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Host-cell-specific glycosylation of HIV-2 envelope glycoprotein

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Neutral complex-type N-glycans of the envelope glycoprotein 120 of HIV-2, propagated in different host cells, display cell-type specific variations. In order to identify typical structural elements, glycans were analysed by gel filtration, by enzymic sequencing and, in part, by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The characteristic substituents of di- tri- and tetraantennary carbohydrate units thus observed include *N*-acetyllactosamine repeats, bisecting *N*-acetylglucosamine and fucose linked to the chitobiose core as well as to *N*-acetyllactosamine antennae. Each glycoprotein preparation displayed a characteristic set of glycoforms.

***Keywords*: glycoprotein, glycosylation, gp120, HIV, MALDI-TOF-MS**

***Abbreviations*: endo H, endo-β-*N*-acetylglucosaminidase H; E-PHA, *Phaseolus vulgaris* agglutinin E_4; GlcNAcOH, *N*-acetyl-glucosaminitol; gp120/HUT78(MOLT4/Mϕ/PBL/U937), external envelope glycoprotein 120 of HIV-2, strain D194, propagated in HUT78 (MOLT4, Mϕ, PBL, U937) cells; gu, glucose units; HPAEC, high-pH anion-exchange chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Mϕ, human monocytes/macrophages; PBL, human peripheral blood lymphocytes; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F**

Introduction

Membrane glycoproteins of enveloped viruses play a key role in infection by mediating receptor recognition, binding of the virion and subsequent fusion of viral and cellular membranes. They also constitute the major target of the host's immune response. Carbohydrate side chains can influence the maintenance of the biologically active conformation, they can protect the polypeptide chain from proteolytic attack and they can influence antigenicity and immunogenicity of viral glycoproteins. Heterogeneous glycosylation pattern of viral glycoproteins might, therefore, be an important parameter determining the chance of success during infection.

In a previous study [1] we have demonstrated that complex-type glycans of HIV-2 envelope glycoprotein 120 (gp120) were the primary objectives for cell-type specific variations in glycosylation – as has been demonstrated for other glycoproteins, as well (see, for example, [2–6]). In these experiments, HIV-2 was propagated in different host cells, *ie* T-cell lines HUT78 and MOLT4, monocytic U937 cells, human peripheral blood lymphocytes (PBL) or monocytes/macrophages (Mϕ), and metabolically labelled with [^{3}H]glucosamine. Viral envelope glycoproteins isolated from the supernatants were proteolytically digested. Glycans were released by endo-β-*N*-acetylglucosaminidase H (endo H) and by peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F (PNGase F) and analysed by chromatographic profiling. In order to investigate the cell-type specific variations observed at a structural level in more detail and to define possible characteristic substituents, we have now studied the neutral complex-type glycans by enzymic sequencing and, in part, by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Materials and methods

Preparation of complex-type oligosaccharides

Isolation of viral external glycoproteins, metabolically labeled with [6-^{3}H]glucosamine, from the supernatants of infected cells (HUT78, MOLT4, U937, PBL, Mϕ), release of corresponding glycans by PNGase F (after removal of endo-H-sensitive species), reduction of oligosaccharides and chromatographic profiling of charged and neutral complex-type oligosaccharide alditols by anion-exchange and high-pH anion-exchange chromatography (HPAEC) are described in detail previously [1]. Separation of oligosaccharides

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by HPLC using a column of LiChrosorb-NH_2 (Merck) was carried out as described elsewhere [4].

Oligosaccharide sequencing

After enzymatic desialylation with neuraminidase from *Vibrio cholerae* [1], neutral glycans were sequentially digested with endo-β-galactosidase from *Bacteroides fragilis*, α-galactosidase from green coffee beans, β-galactosidase and β-*N*-acetylglucosaminidase from *Diplococcus pneumoniae* (all from Boehringer, Germany), β-galactosidase, β-*N*-acetylhexosaminidase and α-mannosidase from jack beans, β-mannosidase from *Helix pomatia* (all from Sigma, Germany) and α-fucosidase from beef kidney (Boehringer) as detailed in [7]. Starting material as well as reaction products were chromatographed on a calibrated Bio-Gel P-4 column ($-$ 400 mesh; 16×600 mm) at 55 °C using 0.02% aqueous sodium azide as eluant [8, 9]. Fractions (0.4 ml) were collected at 0.2 ml min^{-1} and monitored for radioactivity.

Affinity chromatography

A column (5×20 mm) of *Phaseolus vulgaris* agglutinin E_4 (E-PHA) coupled to agarose (Sigma) was equilibrated with 6.7 mM potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl at room temperature. Oligosaccharide samples ($\sim$1000 cpm) were dissolved in 50 µl of buffer, applied to the column and eluted with the same buffer at a flow rate of 0.05 ml min^{-1}. Fractions ($\sim$340 µl) were collected and monitored for radioactivity.

MALDI-TOF-MS

The mass spectrometer used was a VISION 2000 (Finnigan MAT), operating in the positive-ion reflectron mode. Ions generated by a pulsed ultraviolet laser beam (nitrogen laser, $\lambda = 337$ nm) were accelerated at a potential of 5 kV. The laser power density was about 10^6 W cm^{-2}. The matrix used was 2,5-dihydroxybenzoic acid, fucose, 2-hydroxy-5-methoxybenzoic acid (20 : 20 : 1, by mass) dissolved in ethanol: 0.1% aqueous trifluoroacetic acid (1 : 10, by vol) [6]. Prior to analysis, 5–10 µl of the oligosaccharide solution were microdialysed for 20 min on 'V' series membranes (25 nm pore, Millipore, Germany) 'floating' in deionized water [10], applied to the stainless steel slide in 2 µl-portions and dried in vacuum each time to get a small spot. For enzymic digestions, α-fucosidase, endo-β-galactosidase and β-galactosidase from *D. pneumoniae* (see above) were dialysed overnight against 25 mM ammonium acetate buffer, pH 4.5, 5.8, and 6.0, respectively, and concentrated [11]. Portions (1 µl) of dialysed enzymes (0.16 nkat α-fucosidase, 0.07 nkat endo-β-galactosidase or 0.07 nkat β-galactosidase) were added to the dried oligosaccharide spot on the stainless steel slide and incubated overnight at 37 °C in a humid chamber. Samples were dried again in vacuum, redissolved in 1 µl of matrix solution (by careful mixing with a micropipette) and dried in a cold air stream. Mass spectra were obtained by averaging 20–75 single spectra. External mass calibration was provided by $[M + Na]^+$ ions of isomaltosyl oligosaccharides ($n = 5$–20).

Results and discussion

The asialo-oligosaccharide alditols of the various HIV-2 gp120 preparations, obtained after PNGase F treatment, reduction and desialylation were chromatographically characterized by HPAEC [1], HPLC using an aminophase column and gel filtration (see below) in conjunction with authentic oligosaccharide standards. Due to only trace amounts of material being available, subsequent analyses were mostly performed with the whole mixture of glycans rather than with individual oligosaccharide species. Only in the case of gp120/MOLT4 and gp120/HUT78 neutral species were preparatively separated by HPAEC and also individually characterized. Enzymic sequencing was carried out by sequential degradation with α-galactosidase, endo-β-galactosidase, β-galactosidases, β-*N*-acetylhexosaminidases, α-mannosidase, β-mannosidase and α-fucosidase. Reaction products were analysed by Bio-Gel P-4 chromatography (see Figure 1) and hydrodynamic volumes were defined as glucose units (gu) by co-chromatography with glucose oligomers [8, 9]. Putative glycan structures deduced are presented in Table 1. Treatment with α-galactosidase did not influence the elution profile of any sample and is, therefore, omitted from the Table. In the case of gp120/Mϕ, glycans were additionally analysed by MALDI-TOF-MS before and after enzyme treatment (see Figure 2).

The neutral complex-type glycans of gp120/PBL displayed a mixture of di-, tri- and tetraantennary species, part of which ($\sim$15%) are assumed to carry additional *N*-acetyllactosamine repeat(s) due to their sensitivity towards endo-β-galactosidase (see Figure 1 and Table 1). Digestion with β-galactosidase from *D. pneumoniae* confirmed β1-4-linkages for all galactose residues. The elution positions after digestion with β-*N*-acetylglucosaminidase from *D. pneumoniae*, which solely hydrolyses GlcNAcβ1-2Man linkages if the corresponding Man residue is not simultaneously substituted by β1-6 linked GlcNAc [12], revealed the presence of tetraantennary, 2,6- and 2,4-branched isomers of triantennary and diantennary structures. The fucosylated core fragment $Man_3GlcNAc(Fuc)GlcNAcOH$ produced by the enzyme from jack beans (8.2 gu) could be further degraded with α-mannosidase ($-$ 2 Man), β-mannosidase ($-$ 1 Man) and α-fucosidase ($-$ 1 Fuc) (see Table 1).

In the case of gp120/Mϕ, the oligosaccharides chromatographed in gel filtration as multiple peaks (Figure 1) reflecting hydrodynamic volumes mainly from about 21.5 up to more than 26 gu, which are clearly larger than that of a tetraantennary oligosaccharide standard (19.0 gu). Digestion with endo-β-galactosidase resulted in a complete degradation of these oligosaccharides yielding

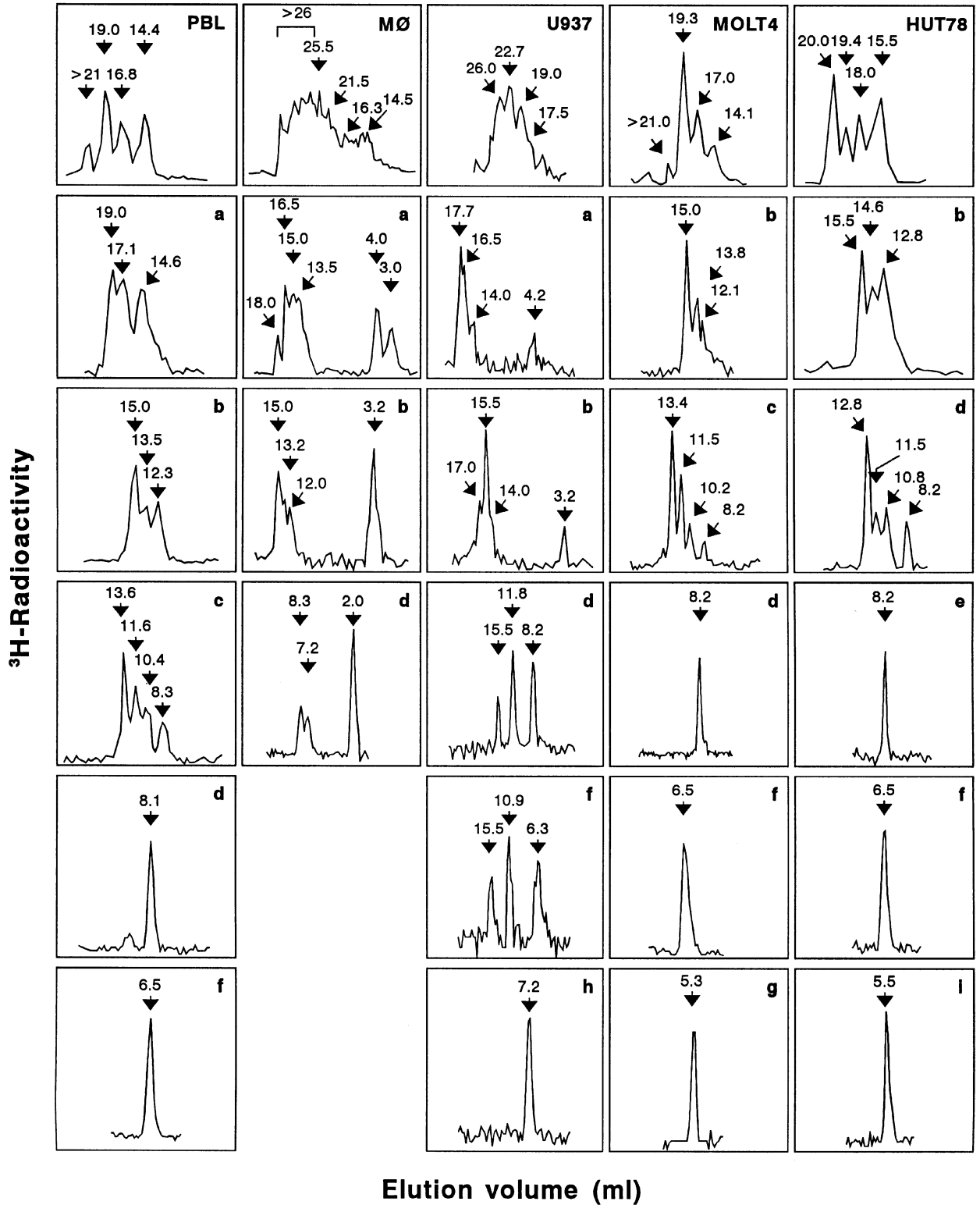
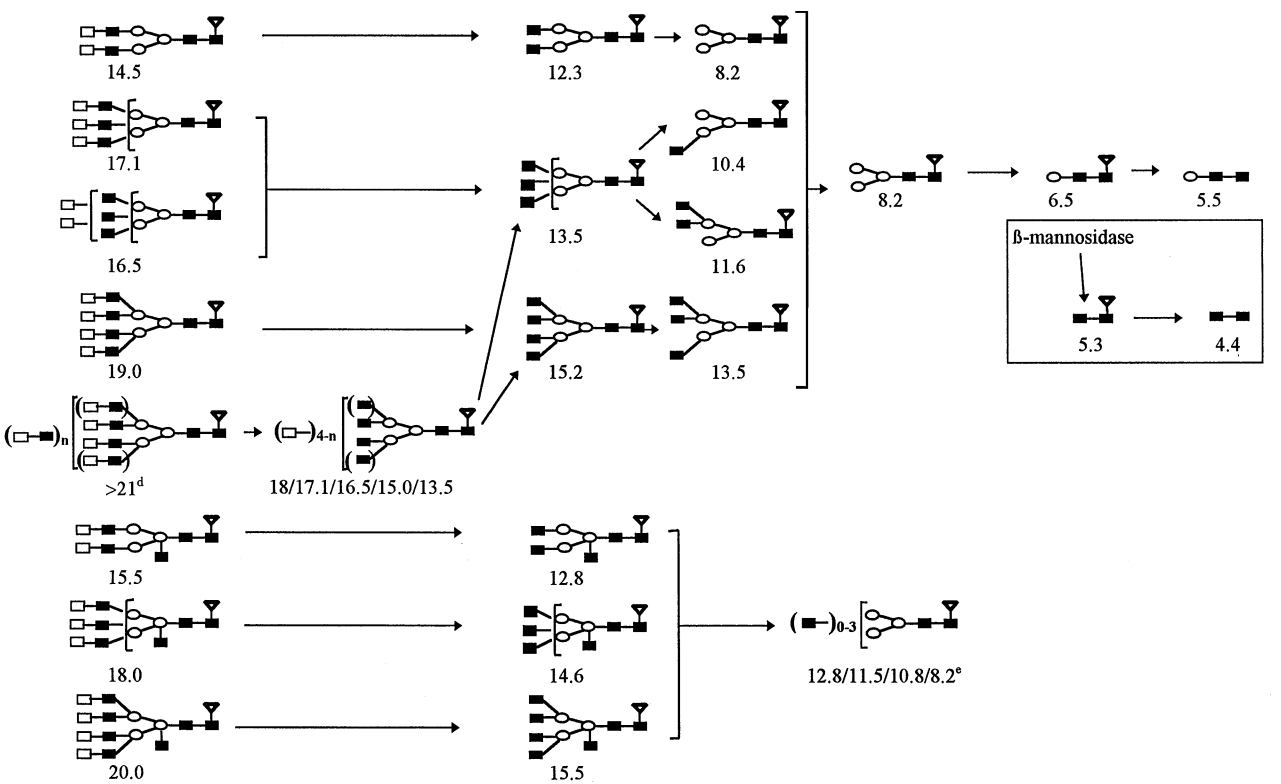


Figure 1. Enzymic sequencing of radiolabelled neutral complex-type glycans from gp120/PBL, gp120/Mϕ, gp120/U937, gp120/MOLT4 and gp120/HUT78. Oligosaccharide alditols were successively digested with endo-β-galactosidase (a), β-galactosidase (b), β-*N*-acetylglucosaminidase from *D. pneumoniae* (c), β-*N*-acetylhexosaminidase from jack beans under standard conditions (d) or with 10-fold amount (e) α-mannosidase (f), β-mannosidase (g), a mixture of β-galactosidase, β-*N*-acetylhexosaminidase and α-fucosidase without α-mannosidase (h) and α-fucosidase without prior digestion with β-mannosidase (i). Aliquots of starting material and reaction products were analysed by gel filtration using a calibrated Bio-Gel P-4 column. Arrows indicate the respective elution volumes as numbers of glucose units [8, 9]. PBL, glycans of gp120/PBL, etc..

Table 1. Schematic illustration of putative complex-type glycan structures of desialylated HIV-2 gp120. Neutral oligosaccharide alditols were digested with the enzymes indicated, and the products were analysed by gel filtration using a Bio-Gel P-4 column. The structures are deduced from the glucose units thus determined [8].

Native structures[a]	*Sequential digestion with*	*Occurrence*				
	endo-β-galactosidase → β-galactosidase[b] → N-*acetylhexosaminidase → α-mannosidase → α-fucosidase* D. pneum → *jack beans*	*PBL*	*Mϕ*	*U937*	*MOLT4*	*HUT78*
		+	(+)[c]		+	
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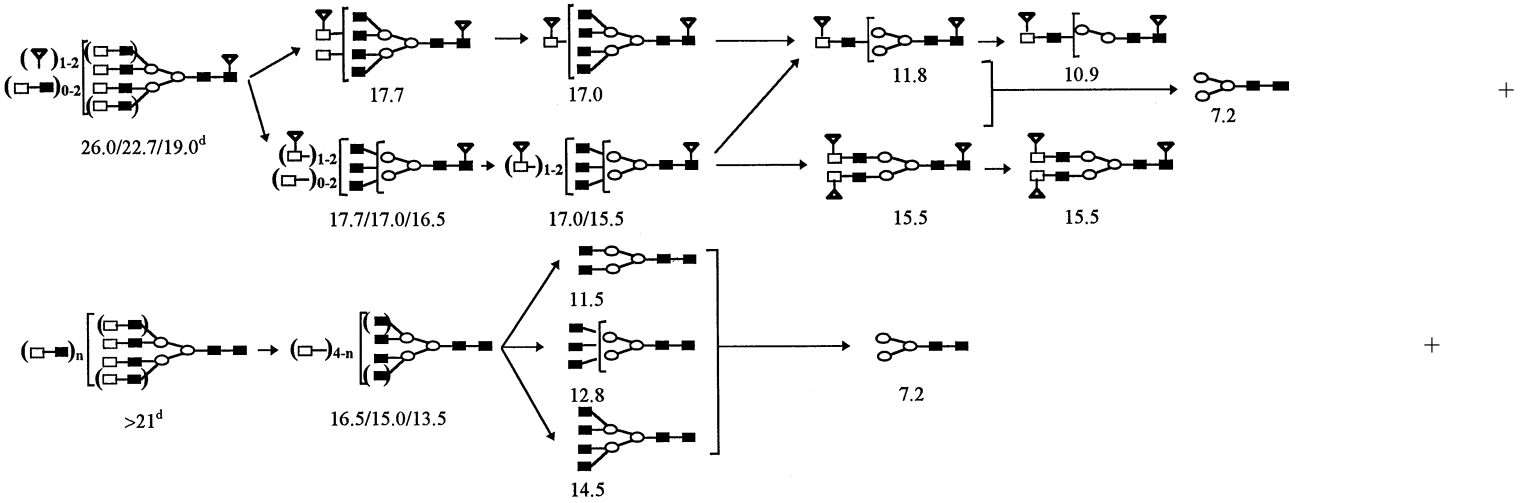

[a]Symbols for monosaccharides: □ = Gal, ■ = GlcNAc, ○ = Man, ▽ = Fuc; in all oligosaccharides the innermost GlcNAc is reduced to GlcNAcOH.
[b]Gp120/PBL, gp120/U937 and gp120/MOLT4 were digested with *β*-galactosidase from *D. pneumoniae* and jack beans yielding the same fragments, respectively.
[c]Minor components.
[d]Due to the enzymatic analysis of the whole mixture of glycans the allocation of substituents to distinct structures is not possible.
[e]Digestion with a 10-fold higher amount of enzyme resulted in the formation of the 8.2 gu fragment only.

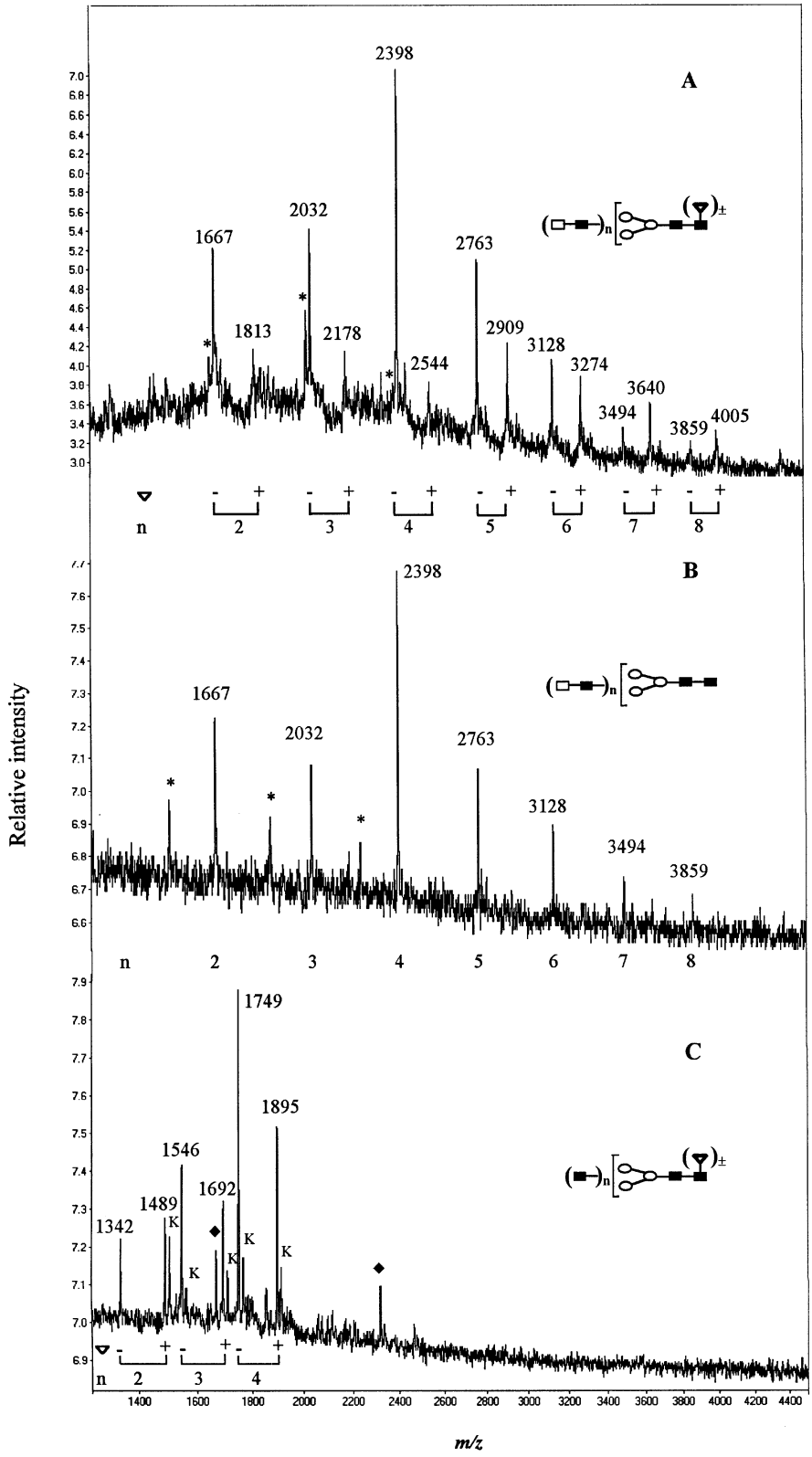

Figure 2. MALDI-TOF-MS spectra of neutral complex-type glycans from gp120/Mϕ. Oligosaccharides were analysed before enzymic treatment (A), after digestion with *a*-fucosidase (B), and after digestion with endo-β-galactosidase and β-galactosidase from *D. pneumoniae* (C). Mass values given are average masses rounded to the next integer. Besides the pseudomolecular ions $[M + Na]^+$ in some cases the corresponding potassium adducts $[M + K]^+$ (peaks marked by 'K' in C) were registered. Peaks labelled by asterisks (*) in A and B indicate incomplete oligosaccharide species. Peaks marked by diamond (◆) in C reflect incomplete degradation products.

a mixture of components eluting around 15.0 gu in addition to fragments which eluted at 3.0 or 4.0 gu representing, most likely, GlcNAcGal disaccharides and GalGlcNAcGal trisaccharides released by the enzyme. The question, however, whether the GlcNAcGal disaccharide units originated from oligo-*N*-acetyllactosamine or from incomplete di-*N*-acetyllactosamine antennae remained open. Subsequent treatment of the whole mixture with *β*-galactosidase reduced the hydrodynamic volumes of the fragments to the typical values for agalacto tetra-, tri- and diantennary glycans. After digestion with *β*-*N*-acetylhexosaminidase from jack beans two products were formed eluting at positions equivalent to the trimannosyl core structures with and without a fucose residue (8.3 and 7.2 gu, respectively). These results indicate that the majority of gp120/M*ϕ* complex-type glycans comprise tri- and tetraantennary species with or without fucose bound to the trimannosyl core structure, which are additionally substituted at some antennae by (poly)-*N*-acetyllactosamine repeats (see Table 1). This conclusion could be confirmed by MALDI-TOF-MS analysis. The positive ion spectrum (Figure 2A) showed a series of peaks in the range of *m*/*z* 1667 up to *m*/*z* 4005. These signals can be mainly grouped into seven pairs of peaks with an average peak interval of *m*/*z* 365, *ie* the increment of one *N*-acetyllactosamine unit. Within each pair, the difference was *m*/*z* 146, *ie* the mass of fucose. The pseudomolecular ions found correspond to the sodium adducts of $(GalGlcNAc)_nMan_3GlcNAc(Fuc)_{\pm}GlcNAcOH$ where n ranges from 2 to 8 and Fuc may be present (+) or not (−). The maximum signal at *m*/*z* 2398, for instance, corresponds to $(GalGlcNAc)_4Man_3GlcNAcGlcNAcOH$, *ie* an unfucosylated glycan with 4 *N*-acetyllactosamine units. Signals at *m*/*z* 1651, 2016 and 2381 (marked by asterisks in Figure 2A) reflect minor components and are consistent with di-, tri- and tetraantennary species carrying one incomplete *N*-acetyllactosamine unit. Starting from extremely low amounts of residual glycans, these assignments could be proven by MALDI-TOF-MS after enzymic treatments. Digestion with α-fucosidase resulted in a clear spectral simplification (Figure 2B) since the peak-doublets had disappeared and only the respective signals of the unfucosylated species $(GalGlcNAc)_{2-8}Man_3GlcNAcGlcNAcOH$ could be detected. Signals at *m*/*z* 1504, 1870 and 2235 (again marked by asterisks) result from the incomplete glycans mentioned above. In a second experiment, glycans were digested with a mixture of endo-*β*-galactosidase and *β*-galactosidase from *D. pneumoniae* (Figure 2C) which caused a drastic reduction in the products' mass. The three pairs of signals (1342/1489; 1546/1692; 1749/1895) differing by *m*/*z* 203, *ie* GlcNAc, correspond to agalacto di-, tri- and tetraantennary species without or with fucose. In some cases, besides the sodium adducts, the respective potassium adducts could be also detected (marked by 'K'). Taken together, MALDI-TOF-MS corroborates the presence of di-, tri- and tetraantennary glycans with 2–8 *N*-acetyllactosamine units, partially substituted by fucose at the trimannosyl core. Species bearing incomplete *N*-acetyllactosamine antennae (lacking 1 Gal) constitute minor components. Taking into account the occurrence of tri- and disaccharide fragments after endo-*β*-galactosidase treatment in about equal amounts (Figure 1) one might assume the presence of species with oligo-*N*-acetyllactosamine units at some of their antennae.

Gel filtration of neutral complex-type oligosaccharides of gp120/U937 revealed the presence of glycan species, a significant proportion of which, carried again *N*-acetyllactosamine repeats as could be deduced from their sensitivity towards endo-*β*-galactosidase and the concomitant appearance of a fragment eluting at about 4.2 gu (Figure 1). In contrast to the respective profile of gp120/M*ϕ*, however, no GlcNAcGal disaccharide was detected. About half of the glycans seemed to be additionally substituted by fucose residues at one (36%) or two (13%) *N*-acetyllactosamine antenna(e). This was concluded for the following reason: Upon exhaustive digestion with *β*-galactosidases and *β*-*N*-acetylhexosaminidases (*D. pneumoniae* and jack beans) about 50% of the material still comprised fragments eluting at 15.5 or 11.8 gu; subsequent addition of α-mannosidase to the reaction mixture resulted in the release of none, one or two mannose residues. Addition of α-fucosidase to the mixture of *β*-galactosidase and *β*-*N*-acetylhexosaminidase led to the complete degradation of all glycans to a fragment eluting at approx. 7 gu, *ie* the elution position of the authentic unfucosylated trimannosyl core structure. Due to the small amounts of material, additional experiments using fucosidases with different specificities could not be performed and thus the exact linkage position of fucose could not be established. The elution profiles after *β*-galactosidase digestions (1 Gal released at most) seem to indicate blocked galactosyl residues as proposed in Table 1. On the other hand, it cannot be ruled out that a substitution of subterminal GlcNAc by fucose might sterically interfere with *β*-galactosidase action and thus different species may be present.

Glycans obtained from gp120/MOLT4 were also subjected to enzymic sequencing after preparative HPAEC. Data obtained demonstrated the presence of typical di- (7%), two isomers of tri- (8% and 9%) and tetraantennary (75%) glycans all of which carried galactose in *β*1-4 linkage and an α-fucosyl residue at the chitobiose unit. A small portion of triantennary species (not detectable in the chromatograms of the mixture in Figure 1) obviously carried incomplete antennae (16.5 gu). In addition, minor species eluting at > 21 gu in gel filtration appeared to comprise an *N*-acetyllactosamine repeat (cf. Table 1).

Analysis of gp120/HUT78 glycans (both as a mixture as well as individual fractions after preparative HPAEC) revealed that most of them were substituted by an additional *N*-acetylglucosamine residue (bisecting GlcNAc) at the innermost, *β*-linked mannosyl residue. Digestion with

β-galactosidase and standard amounts of β-*N*-acetylhexosaminidase converted only a small proportion of the glycans into the trimannosyl core structure eluting at 8.2 gu (Figure 1). Using 10-fold higher concentrations of the enzyme, however, all species yielded this fragment. The presence of bisecting GlcNAc was confirmed by lectin affinity chromatography on an E-PHA column where glycans eluting at 15.5 or 18.0 gu in gel filtration (see, for example, Figure 3A) were, in part, retarded (Figure 3C), whereas the larger oligosaccharides solely appeared in the void volume of the column. In addition, MALDI-TOF-MS of the 15.5 gu species revealed a signal at *m/z* 2016 consistent with a sugar composition of $Gal_2GlcNAc_3Man_3GlcNAc(Fuc)$-GlcNAcOH, *ie* a fucosylated bisected diantennary glycan or a fucosylated incomplete triantennary structure (Figure 3D). The occurrence of both types of glycans could be verified by HPLC on an aminophase column (Figure 3B). In conjunction with the binding specificity of E-PHA [13], the data obtained indicate the presence of bisected diantennary, two isomers of bisected triantennary and bisected tetraantennary glycans in addition to unsubstituted tetraantennary and incomplete triantennary species. All of them carried fucose linked to the core unit.

In conclusion, the results demonstrate that the complex-type *N*-glycans of the envelope glycoprotein of HIV-2 propagated in different host cells display host-cell-dependent variations with regard to the substitution of the trimannosyl core unit (see Table 2). In all glycoproteins di-, tri- and tetraantennary species were found in varying proportions. All sugar chains were substituted by an α-fucosyl residue at the inner chitobiose except for the oligosaccharides from gp120/Mϕ which were, in part, devoid of this sugar. A criterion that distinguished gp120/HUT78 glycans, for instance, from all the others was the high amount of bisecting GlcNAc residues. A characteristic feature of gp120/U937- and gp120/Mϕ-derived carbohydrates, on the other hand, was their high substitution by *N*-acetyllactosamine repeats. In the case of gp120/Mϕ, glycans comprised at least di-*N*-acetyllactosamine and probably also oligo-*N*-acetyllactosamine units. Gp120/U937 glycans were additionally substituted by one or two fucose residues at their *N*-acetyllactosamine antennae. This finding is in agreement with a recent report [14] demonstrating that U937 cells express, albeit in low amounts only, cell-surface glycoproteins with sialyl Lewis^x determinants in addition to *N*-acetyllactosamine repeats. All glycoproteins carried, in varying proportions, sialic acid substituents linked α2-3 or α2-6 to galactose [1].

The function of HIV envelope glycoprotein glycans has been discussed controversially for a long time. Current thinking directs their most significant effects to the early steps in biosynthesis in being indispensible for the creation but dispensible for the maintenance of the functional conformation of gp120 [15, 16]. Especially the lack of complex-type glycans as, for instance, in gp120 expressed in insect cells or in gp120 isolated from cells treated with trimming inhibitors seems to have no effect on major envelope glycoprotein functions like CD4 binding and infectivity [17–19]. However, other properties of the glycoprotein may be influenced by glycans even after proper folding. For example,

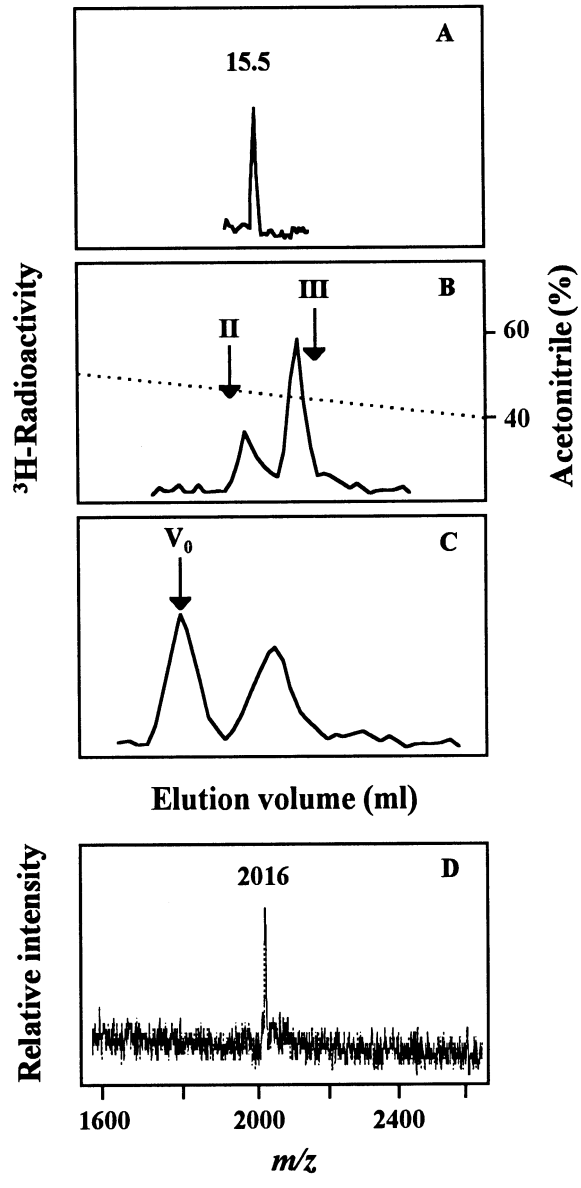

Figure 3. Characterization of an individual glycan fraction from gp120/HUT78 after preparative HPAEC. (A), Analysis of the glycans by gel filtration using a calibrated Bio-Gel P-4 column. The elution volume in glucose units is indicated (compare Figure 1). (B), Subfractionation of the glycans by HPLC on a LiChrosorb-NH_2 column (5 µm, 4.6 × 250 mm) using a gradient of acetonitrile in 15 mM potassium phosphate, pH 5.3. II, III, elution positions of di- and triantennary standard oligosaccharides. (C), Subfractionation of the glycans by affinity chromatography using a column of E-PHA-agarose, V_0, void volume. (D), Analysis of the glycans by MALDI-TOF-MS in the positive-ion reflectron mode. The mass value of the pseudomolecular ion $[M + Na]^+$ is the average mass rounded to the next integer.

Table 2. Characteristic cell-type specific substituents of neutral complex-type glycans from HIV-2 gp120. The presence or absence of the respective structural elements is indicated by + or −, respectively; (+), minor component.

Substituent	*Host cells*				
	PBL	*Mϕ*	*U937*	*MOLT4*	*HUT78*
Inner *α*-Fuc residues	+	+/−	+	+	+
Bisecting GlcNAc	−	−	−	−	+
N-acetyllactosamine repeats	+	+	+	(+)	−
Outer *α*-Fuc residues	−	−	+	−	−
Sialic acid (*α*2-3/6)[a]	+	+	+	+	+

[a]Substitution of complex-type glycans by sialic acid was analysed previously [1].

evidence has been provided that the induction of and the sensitivity towards neutralizing antibodies [20–22] or the host cell tropism of the virus [23] may be modulated by alterations in glycosylation. A certain HIV-1 strain, for instance, passaged through peripheral blood mononuclear cells was not able to infect U937 cells; after propagation of the virus in HUT78 cells, however, U937 cells were effectively infected. Virions lost this property again after backpassage to peripheral blood mononuclear cells. The only difference in gp120 associated with this phenomenon was a (reversible) alteration in size, possibly reflecting changes in glycosylation and/or other posttranslational modifications [23].

In our previous study [1] we demonstrated that the glycosylation pattern of HIV-2 gp120 depends strongly on the host cells used. Differences in the size of gp120 were found to correlate mainly with variations in complex-type *N*-glycans. In the present report, the characteristic substituents imposed by the different host cells were further characterized. One might speculate that substitution of glycan chains by repeating units, bisecting *N*-acetylglucosamine or outer fucose residues may affect their spatial arrangement on the glycoproteins' surface. There is increasing evidence that many functions of the HIV envelope glycoprotein do not reside in one domain of the molecule only, but depend on interactions between several functional domains [24–26]. Hence, structural and spatial changes in the carbohydrate side chains may thus contribute to subtle modulations of functions.

Acknowledgements

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