

Disruption of α -mannosidase processing induces non-canonical hybrid-type glycosylation

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Abstract Golgi α -mannosidase II is essential for the efficient formation of complex-type glycosylation. Here, we demonstrate that the disruption of Golgi α -mannosidase II activity by swainsonine in human embryonic kidney cells is capable of inducing a novel class of hybrid-type glycosylation containing a partially processed mannose moiety. The discovery of 'Man₆-based' hybrid-type glycans reveals a broader *in vivo* specificity of *N*-acetylglucosaminyltransferase I, further defines the arm-specific tolerance of core α 1-6 fucosyltransferase to terminal α 1-2 mannose residues, and suggests that disruption of Golgi α -mannosidase II activity is capable of inducing potentially 'non-self' structures.

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1. Introduction

The complexity of glycosylation is essential for the growth and development of higher eukaryotic organisms. Numerous glycosidases and glycosyltransferases orchestrate the conversion of the glucosylated oligomannose-type glycans of nascent glycoproteins to the hybrid- and complex-type glycosylation characteristic of mature mammalian glycoproteins [1]. With the exception of glycans that are protected from glycosidase

processing by the protein moiety [2], following endoplasmic reticulum (ER) α -glucosidase I and II, many glycans are subjected to ER α -mannosidase I cleavage resulting in D1,D3-Man₈GlcNAc₂ isomer [3] (compound 6, Fig. 1). Golgi α -mannosidase IA, IB, IC, complements this activity by hydrolyzing the remaining D1 and D3-arm α 1-2 mannose residues to yield Man₅GlcNAc₂ [4] (compound 5, Fig. 1). Two ancient enzymatic steps then mediate the main gateway from this oligomannose-type glycan to the hybrid- and complex-type glycans capable of significant heterogeneity.

Classical hybrid-type glycosylation is formed by the transfer of β 1-2-linked GlcNAc to the 3-arm mannose of the trimannosyl core by UDP-*N*-acetyl-D-glucosamine: α -3-D-mannoside β 1,2-*N*-acetylglucosaminyltransferase I (GnT I) [5]. The action of GnT I is permissive for further glycosyltransferase reactions that lead to increased complexity of the hybrid-type structure, such as GnT III and FUT8, which catalyze bisecting β 1-4 GlcNAc and core α 1-6 fucosylation, respectively. Golgi α -mannosidase II hydrolyzes the unsubstituted α 1-3 and α 1-6 mannose residues of the 6-antennae to yield a monoantennary complex-type glycan, which can be further elaborated by numerous glycosyltransferases in the Golgi apparatus.

Homozygotic mutation of *Mgat1*, which encodes GnT I, is embryonically lethal in mice at E9.5 [6,7] whereas disruption of GnT I activity is tolerated in cell lines [8,9]. The importance of hybrid and complex-type glycosylation in development is further supported by the impaired locomotory activity and brain development in *Drosophila melanogaster* [10]. The glycans of GnT I-deficient cell lines, such as Chinese hamster ovary (CHO) *Lec3.2.8.1* and ricin-resistant human embryonic kidney (HEK) 293S cells, are dominated by Man₅GlcNAc₂ [8,9,11,12]. Only a trace amount of α 1-6 fucosylated oligomannose glycans reveal a GnT I-independent glycosyltransferase activity in mammals [13,14].

In contrast, genetic disruption of Golgi α -mannosidase II, which leads to the formation of hybrid-type glycans, is found clinically in humans as congenital dyserythropoietic anemia type II [15,16]. Mice homozygous for deletions of the murine Golgi α -mannosidase II gene display similar dyserythropoiesis and develop systemic autoimmune disease similar to human systemic lupus erythematosus [17]. Chui and others hypothesized that resulting hybrid-type glycans, usually absent or present at very low levels on the cell surface, contribute to the increased autoantibody reactivity [17]. Here, using negative

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Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; CHO, Chinese hamster ovary; CID, collision-induced dissociation; dHex, deoxyhexose; ER, endoplasmic reticulum; ESI, electrospray ionization; Fuc, fucose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GnT, *N*-acetylglucosaminyltransferase; HEK, human embryonic kidney; Hex, hexose; HexNAc, *N*-acetylhexosamine; Man, mannose; Mlg, MAM and Ig domains of RPTP μ ; MS, mass spectrometry; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PNGase F, peptide *N*-glycosidase F; Q, quadrupole; RPTP μ , receptor protein tyrosine phosphatase- μ ; s, soluble; SDS, sodium dodecyl sulfate; Tof, time-of-flight

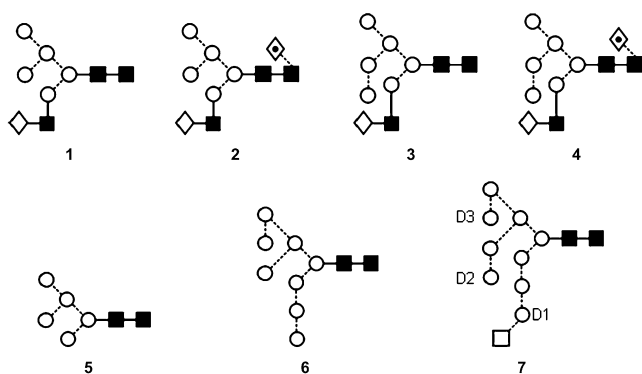


Fig. 1. Structures of the glycans whose fragmentation is discussed in this paper. Key to symbols used to represent monosaccharide constituents in this and subsequent figures: (\diamond) Gal, (\blacksquare) GlcNAc, (\circ) Man, (\diamond) Fuc. The linkage position is shown by the angle of the lines linking the sugar residues (vertical line = 2-link, forward slash = 3-link, horizontal line = 4-link, back slash = 6-link). Anomerism is indicated by full lines for β -bonds and broken lines for α -bonds. Terminal α 1-2 mannose residues of $\text{Man}_6\text{GlcNAc}_2$ are termed the D1, D2 and D3 residues (illustrated on the monoglucosylated $\text{Man}_6\text{GlcNAc}_2$, compound 7) [31–33]. This labelling is distinct from the ion nomenclature of Domon and Costello [28].

ion nano-electrospray ionization mass spectrometry, we demonstrate the biosynthesis in human embryonic kidney cells of hitherto undescribed Man_6 -based hybrid-type glycans. The production of these unusual structures following disruption of Golgi α -mannosidase II activity are consistent with the hypothesis that ‘non-self’ structures are formed [17].

In addition, these observations demonstrate that complete hydrolysis of terminal α 1-2 linked mannose residues is not essential for the formation of hybrid-type glycans. Furthermore, identification of a core α 1-6 fucosylated derivative of the Man_6 -hybrid, reveals that core fucosylation is dependent on the hydrolysis of the D3 mannose, which may prove a powerful tool in the enhancement of antibody-dependent cell-mediated cytotoxicity (ADCC) [18,19].

2. Materials and methods

2.1. Materials

Authentic samples of $\text{Man}_5\text{GlcNAc}_2$ (compound 5; Fig. 1) and D1,D3- $\text{Man}_8\text{GlcNAc}_2$ (compound 6) were obtained from Oxford GlycoSciences Ltd. (Abingdon, UK). $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ (compound 7) was a gift from Dr. M. Mackeen.

2.2. Cloning, expression, and purification of RPTP μ

A cDNA fragment encoding the two soluble (s) N-terminal domains (MAM and Ig, termed sMIg [20]) of human receptor protein tyrosine phosphatase μ , (RPTP μ), extracellular region (residues 1–279), was amplified by polymerase chain reaction (PCR) using the pHFL vector as template [21], a gift from M. Gebbink (University of Utrecht, The Netherlands), and subcloned into the pHLE expression vector [20] in frame with a C-terminal LysHis₆ tag.

HEK 293T (ATCC number CRL-1573), 90% confluent, were transiently transfected with polyethyleneimine [22,23]. The cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma, Poole, Dorset, UK) supplemented with 10% fetal bovine serum (Sigma, Poole, Dorset, UK), L-glutamine and non-essential amino acids (Invitrogen, Paisley, UK). The serum concentration was lowered to 2% immediately after transfection. Swainsonine (Toronto Research Chemicals, North York, Ontario, Canada) was used at a final concentration of 5 μM and added to the HEK 293T culture medium immediately

after transfection. Conditioned media, containing the secreted fusion protein, were collected 4 days post-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 protein was bound to Ni^{2+} -charged chelating Sepharose (Amersham Biosciences, Uppsala, Sweden) in batch mode at 16 $^\circ\text{C}$ 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 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, Uppsala, Sweden) equilibrated in 100 mM Tris-HCl, pH8, 500 mM NaCl. Fractions containing the target protein at >95% purity, as judged by SDS-PAGE, were pooled, quantified and stored at 4 $^\circ\text{C}$.

2.3. Enzymatic release of N-linked glycans

Oligosaccharides were released from MIg with protein N-glycanase (PNGase F (Prozyme, San Leandro, CA, USA) from Coomassie blue-stained SDS-PAGE gels containing the target glycoprotein [24]. Bands containing approximately 10 μg of target glycoprotein were excised from reducing SDS-PAGE gels and washed with 20 mM NaHCO_3 pH 7.0. The washed gel bands were dried in a vacuum centrifuge before rehydration with 30 μl of 30 mM NaHCO_3 pH 7.0, containing 100 Units/ml of peptide N-glycosidase F (PNGase F). After incubation for 12 h at 37 $^\circ\text{C}$, the enzymatically released N-linked glycans were eluted with water. Salts were removed with incubation at RT (5 min) with 200 μl of an acid-activated AG-50W (200–400 mesh) slurry (BioRad, Hercules, CA, USA) which was removed by filtration with a 0.45 μl pore-size filter (Millex-LH, hydrophobic polytetrafluoroethylene).

2.4. Electrospray mass spectrometry

Electrospray mass spectrometry was performed with a Waters-Micromass quadrupole-time-of-flight (Q-ToF) Ultima Global instrument in negative ion mode. Samples in 1:1 (v:v) methanol:water were infused through Protana nanospray capillaries (Protana, Odense, Denmark). The ion source conditions were as follows: temperature, 120 $^\circ\text{C}$; nitrogen flow 50 l/h; infusion needle potential, 1.2 kV; cone voltage 100 V; RF-1 voltage 150 V. Spectra (2 s scans) were acquired with a digitization rate of 4 GHz and accumulated until a satisfactory signal:noise ratio had been obtained. For MS/MS data acquisition, the parent ion was selected at low resolution (about 5 m/z mass window) to allow transmission of isotope peaks and fragmented with argon at a pressure (recorded on the instrument's pressure gauge) of 0.5 mBar. The voltage on the collision cell was adjusted with mass and charge to give an even distribution of fragment ions across the mass scale. Typical values were 80–120 V. Other voltages were as recommended by the manufacturer. Instrument control, data acquisition and processing were performed with MassLynx software Version 4.0 (Water-Micromass, Manchester, UK).

3. Results

3.1. Identification of Man_6 -based hybrid-type glycans

RPTP μ is involved in neural and vascular development and it has been extensively studied for its cell adhesive properties [20,25,26]. We sought to investigate the post-translational processing of the RPTP μ extracellular domains. Recombinant sMIg, containing domains critical for adhesion, was expressed in HEK 293T cells in the presence of 20 μM of the potent Golgi α -mannosidase II inhibitor, swainsonine. Glycans were released by in-gel PNGase F digestion and analyzed by electrospray mass spectrometry. Fig. 1 depicts the structure of the N-linked glycans discussed herein (compounds 1–7). Collision-induced decomposition (CID) of the molecular ions produced non-standard spectra which we here assign to unusual Man_6 -based hybrid structures (compounds 3 and 4). The relative abundance of the Man_6 -based glycans were

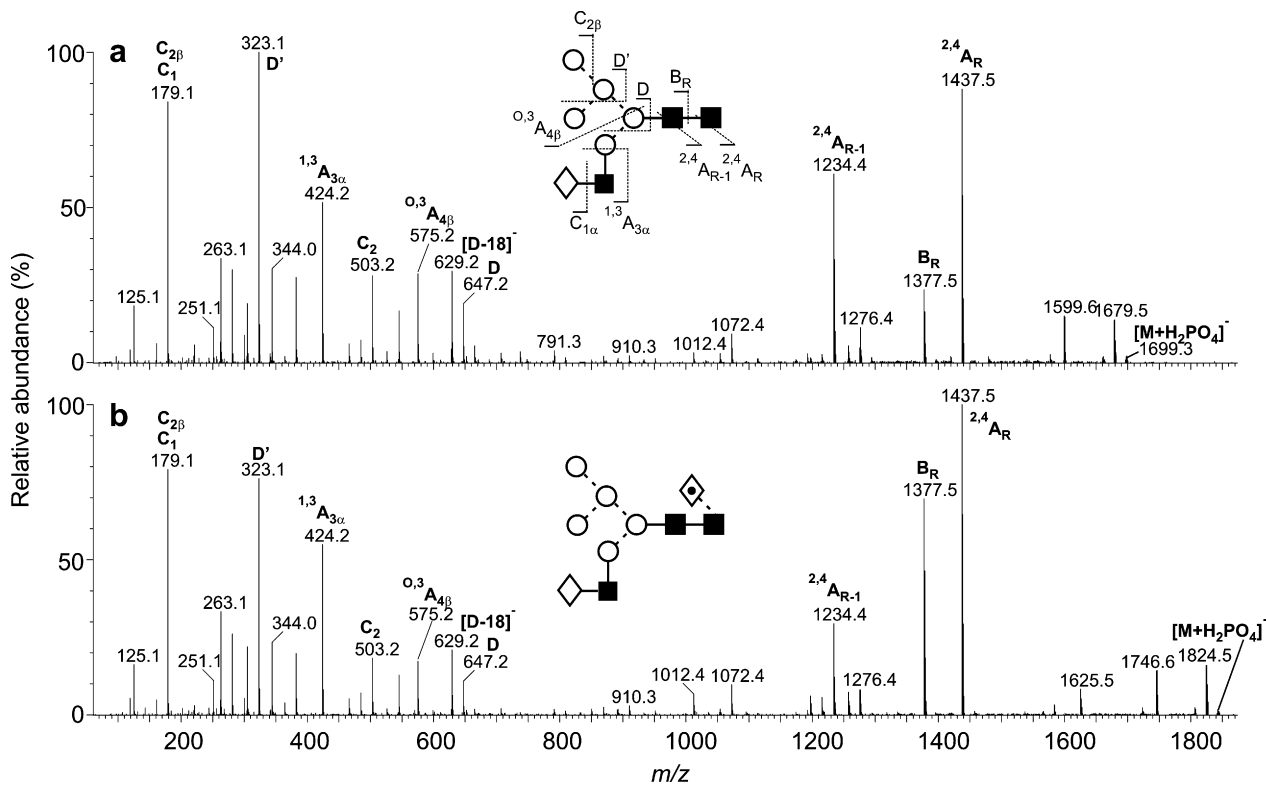


Fig. 2. Negative ion ESI-CID spectrum of classical hybrid-type *N*-linked glycans from sMIg expressed in HEK 293T cells in the presence of swainsonine. Symbols as Fig. 1. Ion nomenclature follows the method of Domon and Costello [28].

between 20% and 30% of the corresponding Man₅-based hybrid-type glycan (data not shown). Fig. 2 shows the negative

ion nano-electrospray MS/MS spectra of classical hybrid *N*-linked oligosaccharides (phosphate adducts) containing five

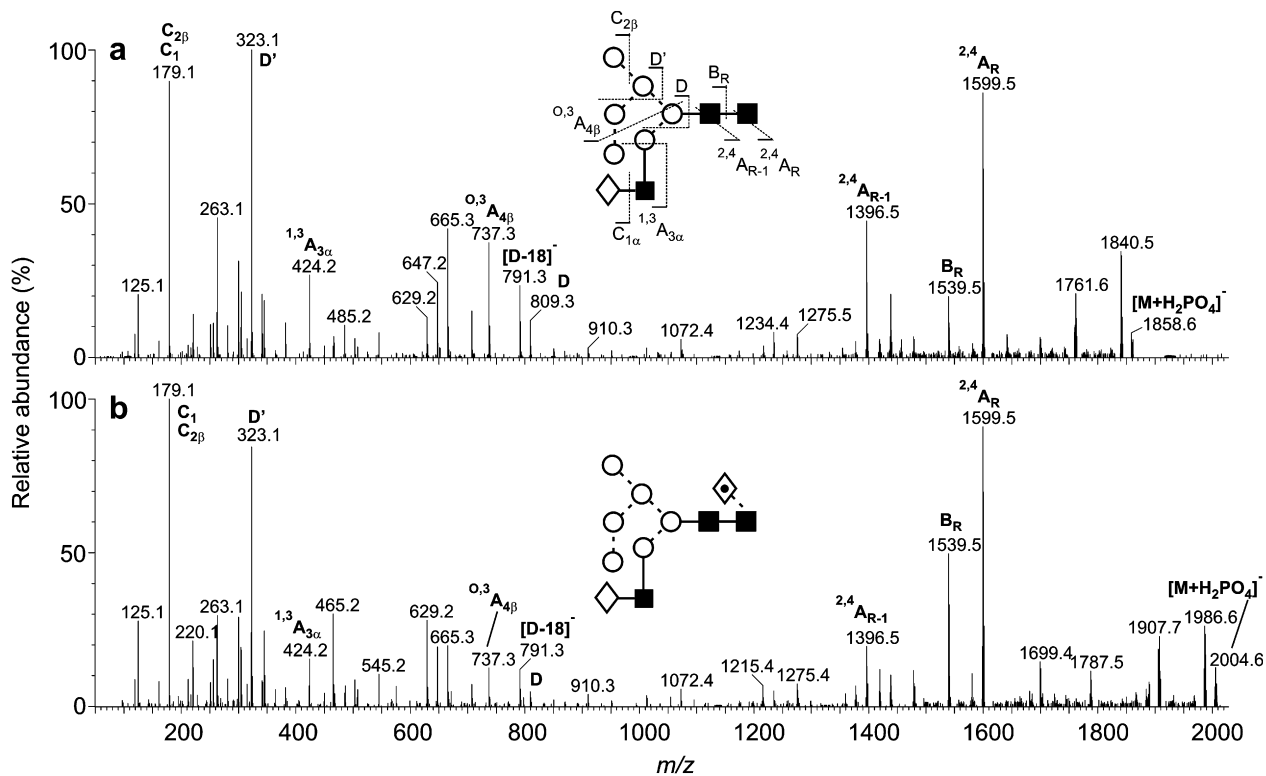


Fig. 3. Negative ion ESI-CID spectrum of non-canonical hybrid-type *N*-linked glycans from sMIg expressed in HEK 293T cells in the presence of swainsonine. Symbols as Fig. 1. Ion nomenclature follows the method of Domon and Costello [28].

mannose residues with and without a fucose residues (Fig. 2a and b, respectively). Similarly, Fig. 3 depicts the MS/MS spectra of the corresponding hybrid-type glycans containing six mannose residues (Fig. 3a and b). The fucose residues in compounds 2 and 4 were located at the 6-position of the reducing-terminal GlcNAc residue as determined by the mass of the $^{2,4}A_R$, B_R and $^{2,4}A_{R-1}$ ions [27], following the nomenclature of Domon and Costello [28], which appeared at the same mass in the spectra of fucosylated and non-fucosylated compounds following loss of the fucose. The ion at m/z 424 is a cross-ring fragment containing hexose–HexNAc and two extra carbon atoms with their substituents from the attached mannose ($^{1,3}A_{3\alpha}$). C_2 ion at m/z 503 (Man_3) and the $C_{3\alpha}$ ion at m/z 665 (Man_4) in the spectra of the compounds 1 and 2 (Fig. 2a and b) and compounds 3 and 4 (Fig. 3a and b), respectively, confirm the hybrid nature of these compounds and the D, $[D-18]^-$ and $^{0,3}A_{R-2}$ ions (m/z 647, 629 and 575, compounds 1 and 2; m/z 809, 791 and 737, compounds 3 and 4) confirm that the mannose residues are in the 6-antenna [23].

3.2. Man_6 -based hybrid-type glycans contain arm-specific mannose

The sixth mannose in compounds 3 and 4 is located on the 3-branch of the 6-antenna based on the following evidence. Because the substitution pattern around the branching mannose of the 6-antenna is the same as that of the core mannose, an ion corresponding to the D ion (containing the core mannose and 6-antenna, Fig. 1) would be expected. This ion, termed the D' ion can be seen at m/z 323 (containing two mannose residues) in the spectra of compound 1 (Fig. 2a) and $Man_5GlcNAc_2$ (compound 5; Fig. 4a). The extra mannose on the 6-branch of the 6-antenna (termed the D3 mannose) in the spectrum of the reference sample of D1,D3- $Man_8GlcNAc_2$ (compound 6; Fig. 4b) shifts this ion to m/z 485. If this mannose were substituted on the 3-branch, no shift would be observed, consistent with the spectrum of $Man_9GlcNAc_2$ (data not shown; [29]) and $Glc_1Man_9GlcNAc_2$ (compound 7; Fig. 4c) where the additional mannose does not cause a peak shift. Although it could be argued that an ion of composition of

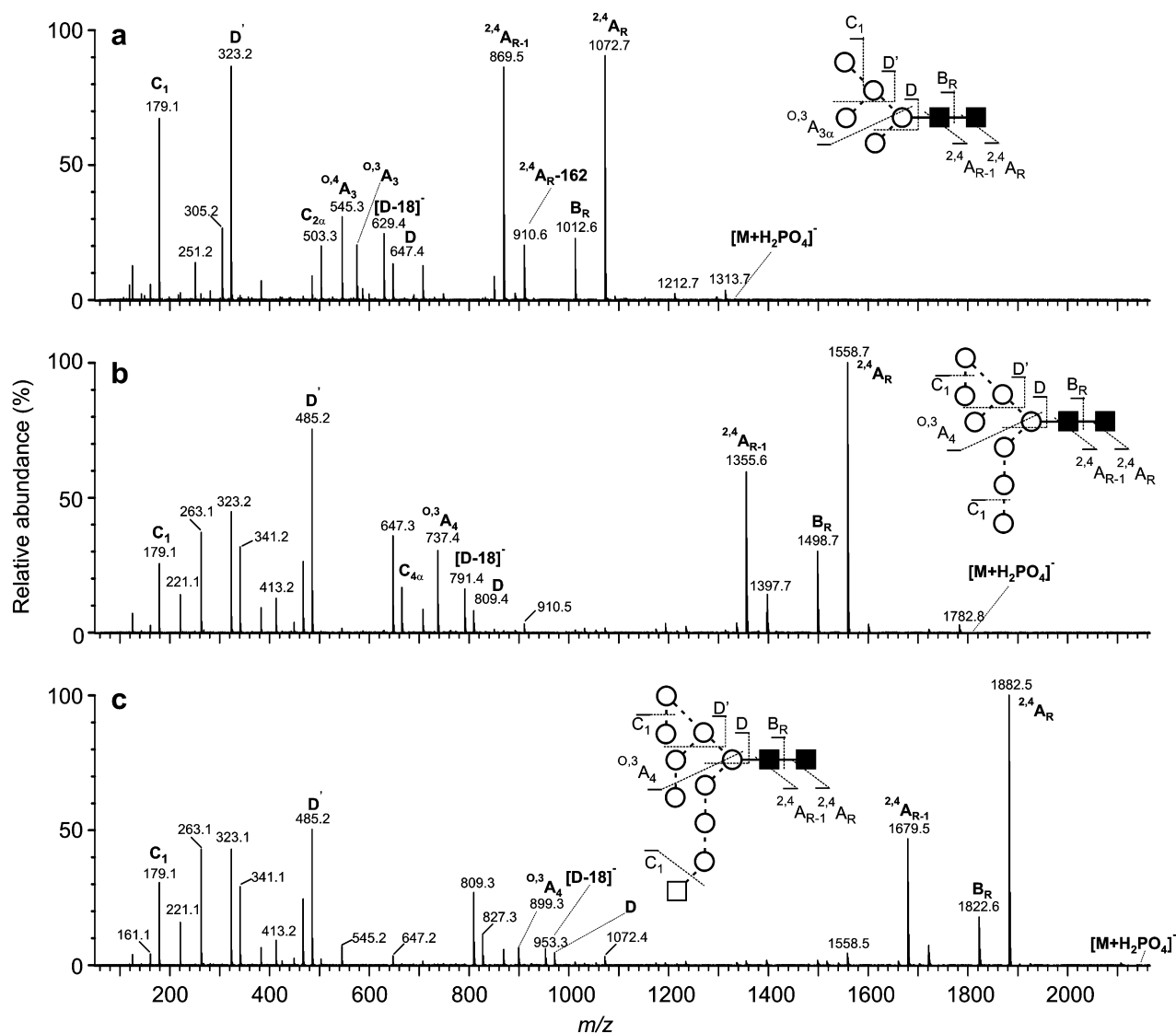


Fig. 4. Negative ion ESI-CID spectra of oligomannose-type *N*-linked glycans: (a) $Man_5GlcNAc_2$; (b) D1,D3- $Man_8GlcNAc_2$; and (c) $Glc_1Man_9GlcNAc_2$. Symbols as Fig. 1. Ion nomenclature follows the method of Domon and Costello [28].

Man₃ (m/z 485) could originate from the 3-antenna in the spectrum of D1,D3-Man₈GlcNAc₂ (compound 6; Fig. 4b) this can be ruled out because the presence of an additional glucose residue in this antenna in Glc₁Man₉GlcNAc₂ (compound 7) does not produce a shift to m/z 647 (Fig. 4c). In the spectra of the hybrid glycans shown in Fig. 3a and b, there is no significant D' ion at m/z 485, but there is a significant ion at m/z 323 (as in the spectra of the Man₅-containing compounds, 1, 2, and 5). Thus the sixth mannose must be located on the 3-branch of the 6-antenna (D2).

4. Discussion

Classical hybrid-type glycans are formed by the action of GnT I on Man₅GlcNAc₂ glycans. The discovery of specific Man₆-based hybrids, demonstrates that lack of processing of the D2 arm is tolerated for GnT I catalysis. Importantly, this activity is supported by *in vitro* studies of recombinant GnT I by Fujiyama and others [30]. Additional enzymological details of the *N*-linked glycosylation pathway are revealed by the discovery of the biosynthesis of fucosylated Man₆-based hybrid-type structures. This further defines the structural basis of the inhibitory effect of 6-antennae α 1-2 linked mannose residues on core α 1-6 fucosylation [14]. Although Man₉GlcNAc₂ is not core fucosylated, core α 1-6 fucosyltransferase can fucosylate Glc₁Man₇GlcNAc₂ and Man₅GlcNAc₂ [14]. Thus, in contrast to the D1-arm, either or both of the D2 or D3 α 1-2 mannose residues suppress core fucosylation [14]. The biosynthesis of core fucosylated D2-Man₆-based hybrids suggests that the hydrolysis of only the D3 mannose is necessary for core α 1-6 fucosylation in mammals. The nuclear magnetic resonance (NMR) [31,32] and crystal structure [33] of Man₉GlcNAc₂ demonstrated that the D3 mannose is located on the same side of the molecule as the C6 position of the reducing terminal GlcNAc and may prevent substrate recognition by α 1-6 fucosyltransferase.

The absence of core α 1-6 fucosylation can significantly enhance ADCC effector function of antibodies [19]. Consequently, cell lines are being developed for the expression of antibodies that are devoid of fucosylation [18]. The potent inhibition of core fucosylation in mammalian cell lines by D3-mannose suggests a further route to the modulation of antibody effector functions. For example, the expression of antibodies in the presence of the α -mannosidase inhibitor, kifunensine [34], would result in oligomannose-type glycans with intact D3-mannose completely devoid of core fucosylation [14]. Interestingly, the oligomannose-type glycans of the Fc would also influence other aspects of the antibody function such as increasing the clearance rate from serum [35].

Although the exact biosynthetic origin of Man₆-based hybrids is unknown, the highest activity for the hydrolysis of the D2 mannose amongst the class I α -mannosidases is exhibited by ER α -mannosidase I, which results in D1,D3-Man₈GlcNAc₂ isomers entering the Golgi apparatus [3]. In contrast, the remaining class I α -mannosidases, Golgi α -mannosidases IA, IB and IC, exhibit the lowest activity to the D2 mannose which is the last residue to be cleaved when Man₉GlcNAc₂ is digested *in vitro* [36,37]. Indeed, comparison of all the class I processing α -mannosidases reveals that in the absence of ER α -mannosidase I cleavage, D2-Man₆GlcNAc₂ is the most abundant Man₆GlcNAc₂ isomer [37]. This suggests that the Man₆-based

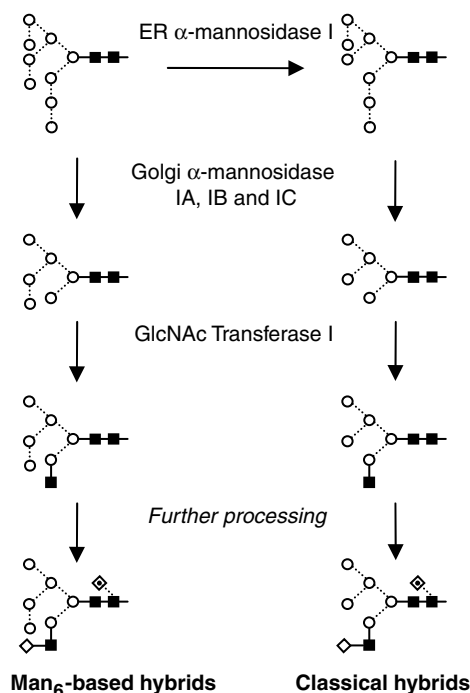


Fig. 5. Proposed pathway for the formation of classic and non-canonical hybrid-type glycans. Symbols as Fig. 1.

hybrids are formed by incomplete digestion by, predominantly, ER α -mannosidase I (Fig. 5).

The induction of novel structures, such as biantennary Lewis X hybrid-type glycans, induced by the putative anti-cancer agent, swainsonine, led Chui and others to propose that they may contribute to the lowering of the threshold for lymphocyte activation and autoimmunity caused by the disruption of Golgi α -mannosidase II activity [17]. Here we expand the repertoire of known swainsonine-induced ‘non-self’ structures to include Man₆-based hybrids.

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