted to nitrocellulose. The blot was analyzed with an antibody directed against the tag epitope. Sf9-cells had been treated as follows: no virus infection (lane 1), infection with authentic AcNPV (lane 2), infection with Bac/HA-virus (lane 3), infection with Bac/tagGNT-virus (lane 4), infection with Bac/tagGNT-and Bac/HA-virus (lane 5), infection with Bac/GNT-virus (lane 6).

Cell extracts prepared 48 h post infection with the Bac/tagGNT-virus were monitored for the presence of the tagged GNT-I protein by SDS–PAGE and subsequent Western blotting. Blots were analyzed using a polyclonal rabbit antibody directed against the tag epitope. A distinct protein band of about 50 kDa is present in cell lysates from Bac/tagGNT-virus infected cells but does not appear in mock-infected cells or cells infected with wild type *Autographa californica nuclear polyhedrosis* baculovirus (AcNPV) (Figure 1). The apparent molecular mass of this band is in good agreement with that predicted from the hGNT-I coding sequence (Kumar *et al.*, 1990). The expression of the GNT-I protein is reduced in cells double-infected with Bac/tagGNT- and Bac/HA-virus as compared to cells only infected with Bac/tagGNT-virus. There is no cross-reactivity detectable between the tag epitope which is derived from a H3 serotype HA molecule and the fowl plague virus (FPV) HA (serotype H7) which is produced in cells infected with Bac/HA virus. When hGNT-I is expressed from Bac/GNT-virus, no protein is detected with the tag antibody indicating that the tag epitope is not an inherent element of the GNT-I-protein. Hence, the tagging strategy described here has proved to be a valuable method for the detection of the hGNT-I protein without raising specific antibodies.

To examine whether human GNT-I is enzymatically active when expressed in Sf9-cells we have modified a previously described *in vitro* assay (Chaney and Stanley, 1986) allowing the evaluation of the GNT-I activity of cell lysates. In this assay, $GlcNAcMan_5GlcNAc_2$ side chains of ovalbumin were converted by treatment with (β-*N*-acetyl-D-glucosaminidase to $Man_5GlcNAc_2$ to act as a substrate for

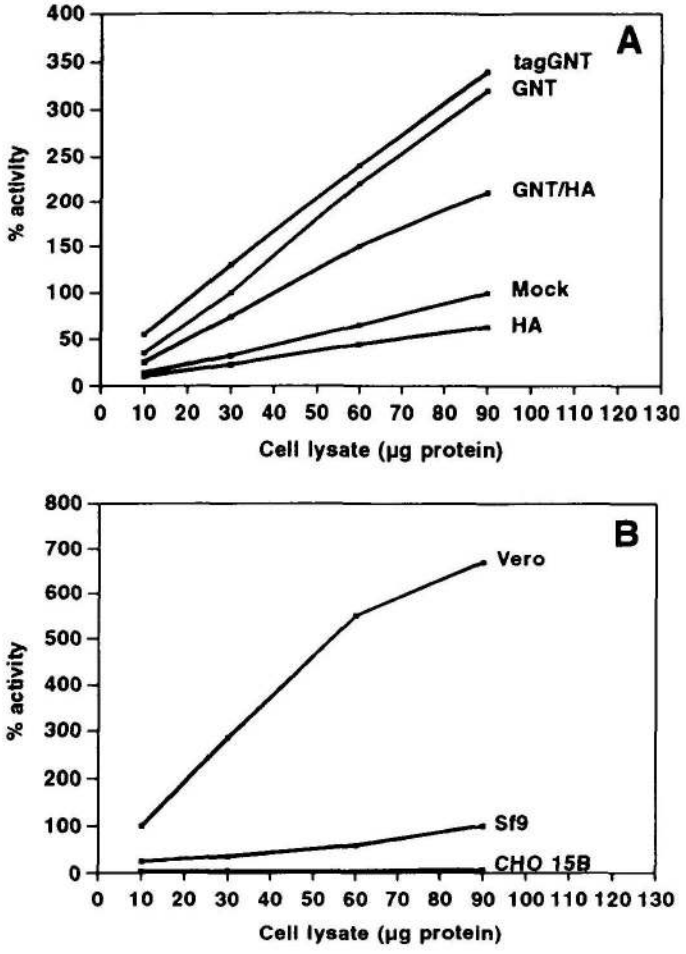

Fig. 2. Analysis of GNT-I activity of Sf9-cells. Lysates from cells infected with different baculoviruses for 48 h were subjected to the GNT-I assay. GNT-I levels are expressed as percent values. Calculation of these relative activities is based on the value obtained with 90 µg of lysate protein from mock-infected Sf9-cells which was set to 100%. Diagram (**A**) shows the results obtained for Sf9-cells infected with Bac/HA-virus (HA), Bac/GNT-virus (GNT), Bac/tagGNT-virus (tagGNT), Bac/GNT- and Bac/HA-virus (GNT/HA), and without viral infection (Mock). In diagram (**B**) GNT-I activity of mock-infected Sf9-cells is compared to that of vero cells and CHO 15B cells.

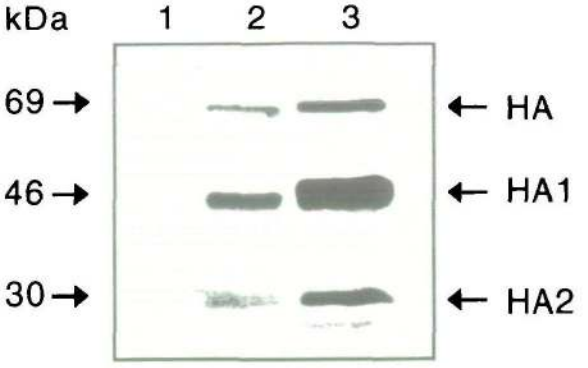

Fig. 3. Identification of terminal GlcNAc-residues on the oligosaccharide side chains of HA by *in vitro* [^{3}H]galactosylation. Sf9-cells were mock-infected (lane 1), singly infected with Bac/HA-virus (lane 2) or doubly infected with Bac/HA- and Bac/GNT-virus (lane 3) for 48 h. Equal amounts of HA-protein were subjected to *in vitro* galactosylation with UDP-[6–^{3}H]galactose. HA was immunoprecipitated using an anti FPV antibody and subsequently analyzed by SDS–PAGE. Protein bands were visualized by fluorography and quantified densitometrically.

the GNT-I-catalyzed transfer of tritiated UDP-GlcNAc. The extent of radioactive label incorporated into ovalbumin was taken as a measure for the GNT-I activity of the tested cell lysates.

Defined amounts of lysates from cells infected with different baculoviruses for 48 h were subjected to this assay (Figure 2). Mock-infected Sf9-cells exhibited a basal inherent GNT-I activity (Figure 2B) as compared to CHO 15B cells which are deficient in GNT-I activity and were therefore taken as a negative control (Gottlieb *et al.*, 1974). The infection of Sf9-cells with a recombinant baculovirus to express FPV HA (Kuroda *et al.*, 1986) markedly reduced this inherent enzymatic activity (Figure 2A) as did the infection with wild type baculovirus (AcNPV) (data not shown). Sf9-cells infected with Bac/GNT-virus exhibited a nearly 3.5-fold increase in GNT-I activity when compared to mock-infected cells, but did not reach the GNT-I activity level of Vero cells which were used as vertebrate positive control cells in this assay (Figure 2B). Coexpression of FPV HA reduced the activity observed in cells only infected with Bac/GNT-virus. Nevertheless, the GNT-I activity of double-infected cells is about four times higher than that of cells only infected with Bac/HA. The presence of swainsonine, a potential mannosidase II inhibitor, had no effect on the extent of ^{3}H-GlcNAc-transfer. This clearly shows that no glycosyltransferases other than GNT-I were active in the tested lysates. Essentially the same results were obtained with Sf9-cells infected with Bac/tagGNT-virus indicating that the N-terminally fused tag-peptide did not interfere with the expression and activity of hGNT-I. Taken together, these results show that expression of human GNT-I in insect cells has been accomplished that is enzymatically active also in the case of coexpression with a second recombinant protein.

In vivo activity of human GNT-I in insect cells

The finding that human GNT-I produced in insect cells displayed enzymatic activity in an *in vitro* assay raised the question whether a similar activity could be detected *in vivo* towards a glycoprotein expressed simultaneously in Sf9-cells. FPV HA was chosen as a model glycoprotein since the structures of its *N*-glycans have already been elucidated after expression in Sf9-cells (Kuroda *et al.*, 1990). Our experimental approach was to identify terminal GlcNAc-residues on the oligosaccharide side chains by *in vitro* galactosylation using tritiated UDP-galactose and galactosyltransferase from human milk (van den Eijnden *et al.*, 1983; Verdon and Berger, 1983). Sf9-cells were infected with Bac/HA-virus alone or doubly infected with Bac/HA- and Bac/GNT-virus. After 48 h the HA content of cell lysates was determined by western blotting and immunostaining of HA bands with a polyclonal serum directed against FPV (data not shown). Equal amounts of HA were then submitted to *in vitro* galactosylation. Subsequently, HA was immunoprecipitated using a polyclonal anti FPV antiserum and applied to SDS–PAGE and autoradiography (Figure 3). Intensities of the HA bands were calculated

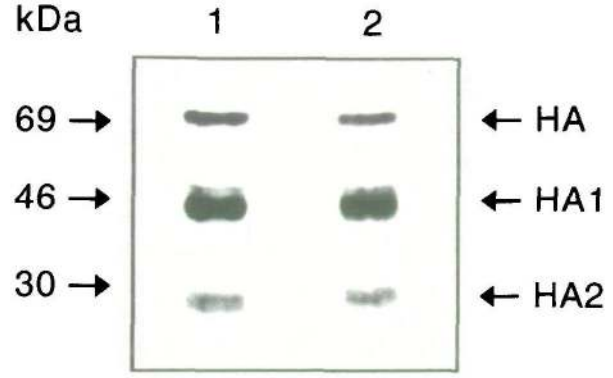

Fig. 4. Purified HA used for oligosaccharide structural analysis. 24 h after infection with either Bac/HA-virus (lane 1) or with Bac/HA- and Bac/GNT-virus (lane 2) Sf9-cells were labeled with [2-^{3}H]mannose for additional 24 h. HA was isolated from cell lysates by immunoaffinity chromatography. Aliquots of the eluates were applied to SDS–PAGE and protein bands were visualized by fluorography.

densitometrically and interpreted for the extend of terminal GlcNAc-residues on its *N*-glycans. No specific protein bands were detected in lysates from mock-infected cells. The HA from cells simultaneously expressing human GNT-I is at least four times more accessible to *in vitro* galactosylation than that from cells only expressing HA thereby revealing an approximately fourfold increase in the amount of terminal GlcNAc-residues on HA from doubly infected cells. As a result of this elongation of the *N*-glycans the molecular weight of the HA1 subunit, which carries 5 of the 7 oligosaccharide side chains of the entire HA molecule, is distinctly increased in doubly infected cells. Identical results were obtained when human GNT-I was expressed from Bac/tagGNT-virus (data not shown) which is in accordance with the data obtained from the *in vitro* activity assay (see Figure 2). These results show that the carbohydrate side chains of FPV HA expressed in Sf9-cells are elongated by coexpression with human GNT-I.

Isolation of oligosaccharide side chains of Sf9-cell derived HA

To determine the amount of side chains carrying terminal GlcNAc-residues and to elucidate their precise structure, *N*-glycans of Sf9-cell derived HA were subjected to structural analysis.

Oligosaccharide side chains of the HA molecule were metabolically labeled by growing infected Sf9-cells in the presence of [2-^{3}H]mannose in the culture medium. Lysates prepared from cells expressing HA with or without simultaneous expression of human GNT-I were subjected to immunoaffinity chromatography. Unbound material was washed from the matrix and specifically bound HA was eluted by alkaline pH. Aliquots of the eluate were analysed for purity by SDS–PAGE and autoradiography (Figure 4). Only HA and its cleavage products HA1 and HA2 were detected, indicating that other labeled proteins were absent in the eluate fraction. Glycopeptides derived from the purified HA were obtained by trypsin treatment and digested with endo-β-*N*-acetylglucosaminidase H (endo H). Endo-H-sensitive *N*-glycans were separated from endo-H-resistant glycopeptides by gel filtration on Biogel

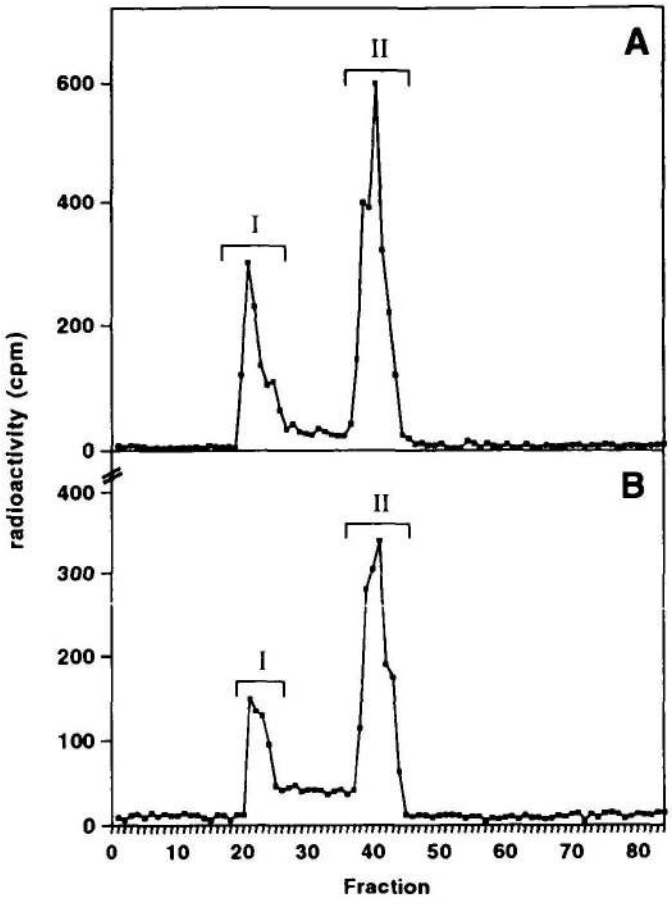

Fig. 5. Separation of endo-H-sensitive oligosaccharides and endo-H-resistant glycopeptides by gel filtration. Tryptic peptides of purified HA from cells infected with Bac/HA-virus (**A**) or Bac/HA- plus Bac/GNT-virus (**B**) were treated with endo H. Resulting products were fractionated on a Biogel P-4 column (-400 mesh; 0.6 cm × 200 cm) with 0.02% aqueous sodium azide as eluant at hydrostatic pressure. Fractions of 850 μl were collected at a flow rate of 0.6 ml/h, and an aliquot of each fraction was monitored for radioactivity by liquid scintillation counting. Fractions comprising endo-H-resistant glycopeptides (I) and endo-H-sensitive oligosaccharides (II) were pooled as indicated by horizontal brackets.

P-4 (Kobata *et al.*, 1987). The proportion of endo-H-resistant (30%) and endo-H-sensitive (70%) material was similar, irrespective of whether the cells had been infected only with Bac/HA-virus or doubly infected with Bac/HA- and Bac/GNT-virus (Figure 5). Thus, coexpression of human GNT-I had no effect on oligosaccharide trimming. Endo-H-resistant glycans were then liberated from glycopeptides by treatment with peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F (*N*-Glycosidase F) (Tarentino *et al.*, 1985). Oligosaccharides released were isolated from remaining peptides by reversed-phase HPLC. Residual peptides were devoid of any radioactivity indicating that the oligosaccharide side chains have been completely removed from the protein. Both the endo-H-sensitive and the endo-H-resistant glycan fractions were reduced with sodium borohydride and characterized as described below.

Analysis of oligosaccharide structures

Part of the endo-H-resistant *N*-glycans from Sf9-cells has been reported to be fucosylated (Kuroda *et al.*, 1990; Staudacher *et al.*, 1992b). To reduce the heterogeneity of these

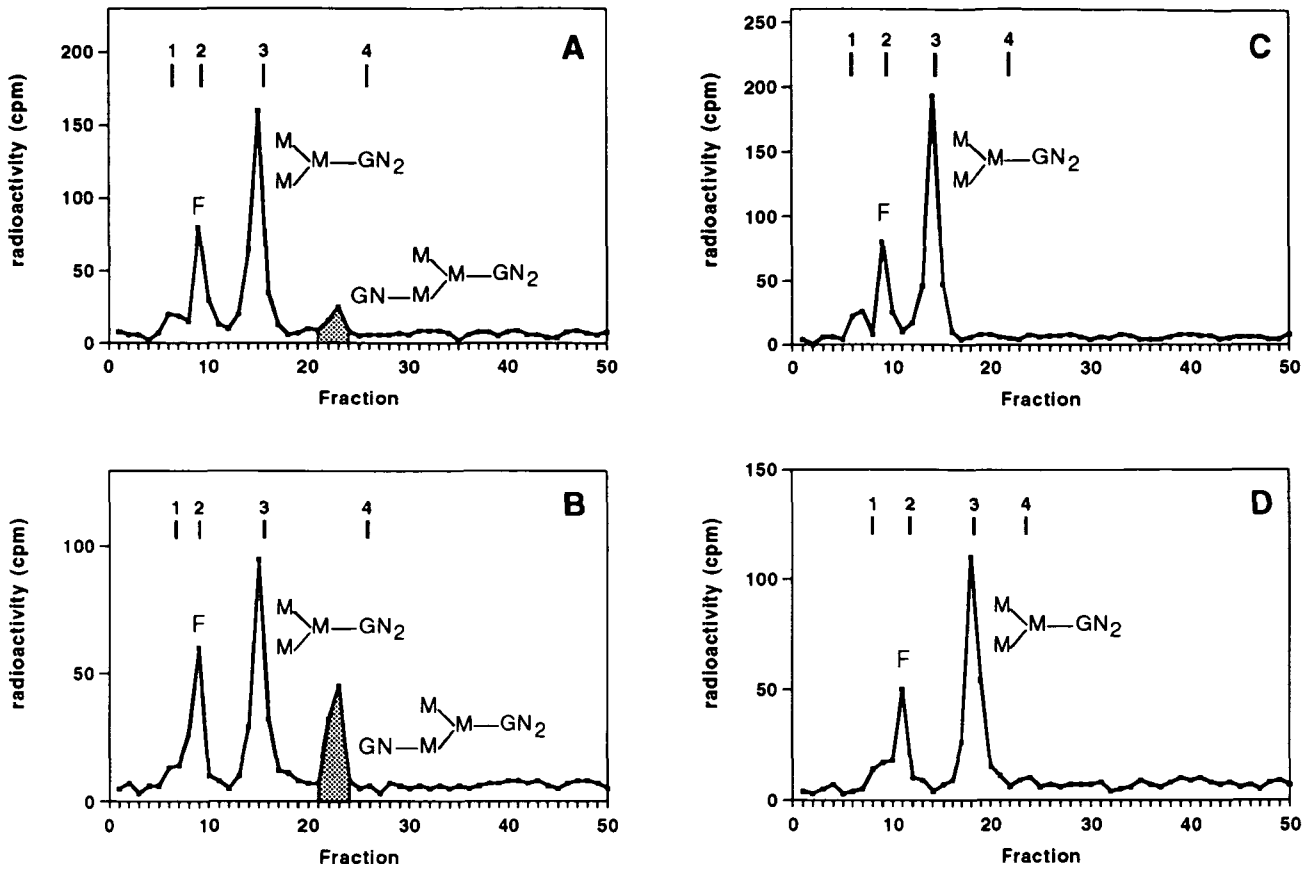

Fig. 6. Fractionation of endo-H-resistant *N*-glycans of HA derived from cells infected with Bac/HA-virus (**A** and **C**) or Bac/HA- plus Bac/GNT-virus (**B** and **D**). Oligosaccharides released from tryptic glycopeptides with *N*-Glycosidase F were treated either with α-fucosidase alone (**A** and **B**) or with α-fucosidase and β-*N*-acetyl-D-glucosaminidase (**C** and **D**). Products were fractionated by HPAEC on a CarboPac PA-100 column (4 × 250 mm) using isocratic conditions for 20 min at 50 mM NaOH followed by a gradient of 50–80 mM NaOH and of 0–50 mM sodium acetate within the next 20 min. Fractions (350 μl) were collected at a flow rate of 1 ml/min and monitored for radioactivity. Numbers (1–4) with bars indicate the elution positions of isomaltosyl oligosaccharide alditols with 1–4 glucose units. Structures of glycans representing individual peaks are depicted in the chromatograms. (Abbreviations: F, fucose; GN, GlcNAc; M, mannose).

oligosaccharides they were therefore treated with α-fucosidase. The resulting glycan fractions were then applied to high-pH anion-exchange chromatography (HPAEC). Three distinct peaks could be detected after this fractionation step (Figure 6A,B). The first peak–eluting at about 1.7 glucose units–represented liberated fucose that had been metabolically labeled by conversion of the employed [2-^{3}H]mannose marker (Strube *et al.*, 1988; Kuroda *et al.*, 1990). The second peak–eluting at 2.9 glucose units–represented the trimannosyl-core structure ($Man_3GlcNAc_2$), whereas the third peak–eluting at 3.7 glucose units–comprised trimannosylstructures with terminal GlcNAc attached in (β1–2)-linkage to the (α1–3)-bound mannose residue ($GlcNAcMan_3GlcNAc_2$). The structures depicted here were confirmed by comparison with the elution profiles of the corresponding authentic reference compounds. These data show that the oligosaccharides with terminal GlcNAc were increased fourfold when HA was coexpressed with human GNT-I (compare hatched areas in Figure 6, A and B). These results closely correspond to those obtained in the *in vitro* galactosylation experiment and clearly demonstrate that the recombinant human GNT-I is enzymatically active on the HA molecule passing through the protein processing pathway of Sf9-cells.

Additional treatment of the *N*-glycans with *N*-acetyl-β-D-glucosaminidase (hexosaminidase) from *Diplococcus pneumoniae* to remove (α1–2) terminally linked GlcNAc-residues (Yamashita *et al.*, 1981) led to the disappearance of the peaks eluting at 3.7 glucose units (Figure 6A,B). Only oligosaccharide peaks representing the trimannosyl-core structure remained. Evidence for the presence of complex type structures was not obtained.

Endo-H-resistant oligosaccharides treated with α-fucosidase and hexosaminidase were also characterized by Biogel P-4-chromatography (Figure 7). In agreement with the results obtained by HPAEC, only one oligosaccharide peak was detected eluting at about 7 glucose units. This material represented the trimannosyl-core structure. The peak occurring at the elution position of about one glucose unit corresponds to liberated fucose-residues. When *N*-glycans were released from HA immunoprecipitated after labeling by *in vitro* galactosylation (see above), trimannosyl species with one terminal GlcNAc-residue could be detected which were elongated by the addition of a single tritiated galactose unit during the *in vitro* galactosylation procedure (data not shown).

Endo-H-sensitive glycans were also fractionated by HPAEC. Five distinct oligosaccharide fractions were ob-

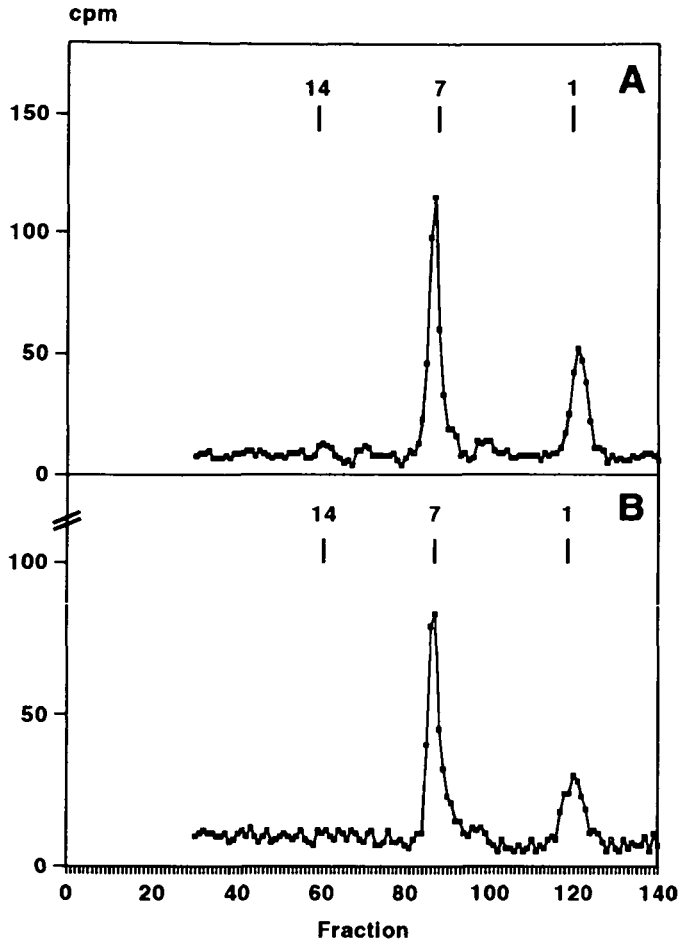

Fig. 7. Size determination of endo-H-resistant *N*-glycans by gel filtration. Oligosaccharides obtained after treatment with α-fucosidase and β-*N*-acetyl-D-glucosaminidase were applied to a Biogel P-4 column (-400 mesh; 1.6 cm × 60 cm) and eluted with 0.02% aqueous sodium azide at a flow rate of 0.2 ml/min. Fractions (850 μl) were collected and monitored for radioactivity by liquid scintillation counting. Panel (**A**) shows the results achieved with the HA-glycans from Sf9-cells infected with Bac/HA-virus, panel (**B**) those obtained for Sf9-cells infected with Bac/HA- plus Bac/GNT-virus. Numbers with bars indicate the elution positions of isomaltosyl oligosaccharide alditols with the given number of glucose units.

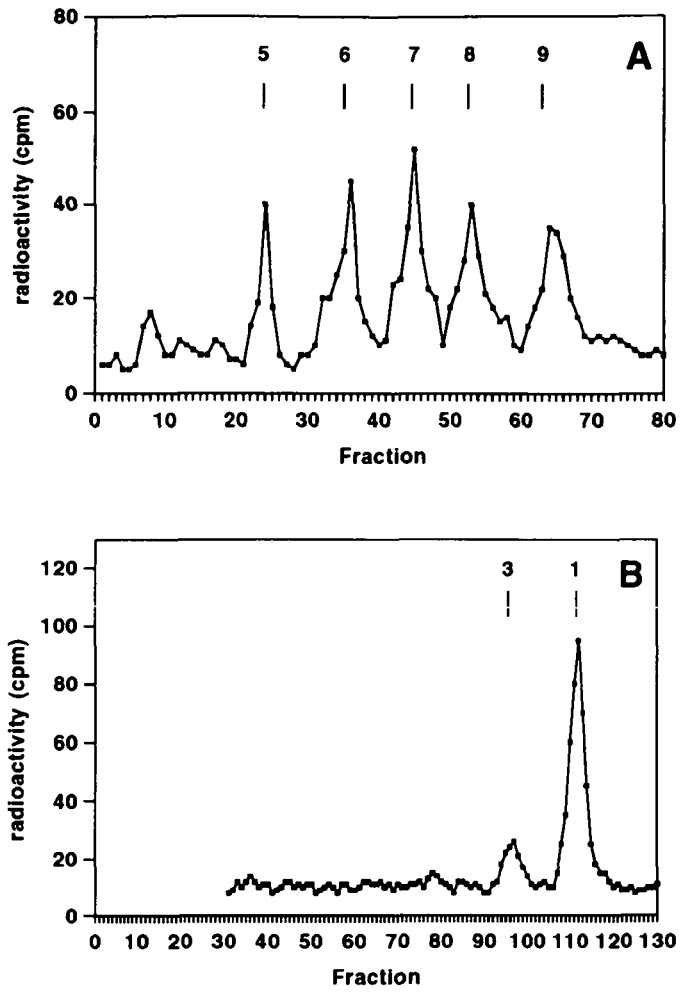

Fig. 8. Characterization of the endo-H-sensitive HA oligosaccharide fraction from Sf9-cells infected with Bac/HA and Bac/GNT. (**A**) HPAEC separation on a CarboPac PA-100 column (4 mm × 250 mm) using a gradient of 10–30 mM sodium acetate in 80 mM NaOH within 70 min. Fractions (350 μl) were collected at a flow rate of 1 ml/min and monitored for radioactivity. Numbers (5–9) with bars indicate the elution positions of oligomannosidic standard alditols $Man_{5-9}GlcNAc_{OH}$. (**B**) Size fractionation by gel filtration of products obtained after treatment of the glycan fraction depicted in (**A**) with (-mannosidase. Samples were applied to a Biogel P-4 column (-400 mesh, 1.6 cm × 60 cm) and eluted with 0.02% aqueous sodium azide at a flow rate of 0.2 ml/min. Fractions (850 μl) were collected and analyzed by liquid scintillation counting. Numbers with bars indicate the elution positions of isomaltosyl oligosaccharide alditols with the given number of glucosyl-residues.

tained by this technique both from cells expressing only the HA molecule (data not shown) and from those expressing HA with human GNT-I (Figure 8A). This again shows that expression of human GNT-I had no effect on the structure of endo-H-sensitive glycans isolated from the HA protein. By comparison with corresponding standards, the five fractions were identified as oligomannosidic oligosaccharides containing five to nine mannose residues. When the endo-H-sensitive glycan fraction was digested with α-mannosidase from jack beans and subsequently analysed by Biogel P-4 chromatography, only one oligosaccharide peak, eluting at about three glucose units, was detected (Figure 8B) which represented the Manβ1–4GlcNAc disaccharide unit. These results show that the endo-H-sensitive glycan fraction contained exclusively oligomannosidic species, all of them being converted to a common truncated disaccharide by treatment with α-mannosidase. Liberated mannose-residues appeared as a peak eluting at of about one glucose unit in the chromatograms. Furthermore, these data demonstrate the absence of terminal monosaccharide constituents other than α-mannosyl-residues in the endo-H-sensitive oligosaccharide fraction, hence ruling out the presence of endo-H-sensitive hybrid type species.

HPAEC performed after hydrolysis of the total HA oligosaccharide fraction revealed that nearly 20% of the radioactive label resided in fucosyl and roughly 80% in mannosyl residues (data not shown), indicating that [2–^{3}H]mannose is solely metabolized into radiolabeled fucose in Sf9-cells. This result was obtained for both cells expressing either HA or HA plus human GNT-I. Based on the assumption that the incorporated [2–^{3}H]mannose label is

equally distributed throughout endo-H-resistant and oligomannosidic structures, it can be deduced that oligomannosidic structures carried in the average about twice as much label as the partially fucosylated trimannosyl structures. Taking into account the total radioactivity of endo-H-resistant (30%) and endo-H-sensitive glycans fractions (70%; see Figure 5), it can be concluded that the molar ratio between these two glycan fractions is roughly 1:1. The relative abundances of carbohydrate species within the endo-H-resistant fractions were evaluated from the data obtained by HPAEC after α-fucosidase treatment (Figure 6). On this basis, species carrying terminal GlcNAc were calculated to represent less than 10% of this glycan fraction when the cells only expressed HA, but amounted to more than 40% when the cells expressed HA plus human GNT-I.

Discussion

Most of the knowledge gathered to date on the protein glycosylation potential of insect cells backs up the current view that these cells are incapable of generating complex type oligosaccharide side chains. We wanted to add a new facet to this subject by examining the effect of a vertebrate cell glycosyltransferase expressed in insect cells. To this end, the human *N*-acetylglucosaminyltransferase I (hGNT-I), an enzyme known to be essential for the synthesis of complex type *N*-glycans in vertebrates (Kornfeld and Kornfeld, 1985), was expressed in the lepidopteran cell line Sf9 mediated by infection with a recombinant baculovirus (AcNPV). Using this approach we were able to increase the apparent enzymatic GNT-I activity of infected Sf9-cells 3.5-fold in comparison to that of uninfected cells as judged by an *in vitro* activity assay. The infection of Sf9-cells with wild type AcNPV or a recombinant baculovirus expressing the influenza virus HA noticeably reduced both the endogenous and the overexpressed GNT-I activity probably due to an generalized detrimental effect of the virus infection on the cell's viability.

The consequences of hGNT-I expression on the protein glycosylation capacities of Sf9-cells were further studied by using coexpressed HA as substrate. The amount of terminal GlcNAc-residues on the oligosaccharide side chains of HA was taken as a measure of the *in vivo* GNT-I activity and was examined by *in vitro* galactosylation with UDP-[^{3}H]galactose and galactosyltransferase. We observed a more than fourfold increase in the amount of terminal GlcNAc-residues on the oligosaccharide side chains of HA expressed simultaneously with hGNT-I compared to that of solely expressed HA. This shows that hGNT-I is enzymatically active within the oligosaccharide processing pathway of Sf9-cells. This concept was further strengthened when a structural analysis of the oligosaccharide side chains of HA was performed. Oligosaccharides metabolically labeled with [2-^{3}H]mannose were isolated from immunoaffinity purified HA and then characterized by sequential enzymatic degradation and subsequent chromatographic procedures. The results revealed that about half of the *N*-glycans are endo-H-sensitive, irrespective of whether they had been processed in the absence or presence of hGNT-I. Furthermore, no significant differences were detected with the individual glycan structures in this fraction. Oligomannosidic chains carrying five to nine mannose residues were present both on solely expressed HA and on HA produced together with hGNT-I. None of these structures carried a terminal GlcNAc-residue indicating that coexpression of hGNT-I did not affect the enzymes involved in trimming of oligomannosidic side chains.

We were not able to detect any stimulatory effect of baculovirus infection on the endogenous GNT-I activity of Sf9-cells. Similar results have been obtained in Sf21-cells by Altman and coworkers (1993). However, activation of GNT-I activity upon baculovirus infection in Sf21-cells is still a matter of controversial discussion, since such an effect has been reported for these cells in another study (Velardo *et al.*, 1993). It has also been reported that baculovirus infection has a stimulating effect on the mannosidase activity of Sf21-cells which acts on the $Man_6GlcNAc_2$-precursor to generate the $Man_5GlcNAc_2$-structure (Davidson *et al.*, 1991). However, no differences in the glycosylation reactions of mock-infected and wild type baculovirus-infected cells were detected when Sf9-cells were used (Kretzschmar *et al.*, 1994). It thus appears that there are cell-specific differences in the response of the glycosylation machinery to baculovirus infection.

The endo-H-resistant glycan fraction comprised unsubstituted trimannosyl core structures ($Man_3GlcNAc_2$) and trimannosyl structures with a terminal GlcNAc-residue attached in (β1–2)-linkage to the (α1–3)-bound mannose residue ($GlcNAcMan_3GlcNAc_2$) as a result of GNT-I action. The latter species was originally described to appear as a minor component of the *N*-linked oligosaccharides of Semliki Forest virus glycoproteins produced in infected mosquito cells (Naim and Koblet, 1992). The amount of this structure is increased at least fourfold on HA from Sf9-cells simultaneously expressing hGNT-I clearly demonstrating that the human enzyme is active *in situ* within the protein glycosylation machinery of Sf9-cells. By coexpression of glycosyltransferases of vertebrate origin it is thus possible to modify the glycosylation pattern of recombinant proteins produced by the baculovirus/insect cell system. Since the two proteins were expressed via two individual recombinant baculoviruses, we cannot rule out the possibility that part of the cells is infected only with one of the two viruses. For the expression of the two Na^+,K^+-ATPase subunits by two different baculoviruses in Sf9-cells, it has already been shown that even at a relatively high multiplicity of infection some cells produced only one of the two subunits (DeTomaso *et al.*, 1993). Hence, HA molecules emerging from cells which do not express hGNT-I would lead to an apparent decrease of the overall hGNT-I effect. By using a double expression vector with both genes of interest it might be possible to further increase the amount of glycans carrying terminal GlcNAc-residues. In a previous study dealing with the glycosylation status of Sf9-cell derived HA without simultaneously expressing hGNT-I, *N*-glycans with terminal GlcNAc-residues were not detected (Kuroda *et al.*, 1990). This might have been due to the limited amounts of starting material subjected to carbohydrate structure analysis which made it impossible to trace this minor oligosaccharide fraction bearing terminal GlcNAc-residues.

A considerable portion of HA protein expressed in Sf9-cells remains unprocessed (Kuroda *et al.*, 1991) presumably owing to inefficient transport to the cell surface. HA of the Rostock strain of fowl plague virus used in this study has been reported before to undergo an irreversible con-

formational change at low pH (Ohuchi *et al.*, 1994). Since the Sf9-cells are cultured at pH 6.3 this mildly acidic milieu might cause partial denaturation of HA and a concomitant decrease in the transport rate. Furthermore, in the case of expression of tissue plasminogen activator (t-PA) in Sf9-cells the addition but not the processing of *N*-glycans was shown to be a prerequisite for efficient t-PA secretion (Jarvis *et al.*, 1990). Accordingly, partial glycosylation of individual HA molecules resulting from incomplete oligosaccharide chain addition to any of the seven glycosylation sites of overexpressed HA might further reduce the transport rate in Sf9-cells. The inability of such HA molecules still residing in the ER to gain access to the trimming enzymes in the Golgi may also contribute to the relatively large quantity of unprocessed *N*-glycans from these cells.

In vertebrate cells, hGNT-I is a crucial processing enzyme catalyzing a committed step in the biosynthetic pathway leading to complex type *N*-glycans. The observation that we did not find complex type glycans on HA from hGNT-I expressing Sf9-cells therefore supports the concept that glycosyltransferases responsible for further elongation are absent in these cells. The $Man_3GlcNAc_2$ structure found as the major component of the endo-H-resistant oligosaccharides in Sf9-cells is usually not present on vertebrate cell glycoproteins and is not an intermediate in the standard oligosaccharide processing cascade (Kornfeld and Kornfeld, 1985). Apart from insect cells, however, this structure has also been found with hen ovomucoid suggesting an alternate pathway for oligosaccharide processing (Yamashita *et al.*, 1983). The enzymatic activities responsible for the occurrence of this type of *N*-glycans in Sf9-cells are under present investigation.

So far, human plasminogen is the only recombinant protein reported to carry complex type oligosaccharide structures after expression in lepidopteran (Sf21-) cells (Davidson *et al.*, 1990). In addition, it has been reported that the pattern of plaminogen *N*-glycosylation depends on the length of time of infection, with the formation of complex type glycans being increased upon prolonged infection (Davidson and Castellino, 1991b). This observation led to the conclusion that the corresponding glycosyltransferases are normally not active in the cells and only become functional upon baculovirus infection. Additionally, the bulk (about 63%) of the carbohydrate side chains of human plasminogen expressed in *Mamestra brassicae* cells appeared to comprise complex type structures (Davidson and Castellino, 1991a). In our system using Sf9-cells we could neither detect a temporal dependence of the nature of *N*-glycans or the induction of enzymatic activities nor maintain baculovirus infected cells in culture for intervals of 96 h as described by Davidson and coworkers (Davidson and Castellino, 1991b). It is reasonable to assume that the infection of Sf9-cells with the recombinant baculoviruses used in our study caused severe cytopathic effects likely to arise from the incorporation of recombinant proteins into cellular membranes.

In summary, we have been able to modify insect cell specific *N*-glycans by expression of hGNT-I in Sf9-cells. It will now be interesting to see whether this change in the glycosylation pattern has any implications on the structural or functional properties of the HA protein. If so, it would be reasonable to stably express hGNT-I in conjunction with further glycosyltransferases in Sf9-cells. This might generate a useful system to thoroughly analyse the effect of individual monosaccharide constituents on the structure and function of a given glycoprotein.

Materials and methods

Cells and viruses

The *Spodoptera frugiperda* clone Sf9 of IPLB-Sf21-AE cells (Vaughn *et al.*, 1977) was maintained as a monolayer culture in TC100 insect cell medium containing 10% FCS (Gibco BRL, Paisley, Great Britain) as described previously (Carstens *et al.*, 1979). Vero cells and CHO 15B cells (kindly provided by Dr. Ari Helenius, New Haven, CT, USA) were grown as monolayer cultures in Dulbecco's minimal essential medium supplemented with 10% FCS (Gibco BRL).

The human GNT-I gene was excised from the pGEM72f+ vector (kindly provided by Dr. Pamela Stanley, New York, USA) by Xho I digestion. The sequence encoding the entire hGNT-I protein including the cytoplasmic tail and the transmembrane segment of this gene was amplified by the polymerase chain reaction (PCR) using the oligonucleotide CTTCCTGGATCCAGG***ATG***CTGAAG as a 5'-forward primer and the oligonucleotide CTGC***TAG***GATCCGTGCTAATTCCA as a 3'-reverse primer. Primers contained the translation start codon ATG and the translation end codon TAG at positions 162 and 1499, respectively, of the hGNT-I gene sequence (Kumar *et al.*, 1990). Both primers contained a BamHI-restriction site. Amplification was performed using a commercially available kit from Perkin Elmar Cetus Instruments, Weiterstadt, Germany. After digestion with BamHI the PCR product was inserted in the BamHI-site of the baculo transfer vector pVL1393 (Dianova, Hamburg, Germany) resulting in the plasmid pVL1393/GNT. To confer the immunogenic tag epitope, the GNT-coding region was excised from the pVL1393 plasmid with BamHI and then inserted into the BamHI-site of the pRS426tag plasmid (kindly provided by Dr. Martin Funk, Marburg, Germany). This plasmid carried a threefold repeat of a sequence encoding the highly immunogenic peptide YPYDVPDYA of a serotype H3 hemagglutinin of influenza virus. The GNT coding region was placed in the pRS426tag plasmid such that the tag epitope was situated right upstream of the GNT sequence. By DNA sequencing the reading frames of both the tag sequence and the GNT sequence were proved to be in frame. The tag-GNT sequence was then obtained from the pRS426tag plasmid by digestion with XbaI and EcoRI and ligated in the equally digested baculo transfer vector pVL1393/linker, thus giving rise to the construct pVL1393/tagGNT. The pVL1393/linker plasmid is a modified version of the pVL1393 with a linker sequence having been introduced between the XbaI- and the EcoRI-site which were too close together to allow the simultaneous digestion with these restriction enzymes within the original plasmid. All work with DNA constructs was done according to methods described earlier (Sambrook *et al.*, 1989) and the enzymes needed (except those for PCR, see above) were purchased from Boehringer (Mannheim, Germany).

The two plasmids pVL1393/GNT and pVL1393/tagGNT were then used for the generation of the recombinant baculoviruses Bac/GNT and Bac/tagGNT, respectively, by cotransfection into Sf9-cells together with Baculogold linearized baculovirus DNA (Dianova) using the lipofectin reagent (BRL Life Technologies, Inc., Gaithersburg, MD, USA). All procedures concerning virus production, selection for recombinant viruses and amplification were performed essentially as outlined before (Summers and Smith, 1987). The construction of the recombinant Bac/HA-virus for the expression of the hemagglutinin of influenza strain A/FPV/Rostock/34 [H7/N1] has already been reported (Kuroda *et al.*, 1986).

Immunostaining of separated proteins

Sf9-cells (1.5×10^6) inoculated with wild type baculovirus (AcNPV) or recombinant baculoviruses at a multiplicity of infection (moi) of 10 PFU/cell were harvested after 48 h and washed three times with cold phosphate-buffered saline (PBS) (135 mM NaCl, 2.5 mM KCl, Na_2HPO_4, KH_2PO_4, pH 7.2). The protein content was determined using the BCA protein assay reagent according to the manufacturer's protocol with bovine serum albumin as a standard (Pierce, Rockford, IL, USA). Proteins were subjected to SDS–PAGE on 10% gels (Laemmli, 1970) and electroblotted to a nitrocellulose membrane. The blot was incubated with a 1:500 dilution of a polyclonal rabbit anti tag epitope (see above) antibody (available from HISS Diagnostics, Freiburg, Germany). Staining of protein bands was achieved using a peroxidase-conjugated polyclonal anti rabbit antibody (1:500 diluted) (purchased from DAKO, Hamburg, Germany) and

the ECL-Western blotting detection reagent as outlined by the manufacturer's manual (Amersham Buchler Life Science, Braunschweig, Germany). Unless stated otherwise, all methods were performed according to Sambrook *et al.*, 1989.

N-Acetylglucosaminyltransferase-assay

Sf9-cells (3×10^6) seeded in 5.3 cm petri dishes were inoculated with baculoviruses (moi = 10) for 48 h. Cells were washed with cold PBS and then lysed in 150 μl of 60 mM HEPES, pH 6.4, containing 1% Nonidet P40. Protein content was determined using the BCA-assay (see above). 300 mg ovalbumin were dissolved in 10 ml of 50 mM sodium-citrate buffer, pH 5, and incubated at 37°C with 30 mU of *N*-acetyl-β-D-glucosaminidase from *Diplococcus pneumoniae* and 2 U of the same enzyme from beef kidney for 24 h (both enzymes available from Boehringer, Mannheim, Germany). Enzyme treatment and incubation was repeated once. The ovalbumin deprived of terminal *N*-acetylglucosaminyl-residues was then transferred to 60 mM HEPES, pH 6.4, by extensive dialysis and heated to 95°C for 10 min to destroy residual hexosaminidase activity; 200 ml of this ovalbumin preparation were incubated with defined amounts of protein from cell lysates. The volume was adjusted to 300 μl with HEPES-buffer and $MnCl_2$ was added to a final concentration of 5 mM. The transfer reaction was started by the addition of 1 μCi of UDP-*N*-acetyl-D-[6-^{3}H]glucosamine (Du Pont de Nemours, Dreieich, Germany). After a 3 h incubation at 27°C the ovalbumin was precipitated with 300 μl of 30% TCA. The pellet was washed three times with water and redissolved in 300 μl of 1N NaOH. Radioactivity of the samples was determined by liquid scintillation counting.

Galactosyltransferase assay

Sf9-cells (3×10^6) were inoculated with recombinant baculoviruses at a MOI of 10 PFU per cell for 48 h. Cells were washed three times with cold PBS and then lysed in 100 μl Tris/HCl, pH 7.0, containing 1.5% Triton X-100. The HA content of aliquots of the lysates was analysed by SDS-PAGE followed by Western blotting and identification of HA-specific protein bands with a polyclonal rabbit anti FPV antibody essentially as outlined above. HA-bands were quantitated densitometrically. Defined volumes of cell lysates bearing identical amounts of HA were then subjected to *in vitro* galactosylation by incubation with 10 μCi of UDP-D-[6-^{3}H]galactose (Amersham) and 10 mU of galactosyltransferase from human milk (Boehringer). Samples were adjusted to 200 μl with Tris-buffer, and $MnCl_2$ was added to a final concentration of 5 mM. Incubation was performed for 3 h at 30°C. Thereafter, 500 μl of RIPA-buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 10 mM EDTA, 1 mM PMSF, 10 mM iodoacetamide, 5000 U aprotinin, 20 mM Tris/HCl, pH 7.6) were added and the lysates centrifuged to remove insoluble debris. HA was immunoprecipitated from the supernatant with a rabbit polyclonal anti-FPV antiserum (1:300) and 30 μl of Protein-A-Sepharose (Sigma Chemie, Deisenhofen, Germany) (1:10; w:w in RIPA-buffer) overnight at 4°C. The immune complexes were washed three times with RIPA-buffer, boiled in electrophoresis sample-buffer, analysed by SDS-PAGE on a 10% gel, and visualized by fluorography.

Immunoaffinity purification of radiolabeled HA

Preparation of the immunoaffinity-matrix was carried out using a modification of the procedure previously described (Schneider *et al.*, 1982). Four milliliters of swollen Protein A-Sepharose beads (1 g of dried material) were incubated at room temperature with 1 ml of a polyclonal rabbit anti FPV antiserum in 0.2 M borate buffer, pH 9.0, for 2 h. Beads were washed three times with 10 volumes of borate buffer to remove unbound material and then resuspended in 50 ml of this buffer. Dimethylpimelimidate hydrochloride (DMP) (Pierce) was added stepwise to a final concentration of 20 mM to covalently crosslink antibodies to the Protein-A-Sepharose beads. The pH of the suspension was readjusted to 9.0 upon each DMP addition. After 1 h of incubation at room temperature the crosslinking reaction was stopped by washing the beads once with 0.2 M ethanolamine, pH 8.0, and then incubating them for 2 h in this solution. Afterwards, the beads were resuspended in PBS with 0.02% sodium azide for long term storage.

Sf9-cells (5×10^7) were inoculated with recombinant baculoviruses at a MOI of 10 PFU/cell in TC100-medium. After 24 h, cells were transferred to medium lacking glucose and tryptose and labeled with [2-^{3}H]mannose (Amersham) (50 μCi/ml) for the following 24 h. The radiolabeled cells were washed three times with cold PBS, and the pelleted cells were solubilized in 10 ml of Triton lysis buffer (200 mM NaCl, 1% Triton X-100, 1% octylglucosid, 10 mM EDTA, 1 mM PMSF, 10 mM iodoacetamide, 5000 units of aprotinin, 20 mM morpholinoethanesulfonic acid, 30 mM Tris/HCl, pH 8.0). The lysate was centrifuged at 15,000 $\times$ *g* to remove debris and applied to the immunoaffinity matrix previously equilibrated in Triton lysis buffer. After 2 h of gentle agitation at 4°C, Protein-A-Sepharose beads were washed in sequence three times with each of the following solutions: (i) 0.5 M NaCl, 0.05 M Tris/HCl pH 8.2, 1 mM EDTA, 0.5% Triton X-100; (ii) 0.15 M NaCl, 0.05 M Tris/HCl pH 8.2, 0.5% Triton X-100; (iii) 0.15 M NaCl, 0.5% deoxycholate. HA protein specifically bound to the matrix was eluted with 50 mM diethylamine, pH 11.5, containing 0.5% deoxycholate. An aliquot of the eluate was dialysed extensively against 10 mM NH_4HCO_3, pH 8.2, containing 0.05% desoxycholate and then lyophilized. The dried material was redissolved in electrophoresis sample-buffer and then applied to SDS-PAGE on a 10% gel and subsequent fluorography. The bulk of the eluate fraction was subjected to carbohydrate structure analysis.

Carbohydrate structure analysis

HA was precipitated from the immunoaffinity eluate with acetone at −20°C overnight. The pellet was redissolved in 0.2 M NH_4HCO_3, pH 8.2, for digestion with trypsin (Serva, Heidelberg, Germany). Resulting glycopeptides were treated with endo-β-*N*-acetylglucosaminidase H (endo H) of *Streptomyces plicatus* (recombinant from *E. coli*) (Boehringer) in 50 mM sodium citrate-phosphate buffer, pH 5.0. Released oligosaccharides were separated from glycopeptides by gel filtration using a Biogel P-4 column (-400 mesh; 0.6 cm $\times$ 200 cm; Bio-Rad, München, Germany) with 0.02% aqueous sodium azide as eluant at hydrostatic pressure. Fractions of 850 μl were collected at a flow rate of 0.6 ml/hour. Glycopeptides were then digested with *N*-glycosidase F from *Flavobacterium meningosepticum* (recombinant from *E.coli*) (Boehringer) in 20 mM sodium phosphate, pH 7.2, to liberate endo-H-resistant *N*-glycans. Oligosaccharides were separated from remaining peptides by reversed-phase HPLC. Separation by preparative gel filtration or reversed-phase HPLC, reduction of oligosaccharides and desalting was done as previously described (Geyer *et al.*, 1992; Geyer and Geyer, 1993).

Aliquots of the oligosaccharide preparations were taken for exoglycosidase digestion. Treatment with α-fucosidase from bovine kidney (Boehringer) was done in 50 mM sodium phosphate-citrate buffer, pH 4.5. Treatment with β-*N*-acetyl-D-glucosaminidase (hexosaminidase) from *Diplococcus pneumoniae* (Boehringer) was performed in 50 mM sodium citrate buffer, pH 5.0. Digestion with α-mannosidase from jack beans (Sigma, München, Germany) was carried out in 50 mM sodium citrate-phosphate buffer, pH 4.5. All incubations were performed at 37°C for 24 h. Enzyme addition and incubation were repeated once.

High-pH anion-exchange chromatography at (HPAEC) was carried out as reported earlier (Pfeiffer *et al.*, 1990; Geyer *et al.*, 1992), except that a CarboPac PA-100 column was employed. Two elution programs were used. (i) t = 0 min, 5% eluant A, 95% eluant C; t = 20–40 min, from 5% to 8% eluant A, from 0% to 5% eluant B and from 95% to 87% eluant C. (ii) Constantly 8% eluant A, eluant B increasing from 1% at t = 0 min to 3% at t = 70 min (Eluant A, 1 M NaOH; eluant B, 1 M sodium acetate; eluant C, water). Program (i) was applied for separation of endo-H-resistant *N*-glycans; program (ii) was applied for separation of oligomannosidic *N*-glycans. Flow rate was 1 ml/min, and fractions of 350 μl were collected and monitored for radioactivity. Isomaltosyl oligosaccharide alditols (IM) obtained after partial acid hydrolysis of dextran and subsequent reduction were used for calibration of the columns in glucose units.

Analytical gel filtration (Kobata *et al.*, 1987) was carried out on a Biogel P-4 column (-400 mesh, 1.6 cm $\times$ 60 cm; Bio-Rad) with distilled water containing 0.02% sodium azide as eluant at 55°C. Fractions of 850 μl were collected at a flow rate of 0.2 ml/min and monitored for radioactivity by liquid scintillation counting.

Acknowledgements

This work was carried out by R.W. in partial fulfilment of the requirements for the Dr. rer. physiol. degree from FB 20, Philipps-Universität, Marburg. We acknowledge support by grants from the Deutsche Forschungsgemeinschaft (SFB 286 and SFB 272) and grants from the Fonds der Chemischen Industrie.

Abbreviations

AcNPV, *Autographa californica nuclear polyhedrosis* virus; endo H, endo-β-*N*-acetylglucosaminidase H; Fuc, fucose; GlcNAc, *N*-acetylglucosamine; FPV HA, fowl plague virus hemagglutinin; hGNT-I, human β1,2-

N-acetylglucosaminyltransferase I; HPAEC, high-pH anion-exchange chromatography; Man, mannose; MOI, multiplicity of infection; *N*-glycosidase F, peptide-N^4-(*N*-acetyl-(-glucosaminyl)asparagine amidase F; Sf9-cells, clonal cell line of *Spodoptera frugiperda*.

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Received on August 30, 1995; revised on October 9, 1995; accepted on October 31, 1995