

## Inhibition of hybrid- and complex-type glycosylation reveals the presence of the GlcNAc transferase I-independent fucosylation pathway

Max Crispin<sup>1–3</sup>, David J. Harvey<sup>2</sup>, Veronica T. Chang<sup>5</sup>,  
Chao Yu<sup>5</sup>, A. Radu Aricescu<sup>4</sup>, E. Yvonne Jones<sup>4</sup>,  
Simon J. Davis<sup>5</sup>, Raymond A. Dwek<sup>2</sup>, and Pauline M. Rudd<sup>2</sup>

<sup>3</sup>Division of Structural Biology and <sup>4</sup>Cancer Research UK Receptor Structure Research Group, Division of Structural Biology, Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Headington, Oxford OX3 7BN, UK; <sup>5</sup>Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DS, UK

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**A mammalian *N*-acetylglucosamine (GlcNAc) transferase I (GnT I)-independent fucosylation pathway is revealed by the use of matrix-assisted laser desorption/ionization (MALDI) and negative-ion nano-electrospray ionization (ESI) mass spectrometry of *N*-linked glycans from natively folded recombinant glycoproteins, expressed in both human embryonic kidney (HEK) 293S and Chinese hamster ovary (CHO) Lec3.2.8.1 cells deficient in GnT I activity. The biosynthesis of core fucosylated Man<sub>5</sub>GlcNAc<sub>2</sub> glycans was enhanced in CHO Lec3.2.8.1 cells by the  $\alpha$ -glucosidase inhibitor, *N*-butyldeoxynojirimycin (NB-DNJ), leading to the increase in core fucosylated Man<sub>5</sub>GlcNAc<sub>2</sub> glycans and the biosynthesis of a novel core fucosylated monoglucosylated oligomannose glycan, Glc<sub>1</sub>Man<sub>7</sub>GlcNAc<sub>2</sub>Fuc. Furthermore, no fucosylated Man<sub>9</sub>GlcNAc<sub>2</sub> glycans were detected following inhibition of  $\alpha$ -mannosidase I with kifunensine. Thus, core fucosylation is prevented by the presence of terminal  $\alpha$ 1–2 mannoses on the 6-antennae but not the 3-antennae of the trimannosyl core. Fucosylated Man<sub>5</sub>GlcNAc<sub>2</sub> glycans were also detected on recombinant glycoprotein from HEK 293T cells following inhibition of Golgi  $\alpha$ -mannosidase II with swainsonine. The paucity of fucosylated oligomannose glycans in wild-type mammalian cells is suggested to be due to kinetic properties of the pathway rather than the absence of the appropriate catalytic activity. The presence of the GnT I-independent fucosylation pathway is an important consideration when engineering mammalian glycosylation.**

**Key words:** electrospray ionization mass spectrometry/fucosyltransferase/matrix-assisted laser desorption/ionization (MALDI)/*N*-linked glycosylation

### Introduction

The *N*-linked glycosylation pathways of higher eukaryotes contain a multitude of biosynthetic reactions that give rise to a wide diversity of glycan structures. Despite this complexity, there are two ancient and highly conserved enzymatic steps that mediate the sequential conversion of oligomannose glycans to hybrid-type and then to complex-type structures. The conversion of oligomannose-type to hybrid-type glycans is initiated by the action of UDP-*N*-acetyl-D-glucosamine: $\alpha$ -3-D-mannoside  $\beta$ 1,2-*N*-acetylglucosaminyltransferase I (GnT I) which transfers *N*-acetylglucosamine (GlcNAc), in a  $\beta$ 1–2 linkage, to the 3-arm mannose residue of the oligomannose substrate, Man<sub>5</sub>GlcNAc<sub>2</sub> (Choi *et al.*, 2003). The transfer of this GlcNAc is the essential step for the generation of complex-type glycans that form after the subsequent cleavage of the two 6-arm mannose residues by Golgi  $\alpha$ 3,6-mannosidase II. Thus, formation of all complex glycans proceeds via the hybrid intermediate, GlcNAc $\beta$ 1–2Man<sub>5</sub>GlcNAc<sub>2</sub>. GnT I activity is often considered absolutely required for the action of other glycosyltransferases, including the mammalian core  $\alpha$ 1,6-fucosyltransferase which catalyses the addition of  $\alpha$ 1–6-linked fucose (Fuc) to the reducing end GlcNAc (Wilson *et al.*, 1976; Longmore and Schachter, 1982; Voynow *et al.*, 1991), as well as the plant and insect core  $\alpha$ 1,3-fucosyltransferases (Johnson and Chrispeels, 1987; von Schaeuwen *et al.*, 1993; Staudacher *et al.*, 1995; Leiter *et al.*, 1999; Strasser *et al.*, 2005).

There are, however, a number of reports of an alternative pathway in which the core fucosylation occurs independently of GnT I activity. For example, fucosylated paucimannose and oligomannose glycans have been identified in the nematode worm *C. elegans* (Haslam and Dell, 2003; Zhang *et al.*, 2003; Zhu *et al.*, 2004). In *C. elegans*, the hydrolysis of the nonreducing terminal GlcNAc following the action of GnT I and Golgi  $\alpha$ 3,6-mannosidase II generates Man<sub>3</sub>GlcNAc<sub>2</sub> which can be core  $\alpha$ 1–3 fucosylated by FUT-1 (Paschinger *et al.*, 2004, 2005). Thus,  $\alpha$ 1–3 fucosylation of paucimannose glycans by *Caenorhabditis* FUT-1 does not require the presence of the GnT I-mediated GlcNAc (Paschinger *et al.*, 2004). Similarly, in *Mgat1*<sup>1/1</sup> *Mgat1*<sup>1</sup>-null *Drosophila* mutants, deficient in GnT I activity, trace fucosylation of paucimannose glycans, but not oligomannose glycans, was observed (Sarkar *et al.*, 2006).

The presence of some  $\alpha$ 1–6 fucosylated mannose structures (with five or fewer mannose residues) has been attributed to GnT I-dependent fucosylation followed by the action of a  $\beta$ -*N*-acetylglucosaminidase (and  $\alpha$ 3,6-mannosidase II for the generation of paucimannose structures) (Zhang *et al.*, 2003). Similar, fucosylated, paucimannose

<sup>1</sup>To whom correspondence should be addressed; e-mail: max@strubi.ox.ac.uk

<sup>2</sup>Present address: Department of Biochemistry, Oxford Glycobiology Institute, University of Oxford, South Parks Road, Oxford OX1 3QU, UK.

structures are common in many invertebrates (reviewed in Staudacher *et al.*, 1999) and are also thought to arise from a GnT I-dependent fucosylation followed by the removal of the terminal  $\beta$ 1-2-GlcNAc by a membrane-bound  $\beta$ -*N*-acetylglucosaminidase (Altmann *et al.*, 1995).

The action of a  $\beta$ -*N*-acetylglucosaminidase has been proposed to account for the unusual monoantennary brain-type glycans that lack  $\beta$ 1-2-GlcNAc on the 3-arm (Chen *et al.*, 1998). Furthermore,  $\beta$ -*N*-acetylglucosaminidase activity could account for the occurrence of both fucosylated Man<sub>5</sub>GlcNAc<sub>2</sub> and paucimannose glycans in lysosomal glycoproteins such as rat liver alkaline phosphatase (Endo *et al.*, 1996), porcine cathepsin D (Takahashi *et al.*, 1983), human  $\beta$ -glucuronidase (Howard *et al.*, 1982), and bovine  $\alpha$ -mannosidase (Faid *et al.*, 2006). Most of these fucosylated oligomannose structures contained five or fewer mannoses, consistent with fucosylation after GnT I activity. However, core fucosylated Man<sub>6</sub>GlcNAc<sub>2</sub> glycans have been identified in human placental arylsulfatase A indicating the presence of GnT I-independent fucosylation (Hoja-Lukowicz *et al.*, 2000).

Lin and others (1994) previously identified GnT I-independent  $\alpha$ 1-6 fucosylation in GnT I-deficient (Lec1) Chinese hamster ovary (CHO) cells. The lack of GnT I activity normally stalls the glycan biosynthesis pathway after the glucosidases I and II and the  $\alpha$ -mannosidase I activities to leave a Man<sub>5</sub>GlcNAc<sub>2</sub> structure. The discovery of a population of core  $\alpha$ 1-6 fucosylated Man<sub>5</sub>GlcNAc<sub>2</sub> and Man<sub>4</sub>GlcNAc<sub>2</sub> structures, produced in Lec1 CHO cells, led to the proposal of an alternative activity of the core fucosyltransferase that was independent of  $\beta$ 1-2-GlcNAc addition by GnT I. However, earlier studies of the  $\alpha$ 1,6-fucosyltransferase could not detect this activity *in vitro* (Wilson *et al.*, 1976; Longmore and Schachter, 1982; Voynow *et al.*, 1991; Shao *et al.*, 1994). Also, fucosylated oligomannose glycans were not observed in GnT I-deficient *Arabidopsis thaliana* (von Schaeuwen *et al.*, 1993; Strasser *et al.*, 2005). Nonetheless, GnT I-independent fucosylation of oligomannose glycans was demonstrated in a *C. elegans* triple null mutant lacking any GnT I activity (Zhu *et al.*, 2004).

In this study, we confirm the presence of core fucosylated Man<sub>5</sub>GlcNAc<sub>2</sub> glycans on a glycoprotein, ligand of inducible co-stimulator (LICOS) (Brodie *et al.*, 2000), expressed in GnT I-deficient CHO Lec3.2.8.1 cells, and we demonstrate that the abundance of this structure is increased upon expression in the presence of the  $\alpha$ -glucosidase inhibitor, *N*-butyldeoxynojirimycin (NB-DNJ). Moreover, we report the biosynthesis of a novel core fucosylated monoglucosylated oligomannose glycan, Glc<sub>1</sub>Man<sub>7</sub>GlcNAc<sub>2</sub>Fuc, induced by NB-DNJ treatment. Furthermore, using human embryonic kidney (HEK) 293 cells, we demonstrate the presence of the human GnT I-independent fucosylation pathway by the structural characterization of *N*-linked glycans from recombinant receptor protein tyrosine phosphatase- $\mu$  (RPTP $\mu$ ) glycoprotein (Ensslen-Craig and Brady-Kalnay, 2004; Aricescu *et al.*, 2006), expressed in the presence of the inhibitors, swainsonine and kifunensine, and in a HEK 293S cell line devoid of GnT I activity (Reeves *et al.*, 2002).

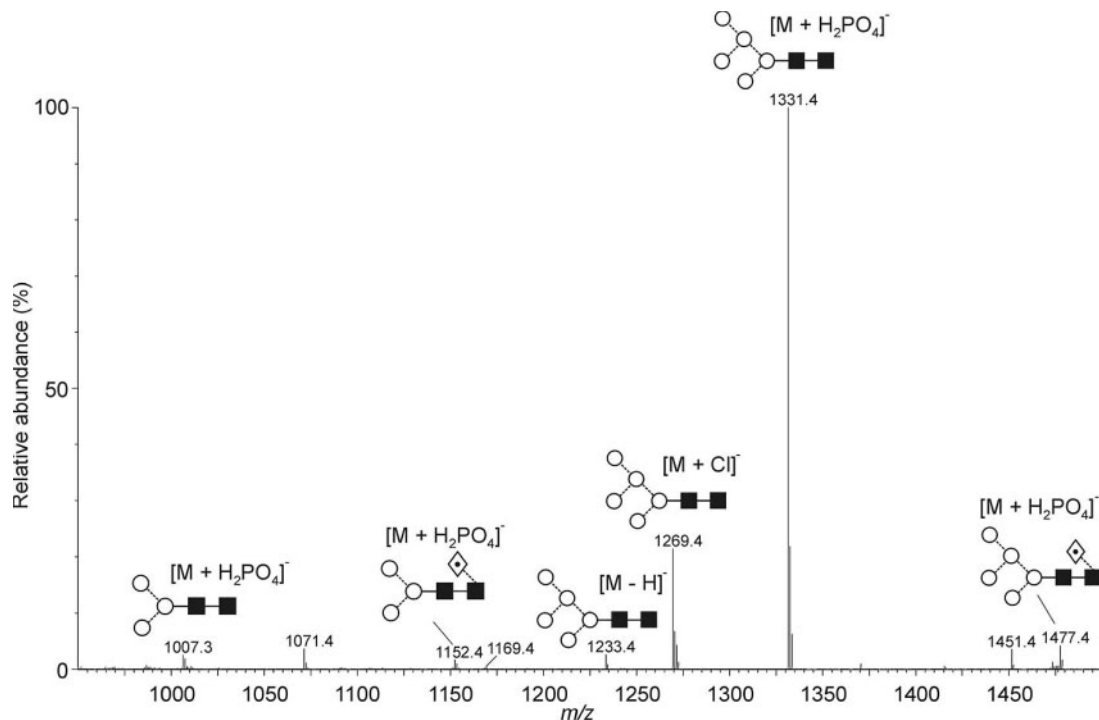
## Results and Discussion

### ESI-MS of CHO Lec3.2.8.1-expressed LICOS

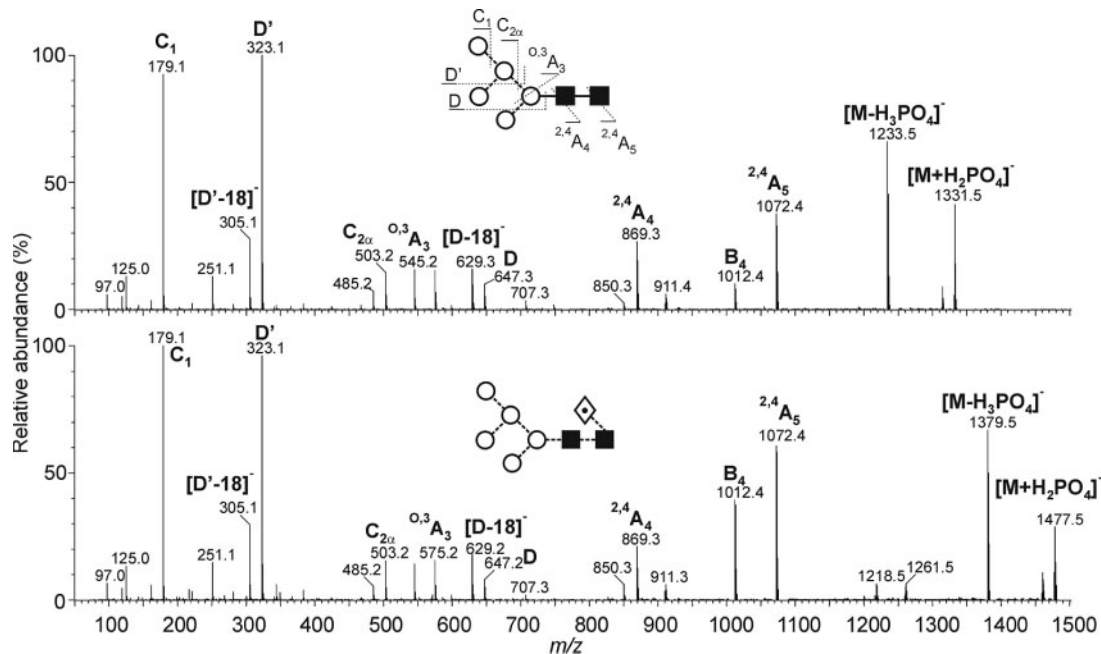
The negative-ion electrospray ionization mass spectrometry (ESI-MS) of the *N*-linked glycans of the extracellular region of LICOS, expressed in CHO Lec3.2.8.1 cells, was dominated by [M - H]<sup>-</sup>, [M + Cl]<sup>-</sup>, and [M + H<sub>2</sub>PO<sub>4</sub>]<sup>-</sup> ions of Hex<sub>5</sub>HexNAc<sub>2</sub> (Figure 1), consistent with the accumulation of the predominant substrate of GnT I, Man<sub>5</sub>GlcNAc<sub>2</sub> (Cl<sup>-</sup> and H<sub>2</sub>PO<sub>4</sub><sup>-</sup> adducts are typically formed from glycans extracted from biological sources and purified with sodium dodecyl sulfate—polyacrylamide gel electrophoresis [SDS-PAGE] gels). [M + H<sub>2</sub>PO<sub>4</sub>]<sup>-</sup> ions corresponding to Hex<sub>5</sub>HexNAc<sub>2</sub>dHex were also observed. The position of the Fuc was assigned using negative-ion collision-induced dissociation (CID) spectra of both the Man<sub>5</sub>GlcNAc<sub>2</sub> and the fucosylated Man<sub>5</sub>GlcNAc<sub>2</sub> (Figure 2). The B<sub>4</sub>, <sup>2,4</sup>A<sub>5</sub>, and <sup>2,4</sup>A<sub>4</sub> ions, following the nomenclature of Domon and Costello (1988), appeared at the same mass in the spectra of both compounds (*m/z* = 1012.4, 1072.4, and 869.3, respectively), indicating that the Fuc modification occurs on the terminal GlcNAc at the C6, consistent with a Fuc $\alpha$ 1-6GlcNAc linkage (Harvey, 2005b). Further, ions defining the nonreducing moiety of both structures were the same in both spectra (Figure 2) and were identical to those in a reference spectrum of Man<sub>5</sub>GlcNAc<sub>2</sub>.

Ions defining both the fucosylated and unfucosylated paucimannose glycan, Man<sub>3</sub>GlcNAc<sub>2</sub>, were also identified in the spectra of LICOS (Figure 1). These paucimannose structures confirm the presence of a GnT I-independent  $\alpha$ -mannosidase activity in CHO Lec3.2.8.1 cells and account for the incomplete digestion by endoglycosidase H (Endo H) of glycoproteins from this expression system (Butters *et al.*, 1999). To prevent the formation of Endo H-resistant glycans, Butters and others (1999) expressed glycoproteins in CHO Lec3.2.8.1 cells in the presence of the  $\alpha$ -glucosidase inhibitor, NB-DNJ. We analyzed the *N*-linked glycans of LICOS expressed in CHO Lec3.2.8.1 cells in the presence of 0.25 mM NB-DNJ (Figure 3). Ions were present in the matrix-assisted laser desorption/ionization (MALDI) mass spectrum defining Hex<sub>5</sub>HexNAc<sub>2</sub> (Man<sub>5</sub>GlcNAc<sub>2</sub>, *m/z* = 1257.4), Hex<sub>8</sub>HexNAc<sub>2</sub> (Glc<sub>1</sub>Man<sub>7</sub>GlcNAc<sub>2</sub>, *m/z* = 1743.5), and Hex<sub>10</sub>HexNAc<sub>2</sub> (Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub>, *m/z* = 2067.7) and the deoxyhexose (dHex) derivatives of Man<sub>5</sub>GlcNAc<sub>2</sub> (*m/z* = 1403.4) and Glc<sub>1</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> (*m/z* = 1889.6). Interestingly, the relative abundance of the fucosylated Man<sub>5</sub>GlcNAc<sub>2</sub> structure, at ~38%, is significantly higher than in the spectrum of the LICOS glycans from untreated CHO Lec3.2.8.1 cells.

CID spectra were generated from the parent ions of both the fucosylated and the unfucosylated Glc<sub>1</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> (Figure 4). The B<sub>6</sub>, <sup>2,4</sup>A<sub>7</sub>, and <sup>2,4</sup>A<sub>6</sub> ions (*m/z* = 1498.5, 1558.5, and 1355.4, respectively; Figure 4), resulting from glycosidic and cross-ring cleavage of the reducing terminal GlcNAc, demonstrated that the dHex (Fuc) is situated at the C6 position on the reducing terminal GlcNAc in an  $\alpha$ 1-6 linkage. D and [D - 18]<sup>-</sup> ions at an *m/z* of 647 and 629, respectively, defined the composition of the 6-antennae (Harvey, 2005a). Other diagnostic ions are indicated in Figure 4. The identification of the Glc<sub>1</sub>Man<sub>7</sub>GlcNAc<sub>2</sub>Fuc



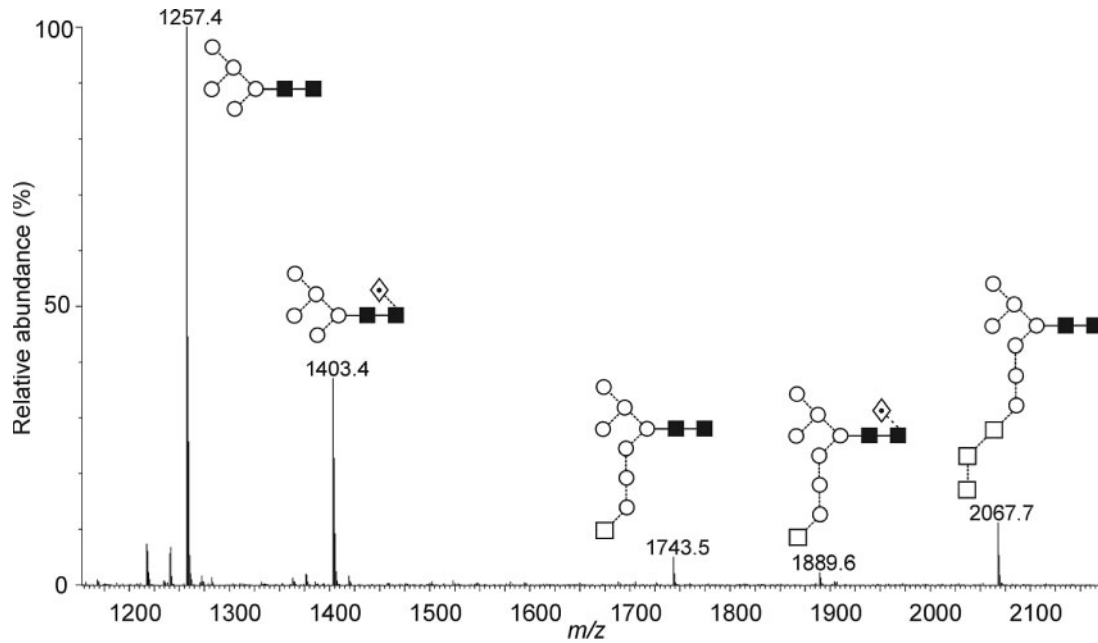
**Fig. 1.** Negative-ion ESI spectrum of *N*-linked glycans from CHO Lec3.2.8.1-expressed LICOS. Key to symbols used to represent monosaccharide constituents in this and subsequent figures: Gal ( $\diamond$ ), GalNAc ( $\blacklozenge$ ), Glc ( $\square$ ), GlcNAc ( $\blacksquare$ ), Man ( $\circ$ ), sialic acid ( $\star$ ), and Fuc ( $\diamond$ ). The angles of the lines linking monosaccharide residues indicate linkage; anomericity is indicated by full lines for  $\beta$ -bonds and broken lines for  $\alpha$ -bonds.



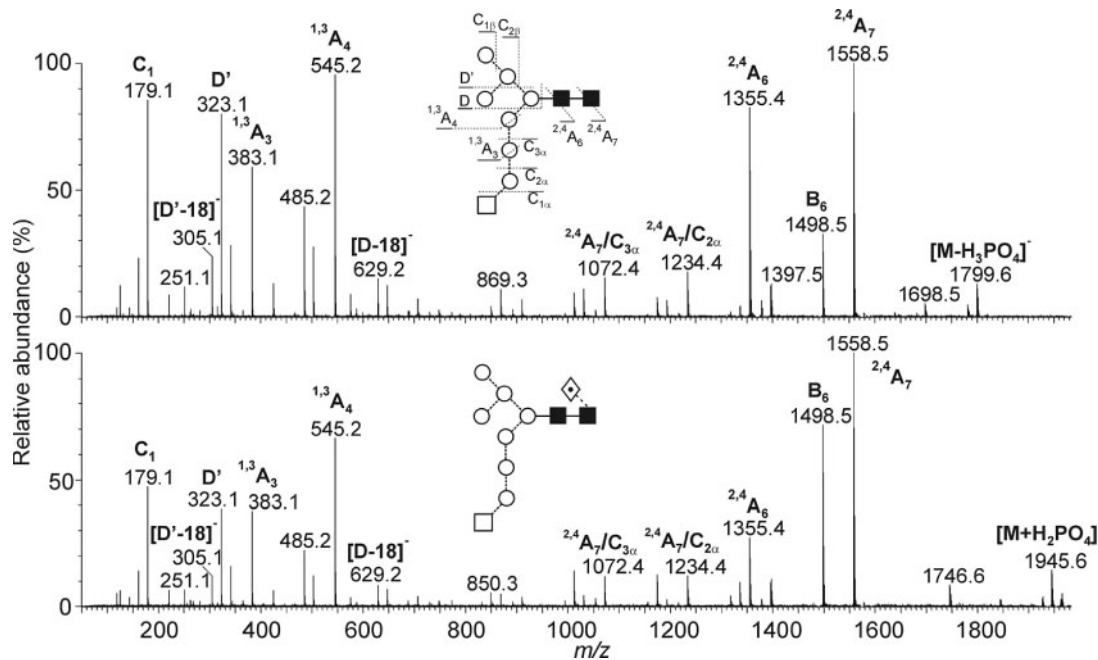
**Fig. 2.** Negative-ion ESI-CID spectra of the phosphate adducts (from naturally occurring phosphate) of oligomannose *N*-linked glycans from CHO Lec3.2.8.1-expressed LICOS: (a)  $\text{Man}_3\text{GlcNAc}_2$  and (b)  $\text{Man}_3\text{GlcNAc}_2\text{Fuc}$ . Symbols as in Figure 1. Ion nomenclature follows the method of Domon and Costello (1988).

structure demonstrates that the  $\text{Glc}\alpha 1\text{-}3\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}2$  saccharides of the 3-antennae do not prevent core fucosylation in CHO cells. Interestingly, there was no evidence of fucosylated  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$  glycans. Glycosylation

sites bearing such triglycosylated structures have not been exposed to calnexin, indicating that core fucosylation may be dependent on the conformation around the glycosylation site.



**Fig. 3.** Positive-ion MALDI-TOF mass spectrum of *N*-linked glycans from LICOS expressed in CHO Lec3.2.8.1 cells in the presence of the  $\alpha$ -glucosidase inhibitor, NB-DNJ. Symbols as in Figure 1.

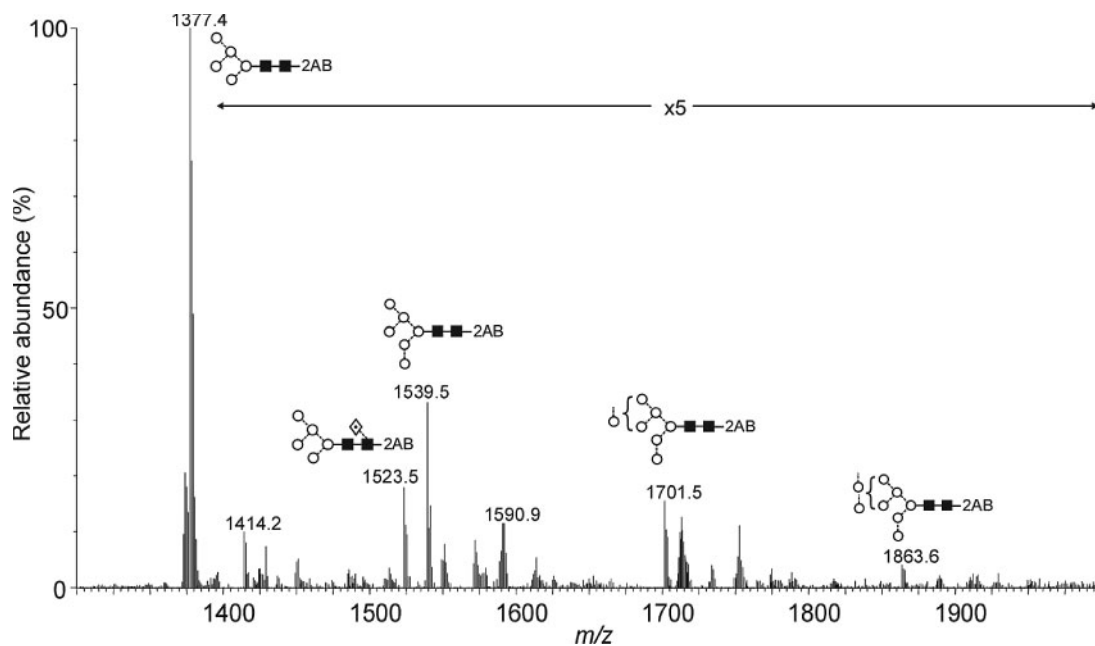


**Fig. 4.** Negative-ion ESI-CID spectra of the phosphate adducts (from naturally occurring phosphate) of monoglucosylated oligomannose *N*-linked glycans from LICOS expressed in CHO Lec3.2.8.1 cells in the presence of NB-DNJ: (a)  $\text{Glc}_1\text{Man}_7\text{GlcNAc}_2$  and (b)  $\text{Glc}_1\text{Man}_7\text{GlcNAc}_2\text{Fuc}$ . Symbols as in Figure 1. Ion nomenclature follows the method of Domon and Costello (1988).

*GnT I-deficient HEK 293S cells produce fucosylated  $\text{Man}_5\text{GlcNAc}_2$  glycans*

MALDI-time-of-flight (TOF) MS analysis of recombinant RPTP $\mu$  expressed in *GnT I*-deficient HEK 293S cells was dominated by the ions from  $\text{Man}_5\text{GlcNAc}_2$ -2-aminobenzamide

(2AB) ( $m/z = 1377.4$ ; Figure 5). The glycans were derivatized with 2AB to facilitate high-performance liquid chromatography (HPLC) analysis (data not shown). No glycans were detected by MALDI MS with an  $m/z$  value below that of the 2AB-derivatized  $\text{Man}_5\text{GlcNAc}_2$  glycan.



**Fig. 5.** Positive-ion MALDI-TOF mass spectrum of 2AB-labeled *N*-linked glycans from RPTP $\mu$  expressed in GnT I<sup>-/-</sup> HEK 293S cells. The relative abundance of the region from *m/z* 1400 has been amplified 5-fold. Symbols as in Figure 1.

This is consistent with a lack of GnT I activity as reported by Reeves and others (2002) and demonstrates that, in contrast to the CHO Lec3.2.8.1 cell line (Figure 1), there is no GnT I-independent  $\alpha$ -mannosidase activity that processes the glycans beyond Man<sub>5</sub>GlcNAc<sub>2</sub>. However, together with the oligomannose series, Man<sub>6-8</sub>GlcNAc<sub>2</sub>, there is an ion at *m/z* = 1523.5 consistent with the presence of fucosylated Man<sub>5</sub>GlcNAc<sub>2</sub>-2AB (Figure 5). The presence of such fucosylated oligomannose structure, together with the lack of GnT I activity and detectable hybrid-type structures, is indicative of a human GnT I-independent fucosylation pathway analogous to that observed in the CHO expression system.

#### *Swainsonine-treated HEK 293T cells produce fucosylated Man<sub>5</sub>GlcNAc<sub>2</sub> glycans*

Man<sub>5</sub>GlcNAc<sub>2</sub>Fuc glycans were also identified following analysis of the *N*-linked glycans of RPTP $\mu$  expressed in HEK 293T cells in the presence of 20  $\mu$ M of the Golgi  $\alpha$ -mannosidase II inhibitor, swainsonine (Figure 6). In contrast to the MALDI-TOF MS analysis of RPTP $\mu$  expressed in GnT I-deficient HEK 293S cells (Figure 5), the presence of Man<sub>5</sub>GlcNAc<sub>2</sub>Fuc arising from the combined action of GnT I and  $\beta$ -*N*-acetylglucosaminidase cannot be excluded. Indeed, numerous hybrid-type glycans dominate the spectrum displaying typical GnT I-dependent modifications such as the subsequent addition of galactose, GnT III-mediated bisecting GlcNAc, and core fucosylation. However, analysis of the hybrid-type glycans revealed no evidence of  $\beta$ -*N*-acetylglucosaminidase activity, such as the identification of bisected oligomannose glycans that could account for GnT I-dependent Man<sub>5</sub>GlcNAc<sub>2</sub>Fuc glycans (Figure 6). Such glycans have been identified in rat brain (Chen *et al.*, 1998), supporting the ability of

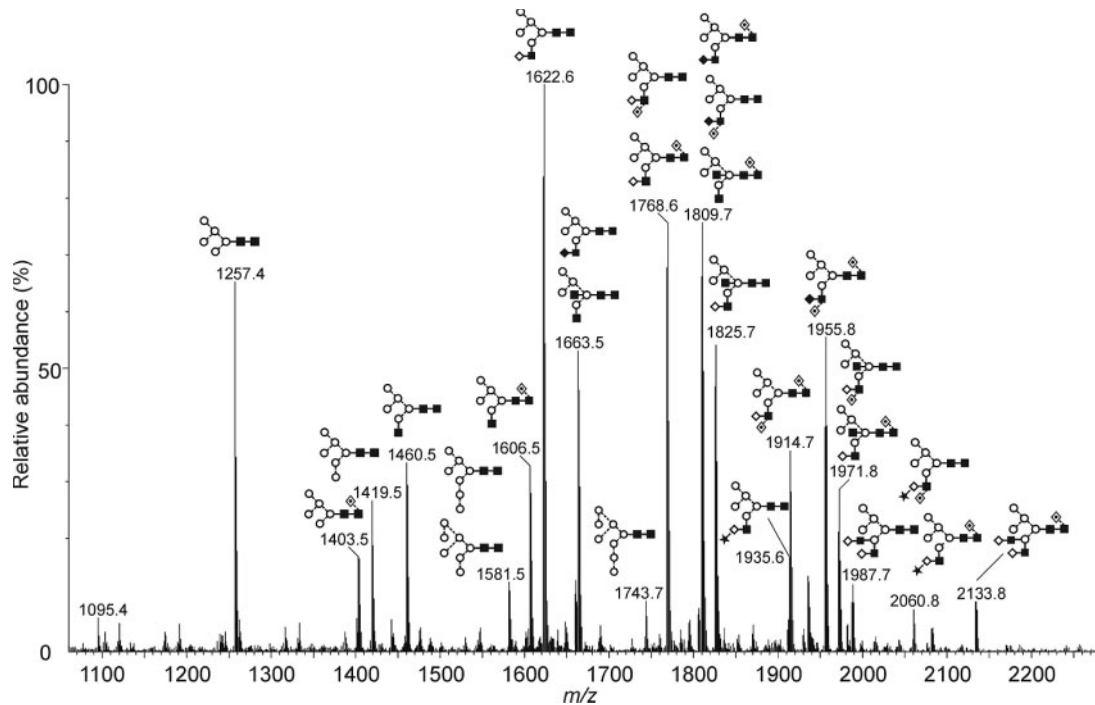
$\beta$ -*N*-acetylglucosaminidase to remove 2-linked GlcNAc in the presence of bisecting GlcNAc.

#### *Inhibition of $\alpha$ -mannosidase I prevents fucosylation in HEK 293T cells*

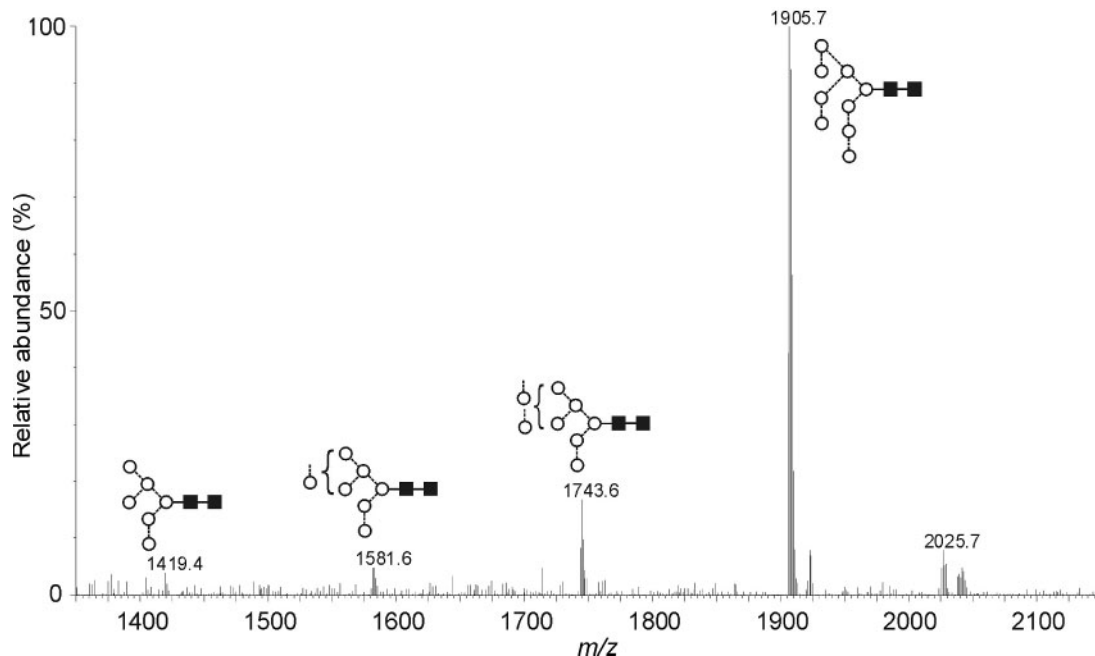
Further details of the substrate specificity of the fucosyltransferase were revealed by the absence of fucosylated oligomannose glycans on RPTP $\mu$  expressed in HEK 293T cells in the presence of 5  $\mu$ M of the  $\alpha$ -mannosidase I inhibitor, kifunensine. MALDI-TOF MS analysis of the *N*-linked glycans was dominated by the oligomannose glycans Man<sub>7-9</sub>GlcNAc<sub>2</sub>, with a trace of Man<sub>6</sub>GlcNAc<sub>2</sub> (Figure 7). Assuming that kifunensine does not inhibit the core fucosyltransferase, the lack of detectable fucosylated derivatives of the Man<sub>7-9</sub>GlcNAc<sub>2</sub> glycans suggests that one or more of the  $\alpha$ 1-2 mannose residues that extend from the Man<sub>5</sub>GlcNAc<sub>2</sub> core disrupt substrate recognition. Taken together with the NB-DNJ-induced Glc<sub>1</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> structure determined in the CHO Lec3.2.8.1 system, these data suggest that  $\alpha$ 1-2 mannoses of the 6-antennae prevent GnT I-independent fucosylation.

#### *A model for the core fucosylation pathway*

The transfer of  $\beta$ 1-2-GlcNAc to the 3-antennae by GnT I is usually regarded as a prerequisite for both core fucosylation and Golgi  $\alpha$ -mannosidase II activity and is normally likely to mask the presence of the GnT I-independent fucosylation pathway. The paucity of fucosylated oligomannose glycans in glycoproteins expressed in wild-type mammalian cells can, thus, be attributed to such GnT I-dependent pathways which act to deplete both the predominant substrate (Man<sub>5</sub>GlcNAc<sub>2</sub>) and the product (Man<sub>5</sub>GlcNAc<sub>2</sub>Fuc) of the GnT I-independent fucosylation pathway (Figure 8). In



**Fig. 6.** Positive-ion MALDI-TOF mass spectrum of oligomannose and hybrid-type *N*-glycans from RPTP $\mu$  expressed in HEK 293T cells in the presence of 20  $\mu$ M of the Golgi  $\alpha$ -mannosidase II inhibitor, swainsonine. Symbols as in Figure 1.



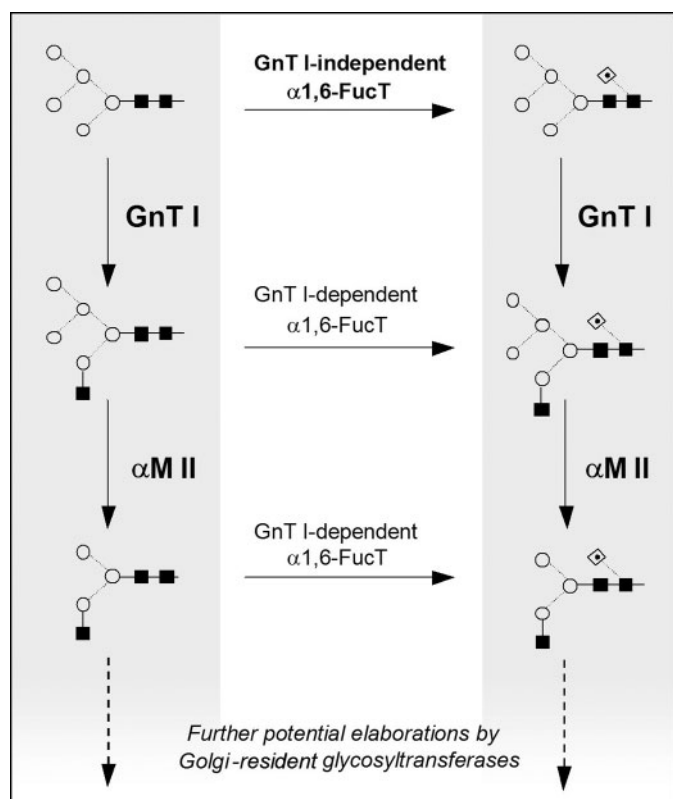
**Fig. 7.** Positive-ion MALDI-TOF spectrum of oligomannose *N*-linked glycans from RPTP $\mu$  expressed in HEK 293S cells in the presence of 5  $\mu$ M of the  $\alpha$ -mannosidase inhibitor, kifunensine. Symbols as in Figure 1.

this work, we have exploited cell lines deficient in GnT I to expose the activity of the GnT I-independent fucosylation pathway. Knowledge of such underlying activities within the mammalian glycosylation pathway is important for the precise engineering of glycoproteins and essential when manufacturing therapeutic glycoproteins with controlled glycosylation.

## Materials and Methods

### *Cloning, expression, and purification of LICOS*

A cDNA fragment encoding human LICOS extracellular region (residues 1–254) was amplified by polymerase chain reaction (PCR) using prLuc-hLICOS vector as template (from J. James in S.J.D.'s laboratory, University of Oxford)



**Fig. 8.** Proposed pathway for the formation, and subsequent depletion, of fucosylated oligomannose *N*-linked glycans. GnT I, GlcNAc transferase I.  $\alpha$ M II,  $\alpha$ -mannosidase II.  $\alpha$ 1,6-FucT,  $\alpha$ 1,6-fucosyltransferase. Symbols as in Figure 1.

and subcloned into the pEE14 vector of the glutamine synthetase-based gene-expression system (Bebbington and Hentschell, 1987) in frame with a C-terminal LysHis6 tag. The LICOS construct was transfected into CHO Lec3.2.8.1 cells (Stanley, 1981; Chen and Stanley, 2003) using FuGENE 6 transfection reagent (Roche Applied Science, East Sussex, UK). Clones resistant to 15  $\mu$ M methionine sulfoximine were selected and screened for LICOS expression using dot blot and western blot. One of these clones which expressed LICOS at a level of  $\sim$ 5 mg/L was selected for large-scale production of protein and was grown in cell factories (Nunc, Roskilde, Denmark) both in the presence and in the absence of 0.25 mM NB-DNJ, as described previously (Butters *et al.*, 1999). The protein secreted into the tissue culture supernatant was concentrated and buffer exchanged to 20 mM Tris-HCl, 0.5 M NaCl, pH 8.0, by ultrafiltration, and loaded onto a Ni-NTA agarose column, which was then eluted with 300 mM imidazole and subjected to size-exclusion chromatography purification.

#### Cloning, expression, and purification of RPTP $\mu$

A cDNA fragment encoding the human RPTP $\mu$  extracellular region (residues 1–742) was amplified by PCR using the pHFL vector as template (Gebbinck *et al.*, 1993), a gift from M. Gebbinck (University of Utrecht, The Netherlands), and subcloned into the pHL expression vector (Aricescu *et al.*, 2006) in frame with a C-terminal LysHis6 tag.

HEK 293T (ATCC number CRL-1573) and HEK 293S GnT I<sup>-/-</sup> cells (a gift from H.G. Khorana and P.J. Reeves, Massachusetts Institute of Technology) (Reeves *et al.*, 2002), 90% confluent, were transiently transfected with polyethyleneimine (Durocher *et al.*, 2002). The cells were cultured in Dulbecco's modified Eagle's medium (Sigma, Poole, Dorset, UK) supplemented with 10% fetal calf serum (Sigma), L-glutamine, and nonessential amino acids (Invitrogen, Paisley, UK). The serum concentration was lowered to 2% immediately after transfection. Swainsonine and kifunensine (Toronto Research Chemicals, North York, Ontario, Canada) were used at a final concentration of 20 and 5  $\mu$ M, respectively, and added to the HEK 293T culture medium immediately after transfection. Conditioned media, containing the secreted fusion proteins, were collected 4 days after transfection, centrifuged, sterile filtered, and then diluted with two volumes of phosphate-buffered saline (PBS). The pH was adjusted to 8.0 with 10 mM Tris-HCl. The His-tagged proteins were bound to Ni<sup>2+</sup>-charged chelating Sepharose (Amersham Biosciences, Uppsala, Sweden) in batch mode at 16°C for 4 h, then the beads were packed in BioRad Econo columns, washed with 20 bed volumes of PBS supplemented with 0.1% Tween-20 followed by 20 bed volumes of PBS. Bound proteins were eluted in 100 mM Tris-HCl, pH 8, 500 mM NaCl, 500 mM imidazole, concentrated and subjected to gel filtration using a Superdex 200 HR column (Amersham Biosciences) equilibrated in 100 mM Tris-HCl, pH 8, 500 mM NaCl. Fractions containing the target proteins at >95% purity, as judged by SDS-PAGE, were pooled, quantified, and kept at 4°C.

#### Enzymatic release of *N*-linked glycans

Oligosaccharides were released with peptide *N*-glycosidase F (PNGase F) (Prozyme, San Leandro, CA) from Coomassie blue-stained SDS-PAGE gels containing the target glycoprotein (Küster *et al.*, 1997). Bands containing  $\sim$ 10  $\mu$ g of target glycoprotein were excised from reducing SDS-PAGE gels and washed with 20 mM NaHCO<sub>3</sub>, pH 7.0. The washed gel fragments were dried in a vacuum centrifuge before rehydration with 30  $\mu$ L of 30 mM NaHCO<sub>3</sub>, pH 7.0, containing 100 U/mL of PNGase F. After incubation for 12 h at 37°C, the enzymatically released *N*-linked glycans were eluted with water. Salts were removed by a 5-min incubation at room temperature with 200  $\mu$ L of an acid-activated AG-50W (200–400 mesh) slurry (BioRad, Hercules, CA), which was removed by filtration with a 0.45  $\mu$ m pore-size filter (Millex-LH, hydrophobic polytetrafluoroethylene). An aliquot of the released glycans was derivatized with 2AB, according to the method described by Bigge and others (1995).

#### MALDI-TOF mass spectrometry

Positive-ion MALDI-TOF mass spectra were recorded with a Waters-Micromass ToFSpec 2E reflectron-TOF mass spectrometer (Waters-Micromass, Manchester, UK) operated under the following conditions: accelerating voltage, 20 kV; pulse voltage, 3.0 kV; time lag focusing delay, 500 ns (setting 39); and laser-repetition rate, 10 Hz. Aqueous glycan samples (0.5  $\mu$ L) were mixed on the MALDI target with the

matrix (0.5  $\mu$ L of a saturated solution of 2,5-dihydroxybenzoic acid in acetonitrile), allowed to dry under ambient conditions, and recrystallized from ethanol (0.2  $\mu$ L).

#### Negative-ion nano-electrospray mass spectrometry and MS/MS

Negative-ion nano-ESI mass spectra were recorded with a Waters-Micromass Q-ToF Ultima Global mass spectrometer (Waters-Micromass). Samples in methanol : water (1:1, v : v) were infused through Proxeon (Proxeon Biosystems, Odense, Denmark) nanospray capillaries. Operating conditions for the mass spectrometer were: ion source temperature, 120°C; desolvation gas (N<sub>2</sub>) flow, 50 L/h; infusion capillary voltage, 1.2 kV; RF-1 potential, 180 V; collision gas (argon) pressure, 0.5 bar; collision cell voltage, appropriate to the mass of the glycan (range 60–90 V); mass window for parent ion selection, about 4 Da; scan time, 1 s; and digitization rate, 4 GHz. Spectra were acquired and processed with MassLynx version 4 software (Waters-Micromass).

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#### Conflict of interest statement

None declared.

#### Abbreviations

2AB, 2-aminobenzamide; CHO, Chinese hamster ovary; CID, collision-induced dissociation; ESI, electrospray ionization; Fuc, fucose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GnT, *N*-acetylglucosaminyltransferase; HEK, human embryonic kidney; Hex, hexose; HexNAc, *N*-acetylhexosamine; LICOS, ligand of inducible co-stimulator (also known as ICOSL, B7h, B7-H2, and B7RP-1); MALDI, matrix-assisted laser desorption/ionization; Man, mannose; MS, mass spectrometry; NB-DNJ, *N*-butyldeoxynojirimycin; PBS, phosphate-buffered saline; RPTP $\mu$ , receptor protein tyrosine phosphatase- $\mu$ ; SDS-PAGE, sodium dodecyl sulfate—polyacrylamide gel electrophoresis; TOF, time-of-flight.

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