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Structures and biosynthesis of the N- and O-glycans of recombinant human oviduct-specific glycoprotein expressed in human embryonic kidney cells

Xiaojing Yang[a], **Shujuan Tao**[b], **Ron Orlando**[b], **Inka Brockhausen**[a,c], and **Frederick W.K. Kan**[a,*]

[a]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, Ontario, Canada, K7L 3N6

[b]Departments of Biochemistry and Molecular Biology, and Chemistry, Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA

[c]Department of Medicine, Faculty of Health Sciences, Queen's University, Kingston, Ontario, Canada, K7L 3N6

Abstract

Oviduct-specific glycoprotein (OVGP1) is a major mucin-like glycoprotein synthesized and secreted exclusively by non-ciliated secretory cells of mammalian oviduct. *In vitro* functional studies showed that OVGP1 plays important roles during fertilization and early embryo development. We have recently produced recombinant human oviduct-specific glycoprotein (rhOVGP1) in human embryonic kidney 293 (HEK293) cells. The present study was undertaken to characterize the structures and determine the biosynthetic pathways of the N- and O-glycans of rhOVGP1. Treatment of the stable rhOVGP1-expressing HEK293 cells with either GalNAcα-Bn to block O-glycan extension, tunicamycin to block N-glycosylation, or neuraminidase increased the electrophoretic mobility of rhOVGP1. A detailed analysis of O- and N-linked glycans of rhOVGP1 by mass spectrometry showed a broad range of many simple and complex glycan structures. In order to identify the enzymes involved in the glycosylation of rhOVGP1, we assayed glycosyltransferase activities involved in the assembly of O- and N-glycans in HEK293 cells, and compared these to those from the immortalized human oviductal cells (OE-E6/E7). Our results demonstrate that HEK293 and OE-E6/E7 cells exhibit a similar spectrum of glycosyltransferase activities that can synthesize elongated and sialylated O-glycans with core 1 and 2 structures, as well as complex multiantennary N-glycans. It is anticipated that the knowledge gained from the present study will facilitate future studies of the role of the glycans of human OVGP1 in fertilization and early embryo development.

Keywords

Oviduct; Oviduct-specific glycoprotein; Oviductin; Glycosyltransferases; Glycan structures; HEK293 cells

*Corresponding author: kanfwk@queensu.ca. Tel.: +1 613 533 2863, Fax: +1 613 533 2022.

1. Introduction

Oviduct-specific glycoprotein (OVGP1), also known as oviductin or MUC9, is a major secretory glycoprotein (mucin) secreted exclusively by the oviduct.[1–3] The synthesis and secretion of OVGP1 have been shown to be under hormonal control.[4, 5] Binding of OVGP1 to the zona pellucida of postovulatory oocytes and early embryos indicates a potential biological role for OVGP1 during fertilization and early embryo development.[6–11] *In vitro* functional studies indicate that OVGP1 has overall positive effects on sperm capacitation, sperm motility and viability, sperm-egg binding, continuing growth of the fertilized oocytes to the blastocyst stage, and prevention of polyspermy (for reviews, see Refs. 1–3).

The cDNA sequences of OVGP1 have been determined in baboon,[12] cow,[13] hamster,[14] human,[15] mouse,[16] pig,[17] rhesus monkey,[18] sheep[19] and goat.[20] These OVGP1s appear to be highly conserved and share 70–78% identity and 76–87% similarity in the overall deduced amino acid sequence.[1] Major sequence differences are found in the carboxy-terminal region which contains many Ser-Thr residues.[17] Significant differences in protein length, amino acids at 141–335, insertions, deletions and truncations have been described in the carboxy-terminal region. The C-terminal region of hamster OVGP1 contains mucin-type variable number of tandem-repeat (VNTR) sequences (six repeats) of 15 amino acids each[21] while a similar region in the mouse OVGP1 contains 21 repeat sequences, each composed of seven amino acids.[16] The C-terminal region of OVGP1 in the human, baboon and rhesus monkey contains only four tandem repeat sequences whereas that of cattle, sheep and pig contains incomplete or no tandem-repeat sequences.[1] In agreement with the reported characteristics of OVGP1 in various species, these VNTR regions are assumed to be heavily O-glycosylated but the structures of these glycans have not yet been determined. The genes encoding VNTR are usually found within one exon.[22] Thus, hamster and mouse OVGP1 may be more closely related to mucins than their counterparts in other species. Human OVGP also has four or more N-glycosylation sites near the C-terminus.

Removal of sialic acid or N-linked glycans from bovine OVGP1 significantly reduces the ability of bovine OVGP1 to maintain sperm viability.[23] Since completely deglycosylated OVGP1 is insoluble, the glycans of OVGP1 appear to play a role in maintaining extracellular solubility.[23] It has been suggested that the carbohydrate moieties of glycoproteins protect molecules from protease degradation and affect protein folding and protein-protein interactions.[24] Differences in tandem repeats, the presence and distribution of N- and O-linked carbohydrate chains, the diversity of side chains, and the length of the C-terminal region may confer species specificity, mediate specific recognition events and regulate the biological activity of OVGP1.[20, 23, 25–27] Despite these advances in our knowledge of OVGP1, its N- and O-linked glycan structures have not been directly analyzed.

The human embryonic kidney (HEK293) cells has been extensively used as an expression tool for recombinant proteins since its biochemical machinery is capable of carrying out most of the post-translational processing required to generate functional, mature proteins from a wide spectrum of mammalian and non-mammalian nucleic acids.[28] We have recently produced recombinant human oviduct-specific glycoprotein (rhOVGP1) in HEK293 cells.[29] Preliminary results obtained from *in vitro* functional studies carried out in our laboratory showed that addition of rhOVGP1 in the capacitating medium can further enhance sperm capacitation evidenced by the increase in tyrosine phosphorylation of human sperm proteins and enhancement of acrosome reaction.[29] In view of these early findings, we postulate that the glycosylation pattern of rhOVGP1 is likely to modulate its bioactivity. In the first step of elucidating the functional properties of carbohydrate moieties of rhOVGP1 in fertilization, the present study was undertaken to characterize, by mass spectrometry (MS), the glycan

structures of rhOVGP1 that we produced and purified from HEK293 cells. The N- and O-linked glycan structures were correlated with their corresponding biosynthetic glycosyltransferases in HEK293 cells. The present results showed that the pathways of N- and O-glycosylation in HEK293 cells are similar to those found in the immortalized human oviductal cell line (OE-E6/E7) which we have previously shown to express both mRNA transcripts and protein of human OVGP1.[30] It is anticipated that the results obtained in the present study will facilitate future studies of the role of specific glycan structures of rhOVGP1 during fertilization.

2. Results and discussion

Several lines of evidence indicate that the mammalian oviduct makes important contributions to the intriguing process of reproduction other than being simply a conduct for gametes and embryos. Previous studies carried out mainly *in vitro* in different species, including the human, have shown that the oviduct secretes a major glycoprotein, known as oviduct-specific glycoprotein or oviductin, which plays important roles in fertilization and early embryo development.[1] Carbohydrates, especially Ser/Thr linked oligosaccharides, have been shown to be responsible for sperm-egg binding, sperm-oviductal adhesion, and induction of acrosome reaction.[31–35] Glycosylation also plays key roles in controlling cellular processes during fertilization.[20, 25–27] For ethical reasons, it would be impossible to isolate and purify sufficient amount of native human OVGP1 for glycan analyses. We therefore need model cells to produce rhOVGP1. Our recent success in producing and purifying rhOVGP1 in HEK293 cells prompted us to characterize the glycan structures of rhOVGP1 and identify the enzymes involved in their synthesis in order to set the stage for using rhOVGP1 for future functional studies. Several cell lines were used initially. HEK293 cells were selected for efficient and stable expression of rhOVGP1, and most of the recombinant glycoprotein was secreted into the culture medium from which rhOVGP1 was successfully purified. One of the objectives in the present study was to determine whether the biosynthetic pathways of glycans in the HEK293 cells are similar to those found in oviductal cells which synthesize and secrete OVGP1 in mammals including the human.[1] The OE-E6/E7 cell line was chosen for a comparison because both human OVGP1 gene expression and the presence of protein have been previously detected in these cells.[30] The OE-E6/E7 cells also display the phenotype characteristic of human primary oviduct cells.[36]

2.1. Glycan structures of rhOVGP1

A major interest in producing rhOVGP1 in mammalian cells was to generate a biologically active protein with a glycosylation pattern that resembles the one in human oviductal cells. To investigate the glycan structures of human OVGP1, the stable rhOVGP1-expressing HEK293 cells were cultured in the presence of glycosylation inhibitors GalNAcα-Bn and tunicamycin, or in the presence of neuraminidase. The expression of OVGP1 in the culture medium was examined by Western blot analysis using polyclonal antibody raised against a peptide mapping near the C-terminus of human OVGP1. As shown in Fig. 1, rhOVGP1 from the inhibitor-treated cells showed a shift in the electrophoretic mobility compared with rhOVGP1 from untreated cells. Treatment with neuraminidase also caused a shift of the mobility. These results suggested that human OVGP1 produced in HEK293 cells was modified by O- and N-glycosylation and that N-linked and/or O-linked glycans are sialylated.

Following trypsin-chymotrypsin digestion of rhOVGP1, the monosaccharide composition analysis of glycopeptides revealed the presence of GalNAc, GlcNAc, Gal, Glc, Man and N-acetylneuraminic acid (Neu5Ac) in the O- and N-glycan fractions (Table 1). Fuc was found only in the N-glycan but not in the O-glycan fraction (Table 1). N-glycolyl-neuraminic acid

(Neu5Gc) was not detected. The latter finding is consistent with the human origin of the HEK293 cells. Small amounts of Man were also found in the O-glycan fraction.

2.1.1. O-glycan structures—A detailed analysis of the glycosylation pattern of rhOVGP1 was performed by tandem mass spectrometry (MS/MS) (Table 2). The Matrix-Assisted Laser-Desorption Time-of-Flight Mass Spectrometry (MALDI-TOF/TOF-MS) spectrum of O-glycans released by β-elimination from rhOVGP1 glycopeptides and the proposed structures are shown in Fig. 2. The glycans detected by MS-MS revealed the presence of simple disaccharide structures (Gal-GalNAc and GlcNAc-GalNAc or GalNAc-GalNAc), as well as mono- and di-sialylated core 1 (Galβ1-3GalNAc-) and core 2 (Galβ1-3[GlcNAcβ1-6]GalNAc-) structures. The most complex O-glycan was a hexasaccharide with an extended core 2 structure carrying two sialic acid residues. It is also possible that small amounts of other structures were also present.

2.1.2. N-glycan structures—The linear ion trap Fourier transform (LTQ-FT) MS/MS spectrum of N-glycans released from rhOVGP1 with N-glycosidase F (PNGase F) is shown in Fig. 3 and in (Tables 2, 3). Further MS analyses of the fragment ions (data not shown) suggested the presence of a variety of N-glycan structures, including high mannose and hybrid chains, and bi-, tri-, and tetra-antennary complex non-bisected and bisected chains, with a majority of bi-antennary N-glycans. Most of the complex (but not the high mannose) chains carried a Fuc residue attached to the core GlcNAc at the reducing end. However, only 5 % of the complex structures had Fuc linked to GlcNAc in the outer antenna. Most of the complex chains contained sialyl-Gal-GlcNAc extensions.

2.2. Glycosyltransferase activities in HEK293 and OE-E6/E7cells

To determine the enzymatic basis for the N- and O-linked oligosaccharide structures of rhOVGP1, HEK293 cell homogenates were assayed for glycosyltransferase activities (Table 4). For a comparison and in order to assess the usefulness of HEK293 cells as an expression system and an alternative model for human oviductal cells, the enzyme activities in OE-E6/E7 cell homogenates were also measured (Table 4).

2.2.1. Glycosyltransferases that synthesize O-glycan core structures—The first reaction of the O-glycosylation pathway is catalyzed by polypeptide GalNAc-transferase (ppGalNAcT) consisted of a large family of enzymes with similar activities but somewhat different in substrate specificity. Using a mucin type acceptor substrate with multiple Thr residues, the activity of ppGalNAcT was found to be 3.9 nmol/h/mg in HEK293 cells but was particularly high in OE-E6/E7 cells (18.7 nmol/h/mg) (Table 4). Another peptide with only one Thr residue as the O-glycosylation site showed high activities in both cell lines. Attempts to compare the kinetic parameters for this substrate indicated that the apparent K_M values were above 4 mM, possibly, because a mixture of enzymes was assayed that may preferably bind to GalNAc-containing peptides. Both cell lines have the activities that synthesize O-glycans with core 1 (core 1 β3Gal-transferase) and the branched core 2 structure (core 2 β6-GlcNAc-transferase, C2GnT). However, C2GnT in OE-E6/E7 cells was 4 times more active (1.2 nmol/h/mg) than its counterpart in HEK293 cells. Like many other cultured cells, the enzyme that synthesizes core 3, GlcNAcβ1-3GalNAc-, (core 3 β3-GlcNAc-transferase) was not detected in either cell line. A low activity of β6-GlcNAc-transferase (C2GnT2) that can convert core 3 to the branched core 4 (GlcNAcβ1-3[GlcNAcβ1-6]GalNAc-) structure was detected in OE-E6/E7 but not in HEK293 cells (Table 4). This suggests that C2GnT1 is expressed in HEK293 cells and that OE-E6/E7 cells, in addition, express low levels of C2GnT2.[37] Thus both cell types can efficiently synthesize O-glycan core 1 and 2 structures, and lack the enzyme activity that synthesizes O-glycan core 3 which is the precursor for the synthesis of core 4 structure.

However, the MS data suggest that there may be a very small amount of core 3 and/or core 4 structures in rhOVGP1 which remains to be confirmed.

2.2.2. Glycosyltransferases that synthesize N-glycan antennae—Both cell types, HEK293 and OE-E6/E7 cells, were shown to contain GlcNAc-transferases involved in the synthesis of complex N-glycan antennae. GlcNAc-transferases I and II involved in the synthesis of biantennary chains were active in HEK293 and OE-E6/E7 cells (Table 4). The *in vitro* activities of GlcNAc-transferases III, IV and V are usually very low, although according to the proposed structures, the enzymes should be active *in vivo* in the synthesis of tri- and tetra-antennary chains and the large proportion of proposed bisected N-glycans. The combined activities of GlcNAc-transferases III, IV and V, as measured by HPLC analysis of enzyme products, were 0.05 nmol/h/mg in both cell types. Using a specific substrate for GlcNAc-transferase V (Table 4), the activities were found to be present in both HEK293 cells (0.09 nmol/h/mg) and OE-E6/E7 cells (0.06 nmol/h/mg). Thus, both cell types are capable of synthesizing multi-antennary N-glycan structures.

2.2.3. Extension and sialylation of N- and O-glycan chains—Using GlcNAcβ-benzyl as the acceptor substrate, Gal-transferases were found to be very active (9.2 nmol/h/mg in HEK293 cells and 25.6 nmol/h/mg in OE-E6/E7 cells) (Table 4). In the presence of the specific β4Gal-transferase inhibitor N-butyryl-glucosamine-S-naphthyl (GlcNBu-S-naphthyl) the activities were reduced to 0.6 and 2.8 nmol/h/mg, respectively. This residual activity is likely due to β3Gal-transferase activities that could elongate either O- or N-linked oligosaccharide chains containing terminal GlcNAc residues.[38] Similar results were obtained using GlcNAcβ1-3GalNAcα-Bn, which is a preferred substrate for β3Gal-transferase.

The sialic acid-containing O-glycans are synthesized by α3- and α6-sialyltransferases. Using O-glycan core 1 as an acceptor substrate, sialyltransferase activities were 1.7 nmol/h/mg in HEK293 cells and 6.1 nmol/h/mg in OE-E6/E7 cells (Table 4). A specific α3-sialyltransferase substrate, Galβ1-3[6-deoxy-]GalNAc-, showed similar activity, indicating that the activity measured with the core 1 acceptor substrate was α3-sialyltransferase. The α3/6-sialyltransferases that act on N-glycans were measured using an N-acetyllactosamine acceptor and found to be 4.1 nmol/h/mg in HEK293 cells and 4.8 nmol/h/mg in OE-E6/E7 cells (Table 4). GalNAcβ1-4GlcNAcβ-Bn was used as a specific substrate for α6-sialyltransferase[39] and showed 1.3 nmol/h/mg in HEK293 cells and 0.5 nmol/h/mg in OE-E6/E7 cells. Thus, in homogenates prepared from both cell types, α3-sialyltransferase was much more active than α6-sialyltransferase. There was no detectable activity of the haematopoietic type α2-Fuc-transferase using Galβ-Bn acceptor. Low activities of the secretory type α2-Fuc-transferase using core 1–Bn acceptor were found, with OE-E6/E7 cells having 4 times higher activity (1.34 nmol/h/mg) than HEK cells (Table 4). The α3-Fuc-transferase activity that participates in Lewis^x synthesis was higher in HEK cells at 0.59 nmol/h/mg (Table 4). In contrast, α4-Fuc-transferase activity was 14-fold higher in OE-E6/E7 cells (1.23 nmol/h/mg). Based on these results, the biosynthetic pathways of O-glycans (Fig. 4) and N-glycans (Fig. 5) in HEK293 and OE-E6/E7 cells were constructed. Clearly, both cell lines exhibit a similar broad spectrum of glycosyltransferase activities that can synthesize and modify N- and O-glycans. However, oviductal cells exhibit the levels of enzymes typical for a secretory type of cell that can O-glycosylate secreted glycoproteins and mucins.[37]

Although HEK293 cells are not derived from the oviduct, studies carried out in our laboratory showed that HEK293 cells transfected with human OVGP1 cDNA yielded ample amounts of rhOVGP1. This indicates that the HEK293 cells are an excellent expression system for producing rhOVGP1. In the present study, the glycosylation pathways in the

immortalized human oviductal cells were found to be similar to those in HEK293 cells, thus validating HEK293 cells as a good model for the production of recombinant human oviduct-specific glycoprotein. The natural human oviductin may have a similar glycosylation pattern although there may also be individual differences. The enzyme study supports the MS data showing that complex N-and O-glycans in both cell types are expected to terminate mainly in sialic acid residues that may play important roles in glycoprotein recognition phenomena or cell surface functions. The MS data also support that the activities of α2, α3 and α4-Fuc-transferases were too low to fucosylate significant numbers of O-glycan chains although the core GlcNAc residues of N-glycans were efficiently fucosylated.

Oviduct-specific glycoprotein or oviductin has been identified and characterized in many mammalian species including the human. However, further exploration of the functional role(s) of OVGP1 in the process of reproduction in humans has been hampered by the unavailability of human OVGP1. We have succeeded in producing human recombinant human OVGP1 in HEK293 cells and have identified possible target enzymes for a modification of glycosylation that might be useful for studies of glycan function. It is hoped that the availability of rhOVGP1 and the information of the structures and biosynthesis of rhOVGP1 provided by the present work will rekindle the interest of researchers in pursuing further the role of human OVGP1 during the early process of reproduction.

3. Experimental Procedures

3.1. Materials

The following chemical agents were purchased from the indicated sources: Dulbecco's Modified Eagle Medium, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham, fetal bovine serum (FBS), penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA); polyclonal antibody against human OVGP1 [oviductin (P-20)], oviductin (P-20) P, donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); Western Lighting-Enhanced Chemiluminescence Substrate (PerkinElmer, Waltham, MA, USA); Sep-Pak C18 cartridges (Waters, Milford, MA, USA); PNGase F (New England Biolabs, Ipswich, MA USA); All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Cell cultivation

The stable rhOVGP1-expressing HEK293 cell clone was previously established in our laboratory.[29] Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, penicillin (50 U/ml), streptomycin (50 μg/ml) and Geneticin (200 μg/ml). For the study of the effects of inhibitors of O- and N-glycosylation as well as sialidase on the electrophoretic mobility of rhOVGP1, the stable rhOVGP1-expressing HEK293 cells were grown in the presence or absence of 3 mM GalNAc α Bn for 48 hours. Some cells were also grown in the presence or absence of neuraminidase (180mU/ml) from *clostridium perfringens* for 48 hours or tunicamycin from *Streptomyces sp.* at various concentrations (10, 100, 1000 ng/ml) for 4 days. Expression of human OVGP1 in the culture medium was examined by Western blot analysis using goat polyclonal antibody raised against a peptide mapping near the C-terminus of human OVGP1 [oviductin (P-20)].

Immortalized human oviductal cells (OE-E6/E7), a gift from Dr. K. F. Lee of the University of Hong Kong, were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham, supplemented with 10% FBS, penicillin (20 U/ml) and streptomycin (20 μg/ml) under standard cell culture conditions (5% CO_2, 37°C).

3.3. SDS-PAGE and Western blot analysis

HEK293 cells were used for efficient expression of rhOVGP1. Details of expression and purification of rhOVGP1 will be described elsewhere. Protein samples of rhOVGP1 were size-fractionated by SDS-PAGE on a 7.5% gel and electrophoretically transferred to polyvinylidene fluoride (PVDF). After incubation in a blocking buffer [5% nonfat milk and 0.05% Tween 20 in sodium phosphate buffer (PBS)] for 1 hour, blots were probed sequentially with a polyclonal antibody to human OVGP1 [Oviductin (P-20)] at a dilution of 1:1000 and peroxidase-labeled donkey anti-goat IgG as a secondary antibody at a 1:2000 dilution.

3.4. Analysis of the monosaccharide composition of N-linked glycans and O-linked glycopeptides by high-performance anion-exchange chromatography (HPAEC)

N-linked glycans and O-linked glycopeptides were prepared according to the method of Aoki et al.[40] Briefly, recombinant human OVGP1 (~435 μg) was digested with trypsin and chymotrypsin. The tryptic-chymotryptic digest was passed through a C18-Sep-Pak cartridge, washed with 5% acetic acid, and glycopeptides/peptides were eluted subsequently in series with 20% isopropanol in 5% acetic acid, 40% isopropanol in 5% acetic acid and 100% isopropanol. The eluate was dried initially under a stream of nitrogen (N_2) gas and lyophilized. The dried eluate was dissolved with 20 mM PBS (pH 7.5) and treated with PNGase F and incubated at 37°C overnight to release the N-linked glycans. The N-linked carbohydrate fraction was eluted with 5% acetic acid, and the O-linked glycopeptides/peptides were eluted in series with 20% isopropanol in 5% acetic acid, 40% isopropanol in 5% acetic acid and 100% isopropanol. The N-glycan fraction was frozen on dry ice and lyophilized immediately whereas the O-linked glycopeptides/peptides i.e. the isopropanol fractions, were dried initially under a stream of N_2 gas to evaporate the isopropanol followed by lyophilization and then combined in one tube.

The N-glycan fraction was dissolved in 200 μl bi-distilled water and divided into two equal aliquots (one for the analysis of neutral and amino sugars, and the other aliquot for the analysis of sialic acid residues). The O-glycopeptide fraction was dissolved in 400 μl bi-distilled water; 100 μl were allocated for the analysis of neutral and amino sugars, 100 μl were allocated for sialic acid analysis, and the remainder was lyophilized and the O-glycans released by β-elimination. The aliquots intended for the analysis of neutral and amino sugars were hydrolyzed with 400 μl of 2.0 N trifluoroacetic acid (TFA) at 100°C for 4 hours whereas the aliquots for sialic acid analysis were hydrolyzed with 400 μl of 2.0 M acetic acid at 80°C for 3 hours. All hydrolysates were lyophilized, resuspended in bi-distilled water, sonicated for 7 minutes on ice and then transferred to injection vials. Mixtures of standards for neutral and amino sugars and for sialic acid were hydrolyzed in the same manner as the samples. Four concentrations of standard mixtures were prepared to establish a calibration equation. The number of moles of each sugar residue in the sample was quantified by linear intrapolation from the calibration equation. The neutral and amino sugars as well as sialic acid were analyzed by HPAEC using a Dionex ICS3000 system equipped with a gradient pump, an electrochemical detector, and an auto-sampler (Dionex, Bannockburn, IL, USA). The individual neutral and amino sugars and sialic acid were separated by a Dionex CarboPac PA20 (3 × 150 mm) analytical column (Dionex, Bannockburn, IL, USA) with an amino trap. The gradient programs used the following mobile phase eluents - A: degassed nanopure water; B: 200 mM NaOH for neutral and amino sugars; C: 100 mM NaOH; D: 1 M sodium acetate in 100 mM NaOH for sialic acid. All methods employed were based on protocols described by Hardy and Townsend.[41]

3.5. O-Glycan analysis

3.5.1. Release of O-linked glycans by β-elimination—O-linked carbohydrates were cleaved from glycopeptides by β-elimination. Briefly, 250 μl of 50 mM NaOH were added to the dried O-glycopeptide fraction. Another 250 μl of 50 mM NaOH containing 19 mg of sodium borohydride were then added to the sample followed by incubation at 45°C overnight. The solution was neutralized with 10% acetic acid and desalted by passing through a packed column of Dowex resins (50 W × 8–100, Sigma Aldrich, St. Louise, MO, USA) and subsequently lyophilized. The dried sample was cleaned of borate with methanol:acetic acid (9:1) under a stream of N_2 gas before permethylation.

3.5.2. Per-O-methylation of carbohydrates—The O-linked glycans prepared from rhOVGP1 were permethylated for structural characterization by MS.[42] The dried eluate was dissolved in dimethyl sulfoxide (DMSO) and permethylated with NaOH and methyl iodide. The reaction was quenched with water and per-O-methylated carbohydrates were extracted with methylene chloride and dried under N_2 gas.

3.5.3. Profiling of oligosaccharides associated with O-linked glycans—The permethylated glycans prepared from rhOVPG1 were dissolved in methanol and crystallized with α-dihyroxybenzoic acid (DHBA, 20 mg/ml in 50% methanol in bi-distilled water) matrix. Analysis of glycans present in the samples was performed in the positive ion mode by MALDI-TOF/TOF-MS using AB SCIEX TOF/TOF 5800 (Applied Biosystem/MDS Analytical Technologies, Foster City, CA, USA).

3.6. N-Glycan analysis

The rhOVGP1 sample (100 μg) was digested with trypsin. A small volume (3 μl) of PNGase F (500,000 U/ml) was then added to release the N-glycans. A C18-Sep-Pak cartridge was used to separate the released N-glycans and peptides. The N-glycan eluate was dried and permethylated with methyl iodide.[43] Subsequently, a C18-Sep-Pak cartridge was used to clean the permethylated N-glycans. The MS analysis was carried out on a hybrid LTQ-FT mass spectrometry (Thermo Electron Corporation, San Jose, CA, USA) by direct infusion. The MS data were generated by FT, and MS/MS data were generated by LTQ. Each composition of the N-glycans was determined based on its molecular weight, isotopic peak distribution and MS/MS fragment assignment.

3.7. Glycosyltransferase assays

HEK293 and OE-E6/E7 cells released from the culture plates were washed three times with PBS, scraped from the plates in the same buffer, and then hand-homogenized with a Potter Elvejehm homogenizer in 5 to 10 volumes of 0.25 M sucrose. Homogenates were stored in aliquots at −80°C. Glycosyltransferase activities were assayed by ion exchange (AG 1×8), C18-Sep-Pak cartridge and/or ion exchange followed by high performance liquid chromatography (HPLC), using specific substrates as previously described.[38, 44–47] Enzyme assays were performed in at least duplicate determinations with <10 variation between duplicates. Rat colon homogenate was used as the positive control tissue for all glycosyltransferase assays.[44]

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Abbreviations

HEK293	human embryonic kidney 293
HPLC	high-performance liquid chromatography
MS	mass spectrometry
OE-E6/E7	immortalized human oviductal cells
PBS	phosphate-buffered saline
pnp	*p*-nitro-phenyl
rhOVGP1	recombinant human oviduct-specific glycoprotein

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- N- and O-glycans of recombinant human oviductin (rhOVGP1) were analyzed.
- Their pathways are similar to those present in immortalized human oviductal cells.
- The work was the first to analyze N- and O-linked glycans of rhOVGP1.

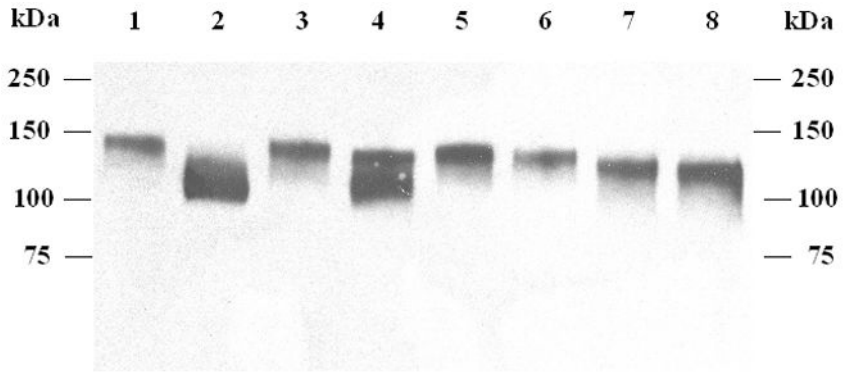

Figure 1.

Effects of O-glycan and N-glycan inhibitors and treatment with neuraminidase on rhOVGP1. The stable rhOVGP1-expressing HEK-293 cell clone was grown in the absence (lanes 1, 3, and 5) and presence of 3 mM GalNAcα-Bn (lane 2), 180 mU/ml neuraminidase (lane 4) for 48 hours or with 10, 100, 1000 ng/ml tunicamycin (lanes 6, 7, and 8, respectively) for 4 days. The expression of OVGP1 in the culture medium was examined by Western blot analysis using goat polyclonal antibody raised against a peptide mapping near the C-terminus of human OVGP1.

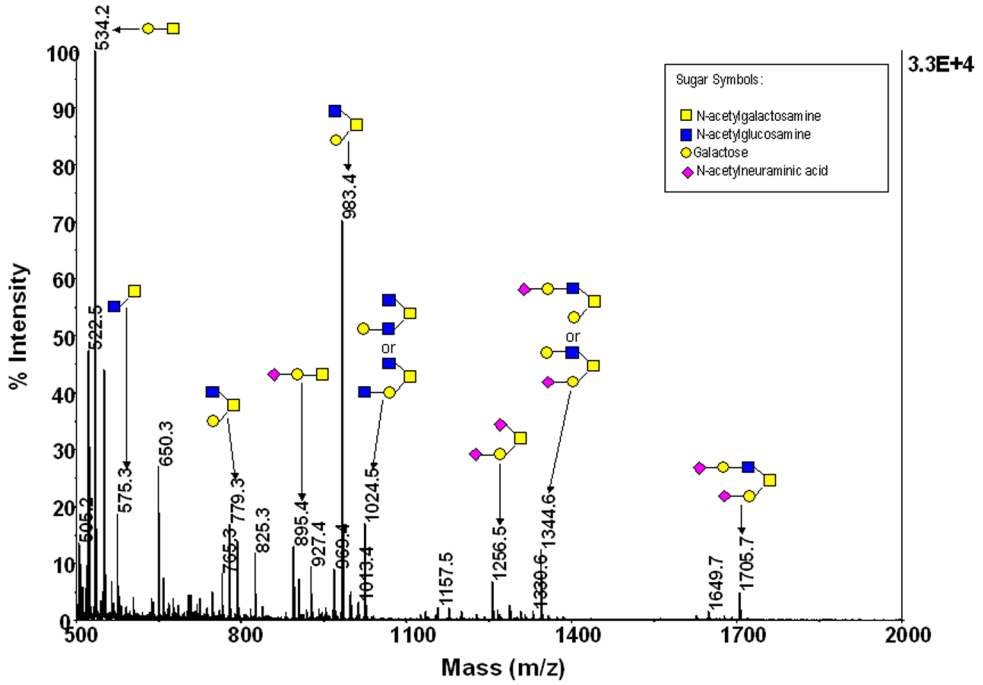

Figure 2.
MALDI-TOF/TOF MS spectrum of permethylated O-linked glycans cleaved by β-elimination from rhOVGP1. Proposed structures are based on common biosynthetic pathways of O-glycans.

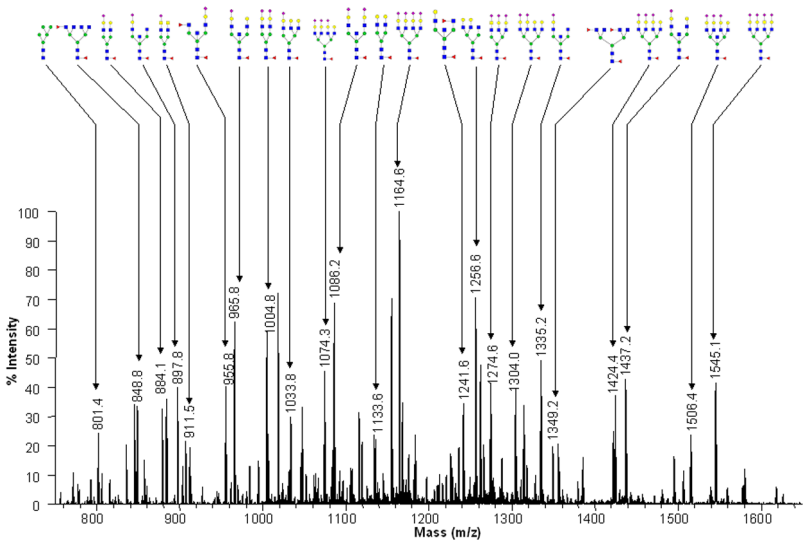

Figure 3.
LTQ-FT MS/MS spectrum of N-glycans cleaved with PNGase F from rhOVGP1. Proposed most abundant N-glycans of rhOVGP1. For sugar symbols see Figure 2. Mannose.

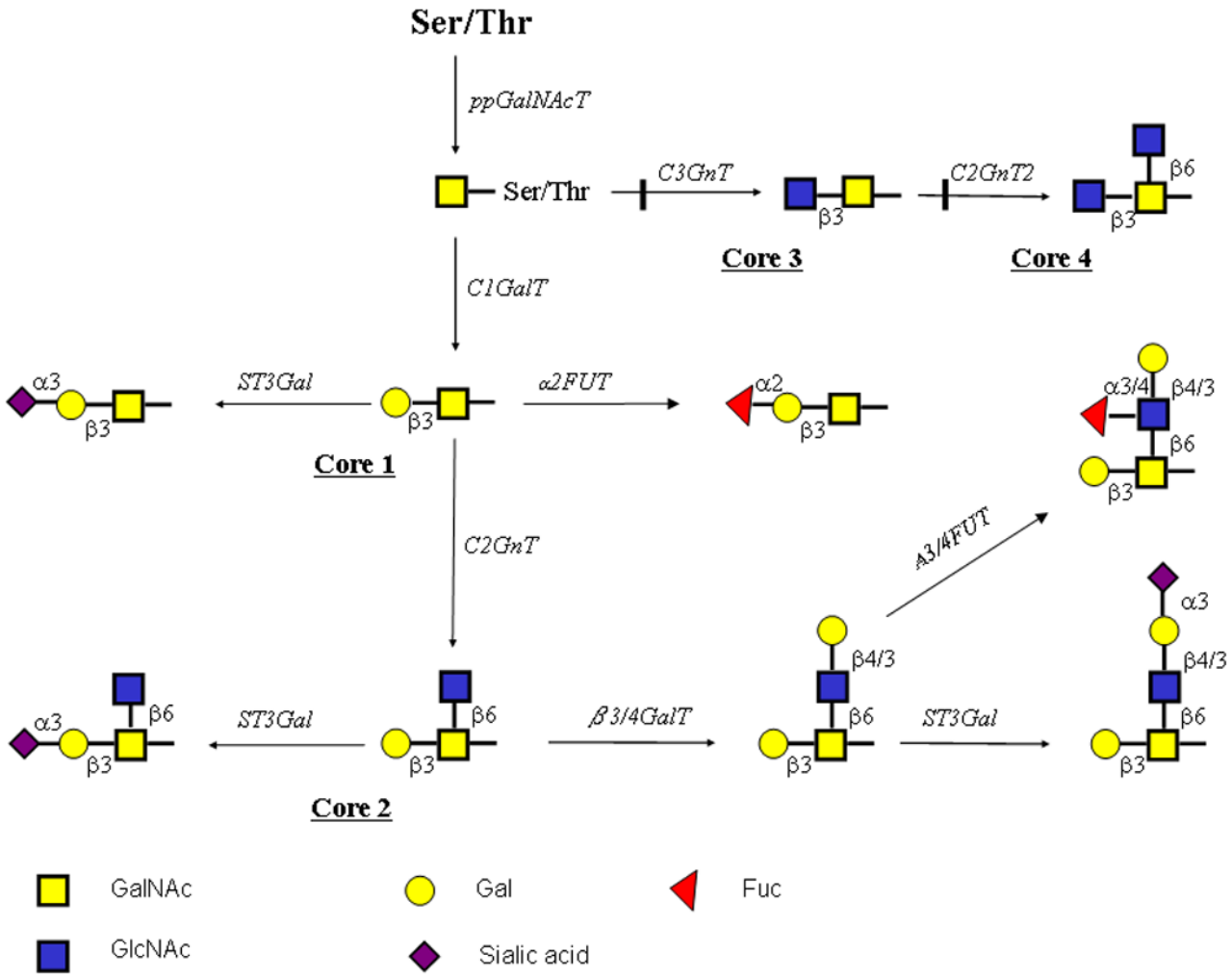

Figure 4.

Proposed biosynthetic pathways of O-glycans in HEK293 and OE-E6/E7 cells. Human oviductin is a mucin-like molecule with many potential O-glycosylation sites. The enzymes that synthesize O-glycan core 1, polypeptide GalNAc-transferase (ppGalNAcT) and core 1 β3Gal-transferase (C1GalT) as well as core 2 β6-GlcNAc-transferase (C2GnT) that synthesizes the branched core 2 structure, are active in HEK293 and OE-E6/E7 cells. Core 2 can also be extended by β3/4-Gal-transferase. These structures can be extended by α3-sialyltransferases (ST3Gal) or by Fuc-transferases (FUT). The activities of the enzymes that synthesize core 3 (C3GnT) and core 4 (C2GnT2) structures were not detected in HEK cells. E6/E7 cells, however, showed a low activity of C2GnT2. A bar through the arrow indicates that the path is blocked. The proposed biosynthetic pathways between HEK293 cells and OE-E6/E7 cells are similar.

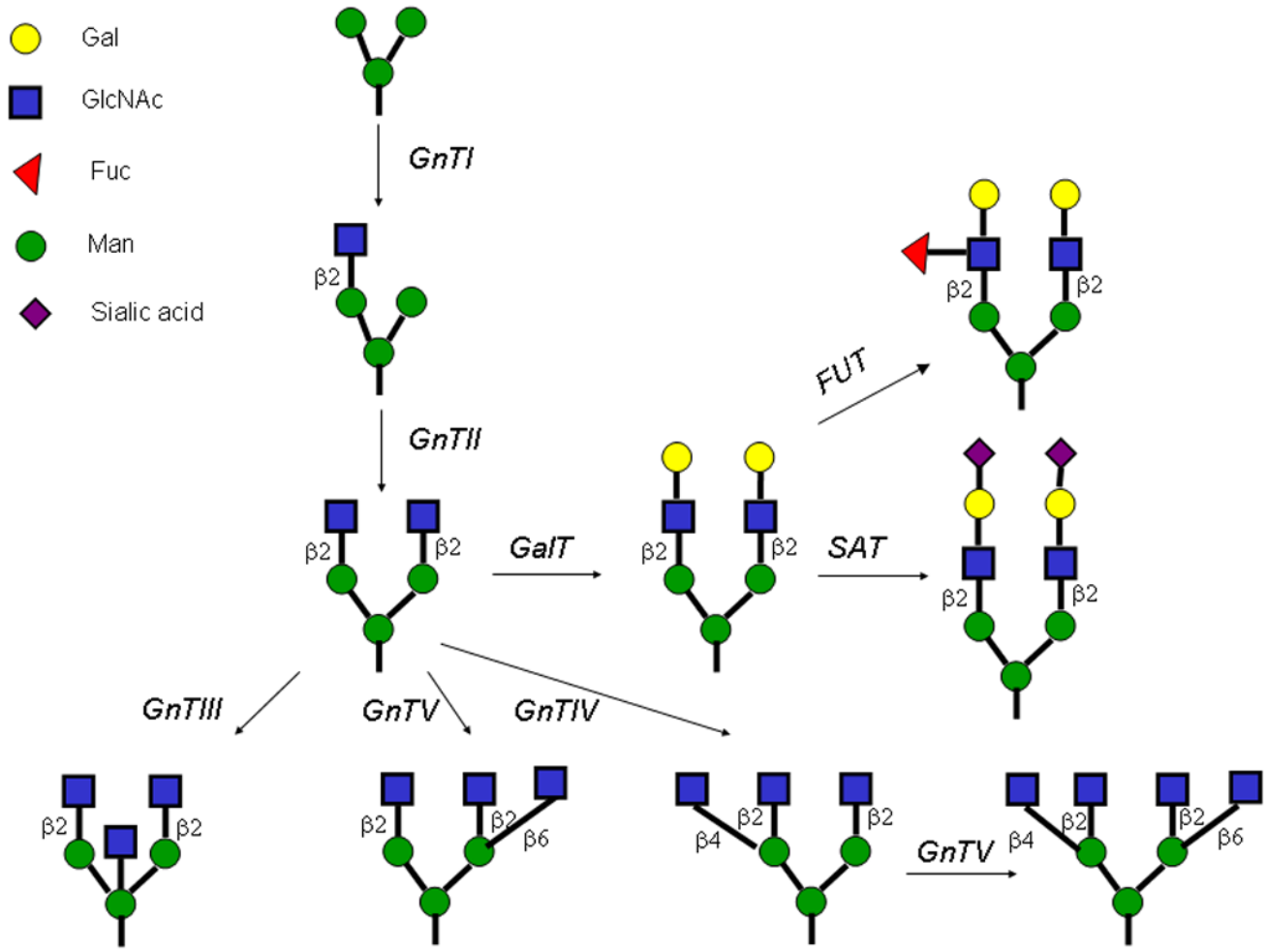

Figure 5.

Proposed biosynthetic pathways of N-glycans in HEK293 and OE-E6/E7 cells. Human oviductin has at least 4 potential N-glycosylation sites. In the enzyme assays the tri-Mannose structure was converted to mono- and bi-antennary structures due to the activities of GlcNAc-transferases I and II (GnTI, GnTII). Low activities of GlcNAc-transferases (III, IV and V) (GnTIII, GnTIV, GnTV) that synthesize tri- and tetra-antennary structures were also detected. The GlcNAc antennae can be extended by Gal-transferases (GalT) and further by sialyltransferases (SAT) and Fuc-transferases (FUT).

Table 1

Monosaccharide composition analysis of rhOVGP1 O-linked glycopeptides and N-linked glycans by HPAEC. Recombinant human OVGP1 (~435 μg) was subjected to this analysis.

		Total amount of sugars in hydrolyzed fractions		
	Glycosyl residue	**nanomoles**	**μg**	**%, by mole**
O-glycopepetides	Fucose (Fuc)	nd	-	-
	N-acetyl galactosamine (GalNAc	4.3779	0.9684	14.2
	N-acetyl glucosamine (GlcNAc)	4.5710	1.0111	14.8
	Galactose (Gal)	13.4877	2.4305	43.7
	Glucose (Glc)	1.0384	0.1871	3.4
	Mannose (Man)	0.2875	0.0518	0.9
	N-acetyl neuraminic acid (NANA)	7.1128	2.1998	23.0
	N-glycolyl neuraminic acid (NGNA)	nd	-	-
N-linked glycans	Fucose (Fuc)	3.5406	0.5814	8.5
	N-acetyl galactosamine (GalNAc)	2.5475	0.5635	6.1
	N-acetyl glucosamine (GlcNAc)	12.9184	2.8577	30.9
	Galactose (Gal)	9.1292	1.6451	21.9
	Glucose (Glc)	2.5775	0.4644	6.2
	Mannose (Man)	6.5783	1.1854	15.8
	N-acetyl neuraminic acid (NANA)	4.4618	1.3799	10.7
	N-glycolyl neuraminic acid (NGNA)	nd	-	-

Table 2

MALDI-TOF/TOF MS signals and composition of permethylated O-linked glycans list on figure 2.

m/z	Composition[1]
534.2	$Gal_1HexNAc_1$
575.3	$HexNAc_2$
779.3	$Gal_1HexNAc_2$
895.4	$NeuAc_1Gal_1HexNAc_1$
983.4	$Gal_2HexNAc_2$
1024.5	$Gal_1HexNAc_3$
1256.5	$NeuAc_2Gal_1HexNAc_1$
1344.6	$NeuAc_1Gal_2HexNAc_2$
1705.7	$NeuAc_2Gal_2HexNAc_2$

[1]Gal=galactose, HexNAc=N-acetylhexosamine, and NeuAc=N-acetylneuraminic acid.

Table 3

ESI FT MS signals and composition of permethylated N-linked glycan list on figure 3.

Monoisotopic peak (m/z)	Charge state (z)	Calculated mass (Da)	Theoretical mass (Da)	Composition[1]
801.3946	2	1556.8096	1556.7935	$HexNAc_2Hex_5$
848.7586	3	2477.3064	2477.2775	$Deoxyhex_2HexNAc_6Hex_3$
883.7694	3	2582.3388	2582.309	$Deoxyhex_1HexNAc_4NeuAc_1Hex_5$
897.4452	3	2623.3662	2623.3355	$Deoxyhex_1HexNAc_5NeuAc_1Hex_4$
911.122	3	2664.3966	2664.362	$Deoxyhex_1HexNAc_6NeuAc_1Hex_3$
955.4758	3	2797.458	2797.4247	$Deoxyhex_2HexNAc_5NeuAc_1Hex_4$
965.4797	3	2827.4697	2827.4353	$Deoxyhex_1HexNAc_5NeuAc_1Hex_5$
1004.1627	3	2943.5187	2943.4827	$Deoxyhex_1HexNAc_4NeuAc_2Hex_5$
1033.5146	3	3031.5744	3031.5351	$Deoxyhex_1HexNAc_5NeuAc_1Hex_6$
1073.7811	4	4203.1652	4203.1086	$Deoxyhex_1HexNAc_6NeuAc_3Hex_7$
1085.8743	3	3188.6535	3188.609	$Deoxyhex_1HexNAc_5NeuAc_2Hex_5$
1133.5742	2	2221.1688	2221.1353	$Deoxyhex_1HexNAc_4Hex_5$
1164.0770	4	4564.3488	4564.2823	$Deoxyhex_1HexNAc_6NeuAc_4Hex_7$
1241.1371	2	2436.2946	2436.251	$Deoxyhex_2HexNAc_5Hex_4$
1256.1407	2	2466.3018	2466.2616	$Deoxyhex_1HexNAc_5Hex_5$
1274.3049	3	3753.9453	3753.8825	$Deoxyhex_1HexNAc_5NeuAc_3Hex_6$
1303.6571	3	3842.0019	3841.9349	$Deoxyhex_1HexNAc_6NeuAc_2Hex_7$
1334.6800	2	2623.3804	2623.3355	$Deoxyhex_1HexNAc_5NeuAc_1Hex_4$
1348.6935	2	2651.4074	2651.3667	$Deoxyhex_3HexNAc_6Hex_3$
1424.052	3	4203.1866	4203.1086	$Deoxyhex_1HexNAc_6NeuAc_3Hex_7$
1436.7343	2	2827.489	2827.4353	$Deoxyhex_1HexNAc_5NeuAc_1Hex_5$
1505.7632	3	4448.3202	4448.2349	$Deoxyhex_1HexNAc_7NeuAc_3Hex_7$
1544.4464	3	4564.3698	4564.2823	$Deoxyhex_1HexNAc_6NeuAc_4Hex_7$

[1] Deoxyhex=fucose, Hex=galactose or mannose, HexNAc=N-acetylhexosamine, and NeuAc=N-acetylneuraminic acid.

Monoisotopic peaks (m/z) were observed from the mass spectrum, which were sodiated adducts of permethylated N-glcyans. The theoretical masses were generated by GlycanMass (http://web.expasy.org/glycanmass/).

Table 4

Glycosyltransferase activities involved in O- and N-glycan synthesis in HEK293 and OE-E6/E7 cells. Assays were carried out as described in references [44–47] by the AG1×8, C18 Sep-Pak or HPLC method.

Enzyme	Type of Assay	Substrate	Activity (nmol/h/mg)	
			HEK293	OE-E6/E7
Polypeptide α-GalNAc-transferase	AG 1×8	0.5 mM TTTVTPTPTG	3.90	18.70
Polypeptide α-GalNAc-transferase	AG 1×8	0.5 mM AQPTPPP	13.45	7.53
Core 1 β3-Gal-transferase	AG 1×8	2 mM GalNAcα-Bn	15.60	16.90
Core 2 β6-GlcNAc-transferase	HPLC	2 mM Galβ3GalNAcα-Bn	0.27	1.18
Core 3 β3-GlcNAc-transferase	HPLC	2 mM GalNAcα-Bn	nd	nd
Core 4 β6-GlcNAc-transferase	HPLC	2 mM GlcNAcβ3GalNAcα-pnp	nd	0.17
β3/4-Gal-transferase	AG 1×8	1 mM GlcNAcβ-Bn	9.20	25.60
β3-Gal-transferase	AG 1×8	1 mM GlcNAcβ-Bn + GlcNBuβ-S-naphthyl	0.60	2.80
β3/4-Gal-transferase	AG 1×8	1 mM GlcNAcβ3GalNAcα-Bn	10.73	29.20
β3-Gal-transferase	AG 1×8	1 mM GlcNAcβ-Bn + cpd612	0.85	2.81
GlcNAc-transferase I	HPLC	0.5 mM Manα6(Manα3)Manβ-octyl	1.10	1.71
GlcNAc-transferase II	HPLC	0.5 mM Manα6(GlcNAcβ2Manα3)Manβ-octyl	3.77	1.58
GlcNAc-transferase III + IV + V	HPLC	0.5 mM GlcNAcβ2Manα6(GlcNAcβ2Manα3) Manβ-octyl	0.05	0.05
GlcNAc-transferase V	HPLC	1 mM GlcNAcβ2Manα6(GlcNAcβ2[4-deoxy-] Manα3)4-deoxy-β-octyl Man	0.09	0.06
α3/6-Sialyltransferase	C18 Sep-Pak	1mM Galβ3GalNAcα-Bn	1.70	6.10
α3-Sialyltransferase	C18 Sep-Pak	1 mM Galβ3(6-deoxy)GalNAcα-Bn	1.80	6.20
α3/6-Sialyltransferase	C18 Sep-Pak	1 mM Galβ4GlcAcβ-Bn	4.10	4.80
α6-Sialyltransferase	C18 Sep-Pak	1 mM GalNAcβ4GlcNAcβ-Bn	1.30	0.50
α2/3 Fucosyl-transferase	HPLC	2.8 mM Galβ-Bn	nd	nd
α2 Fucosyl-transferase	HPLC	2.0 mM Galβ3GalNAcα-Bn	0.26	1.34
α3 Fucosyl-transferase	HPLC	2.0 mM GalNAcβ4GlcNAcβ-Bn	0.59	0.24
α4 Fucosyl-transferase	HPLC	1.4 mM 2–O–Me–Galβ1-3GlcNAcβ-Bn	0.09	1.23

pnp: *p*-nitrophenol; nd: not detected; cpd612, N-butyryl-glucosamine-beta-thio-2-naphthyl.