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Structural characterization of the N-linked oligosaccharides derived from HIVgp120 expressed in lepidopteran cells

Terry D. Butters[1]*, Ben Yudkin[2], Gary S. Jacob[3] and Ian M. Jones[4]

[1]*Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK*
[2]*Genetics, Department of Biochemistry, University of Oxford, OX1 3QU, UK*
[3]*Monsanto Co., Chesterfield, St. Louis, Missouri, USA*
[4]*NERC Institute of Virology, Mansfield Road, Oxford, UK*

The oligosaccharides of recombinant HIV gp120 expressed in lepidopteran Sf9 cells were analysed after hydrazine release by gel permeation and high pH anion exchange chromatography. N-Linked glycans were exclusively of the oligomannose series and no evidence for charged complex or hybrid type glycans was found. However a glycosylation reaction similar to those found in vertebrates was evident. The major glycoform of gp120, that comprised 30% of all the species analysed, was structurally identified by exoglycosidase digestion and found to be a core fucosylated structure, Manα1,6(Manα1,3)Manβ1,4GlcNAc(Fucα1,6)GlcNAc. Further confirmation of the ability of lepidopteran cells to fucosylate N-linked glycans was provided by an *in vitro* analysis of this reaction using authentic oligosaccharide substrates.

***Keywords*: fucosylated glycans, recombinant glycoprotein, insect cells**

Introduction

The majority of glycoproteins analysed from insect tissues, for example cultured mosquito cells [1, 2] and *Drosophila* larvae [3], are of the oligomannosidic type and no compelling evidence for the normal synthesis of complex or hybrid sialylated glycans has been produced. Fucosylation of the innermost GlcNAc residue has been observed in glycoproteins derived from *Drosophila* [3] and in *Apis* (honeybee) [4, 5] and in the latter case are often bifucosylated in α1,3- and α1,6-linkages. The ability to fucosylate in both positions is species dependent within the insect class and has been detected using *in vitro* assays in *Apis* and some lepidopteran cell lines (*Mamestra brassicae*) but is absent in lines derived from *Spodoptera frugiperda* and *Bombyx mori* [6]. The appearance of an oligomannose structure containing core α1,6-linked fucose was noted during expression of viral proteins in lepidopteran Sf9 cells [7, 8]. The use of recombinant baculovirus infected lepidopteran cells for heterologous expression of proteins has extended the debate regarding the glycosylation potential of insect cells, particularly as some experiments have revealed the presence of sialylated, complex type N-linked oligosaccharides [9–13]. However, the acquisition of complex-type glycans does not agree with the majority of reports describing the heterologous expression of recombinant proteins in a variety of insect cell lines. The sensitivity of many structures derived from Sf9 cells to endoglycosidase H reveals a predominant presence of oligomannose (and/or hybrid type) [14–16]. Additional carbohydrate compositional analysis has confirmed the absence of galactose and sialic acid [17–19] but the presence of fucose has been established, linked to the chitobiose core [7, 19–22]. In some of these studies an incomplete sensitivity to endo-H indicated that further processing of the oligomannose glycan had occurred. The generation of small fucosylated N-linked oligosaccharides may explain these data since the chitobiose unit of $Man_3(Fuc)GlcNAc_2$ is resistant to hydrolysis by endo-H [19].

In contrast to the glycosylation profile in mammalian cells [23–26], the expression of HIV envelope glycoproteins in insect cell lines has revealed less diversity of structure. Firstly, the majority of the oligosaccharides are hydrolysed with endo-H, with a subset of structures (not sequenced) that appear resistant [27]. Secondly, a site specific analysis has indicated that all 22 of the potential glycosylation sites in the recombinant protein are exclusively occupied by an oligomannose type series of glycans, Man_5Glc

* To whom correspondence should be addressed. Tel: + 44 1865 275725; Fax: + 44 1865 275216; E-mail: terry@oxglua.glycob.ox.ac.uk.

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NAc_2 to $Man_9GlcNAc_2$ [28]. The present work uses hydrazine release, Bio-Gel P-4 and high pH anion exchange chromatography coupled with exoglycosidase digestion to analyse the type of glycans in gp120 expressed in lepidopteran (Sf9) cells. In addition, we have identified a fucosyltransferase activity responsible for generating mannosylated structures containing core fucose.

Materials and methods

Materials

Recombinant baculovirus infected Sf9 cells expressing soluble gp120 at approximately 1–2 mg/l were propagated as previously described [29]. Radiolabelled N-linked oligosaccharide standards were purchased from Oxford GlycoSystems, Abingdon, or were kindly donated by Dr Phil Williams, Glycobiology Institute. *Aspergillus pheonicis* α1,2-Mannosidase was purified as previously described [30]. α-Fucosidase from *Charonia lampas* was purified according to published methods [31].

Isolation and characterization of gp120 oligosaccharide

Oligosaccharides from gp120 (275 μg protein) were released by hydrazinolysis, re-N-acetylated, labelled with NaB^3H_4 and purified by paper chromatography and preparatively separated by high voltage electrophoresis (HVE). Further separation using BioGel P-4 chromatography and high pH anion exchange chromatography (HPAEC) were performed as previously described [31].

Enzyme assays

Solubilization of cells

Cells were extracted with 2.5% (v/v) Triton X-100 for 30 min at 4 °C and the extract centrifuged at 13 000 × **g** for 10 min. The supernatant was then used as an enzyme source in the following assay.

α-Fucosyltransferase assay

The assay mixture contained either 1 mM N2 (GlcNAcβ1,2Manα1,6[GlcNAcβ1,2Manα1,3]Manβ1,4GlcNAcβ1,4GlcNAc), M3 (Manα1,6[Manα1,3]Manβ1, 4GlcNAcβ1,4GlcNAc) or M5 (Manα1,6[Manα1,3]Manα1, 6[Manα1,3]Manβ1,4GlcNAcβ1,4GlcNAc) with 0.1 M sodium cacodylate buffer, pH 6.8, 200 mM GlcNAc, 10 mM $MnCl_2$, 5 mM AMP, 0.5 nmol GDP-[^{14}C]Fucose (268 mCi/mmol, Amersham) and an aliquot of detergent solubilized cell extract in a total volume of 20 μl. After incubation at 30 °C for Sf9 cells and 37 °C for Molt 4 cells for 1–5 h the reaction was stopped with 20 mM sodium borate and 2 mM EDTA. Reaction products were desalted with 0.5 ml AG1-X8 (Cl^- form) to remove free radiolabelled nucleotide sugar and aliquots taken for the determination of radioactivity.

Results and discussion

Oligosaccharide analysis of gp120

Hydrazine release and borotritiide reduction of the oligosaccharides derived from HIV gp120 were analysed by HVE. Labelled oligosaccharides migrated to a position consistent with a neutral oligosaccharide standard, lactose, indicating the absence of sialylated structures (Figure 1, insert). Separation of the neutral oligosaccharides using BioGel P-4 revealed a series of glycans differing by a single glucose unit from 7.5 to 12.5 glucose units (Figure 1). Further fractionation of the seven glycan pools was obtained by HPAEC. By reference to radiolabelled alditol standards, these seven fractions eluted at positions consistent with an oligomannose-type series of glycans. Most of the glycans derived from pools from the gel filtration column contained a single species when analysed by HPAEC. Fractions I, II, III, IV, and V were preparatively isolated by HPAEC to yield a single chromatographic species (Figure 2a) and then subjected to *A. pheonicis* α1,2-mannosidase digestion. Re-chromatography of the reaction products revealed that all of these fractions except Fraction V

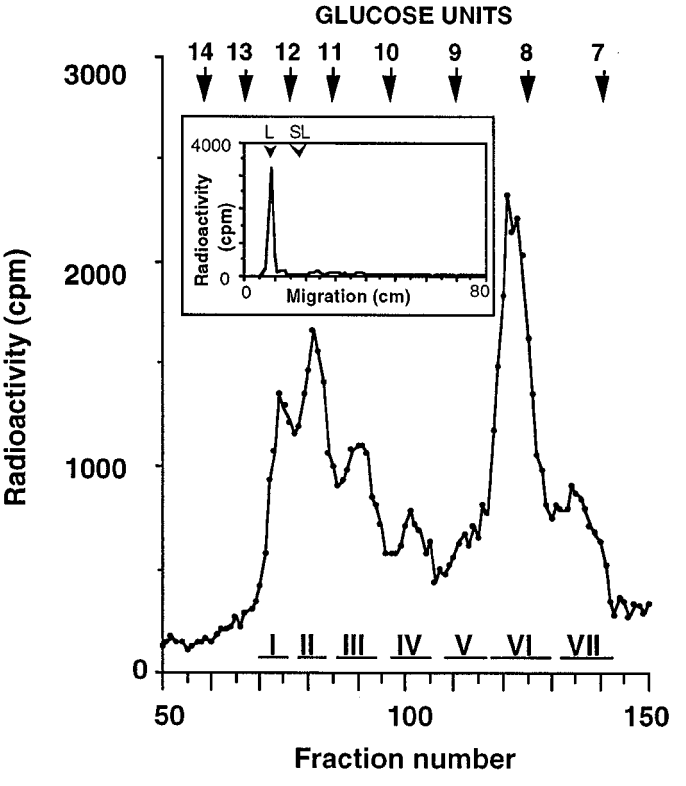


Figure 1. Bio-Gel P-4 Chromatography of gp120 Glycans. Radiolabelled oligosaccharides were applied to the column (2.6 × 95 cm) and aliquots of each fraction collected analysed for radioactivity. The position of an internal standard (dextran) and the pooled fractions I–VII is shown. The insert shows HVE chromatography of hydrazine released borotritiide labelled oligosaccharides. The migration of radiolabelled standards, lactitol (L) and sialyllactitol (SL) is shown.

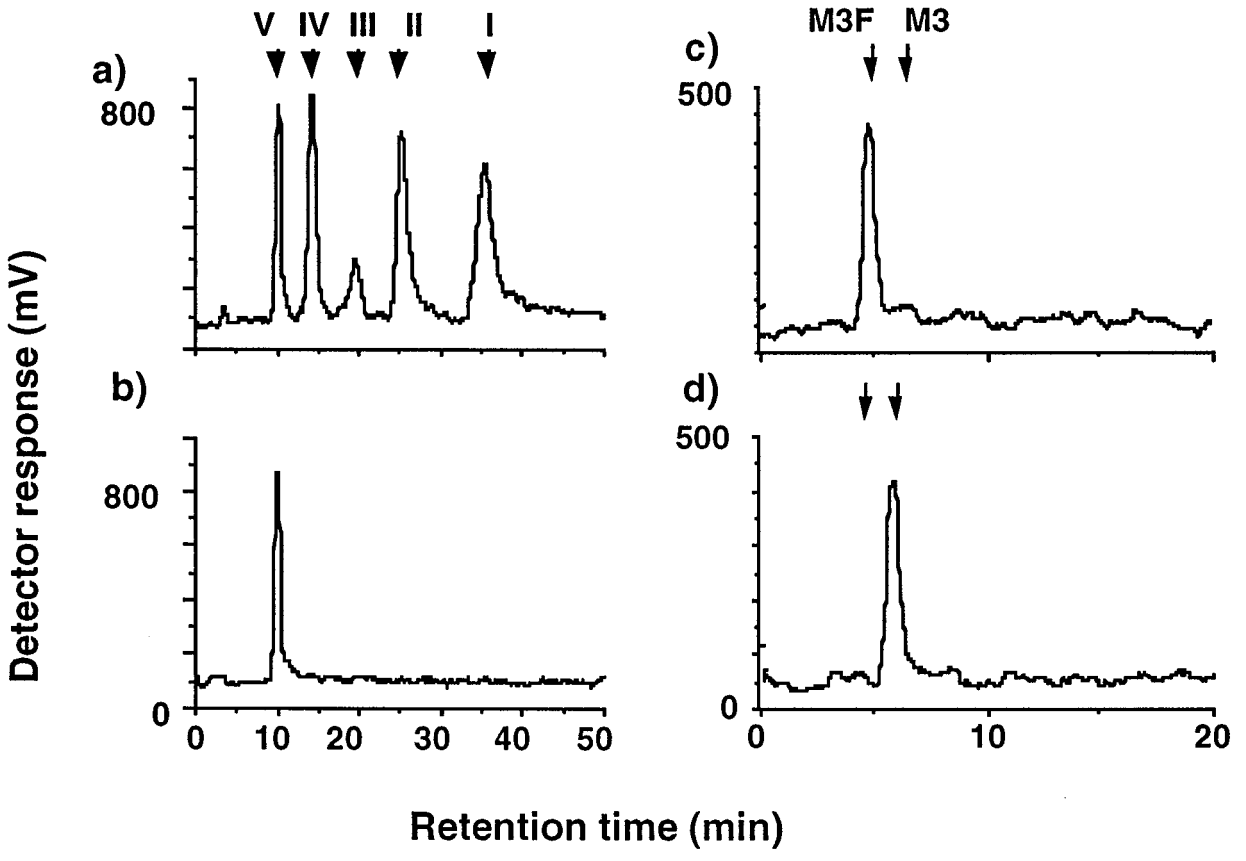
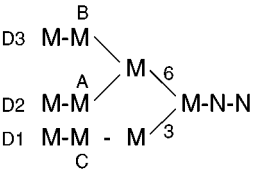
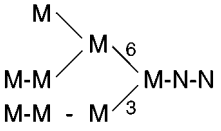
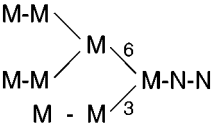
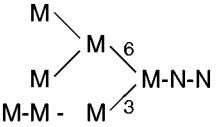
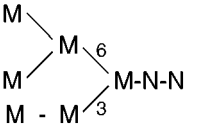
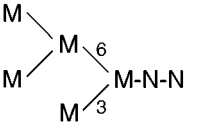
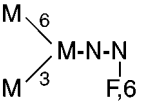
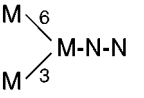


Figure 2. HPAEC separation of Bio-Gel P-4 fractions. Bio-Gel P-4 fractions I, II, III, IV and V (10^5 cpm) before (a) and after (b) incubation with *A. pheonicis* α1,2 specific mannosidase (10 mU/ml) for 16 h at 37 °C, were chromatographed using a Dionex PA-1 column eluted with 200 mM NaOH. Fraction VI before (c) and after (d), incubation with *C. lampas* α-fucosidase (4.5 mU/ml) for 16 h at 37 °C were eluted from the column with 175 mM NaOH. The relative elution positions of standard alditols, M3F ($Man_3(Fuc)GlcNAc_2$-ol) and M3 ($Man_3GlcNAc_2$-ol) are shown. Radioactivity was measured by an on-line detector.

contained labile α1,2-linked-mannose residues and eluted with a retention time identical to authentic $Man_5GlcNAc_2$-ol (Figure 2b). The HPAEC system often provides exceptional resolution of isomeric oligosaccharides and in particular the presence of fucose residues imparts weaker ionization leading to decreased retention times. To determine the presence of fucose, Fraction VI was preparatively isolated by HPAEC and hydrolysed with α1,6-linked fucose specific concentrations of *C. lampas* α-fucosidase [31], and the reaction products re-chromatographed by HPAEC and compared with Fraction VII. Fraction VII was eluted with a retention time identical to an authentic $Man_3GlcNAc_2$-ol glycan isolated from *Drosophila* larvae [3] and Fraction VI eluted with a retention time identical to $Man_3(Fuc)GlcNAc_2$-ol (obtained from the source above) and at least 1 min earlier than $Man_3GlcNAc_2$-ol (Figure 2c). Upon treatment with *C. lampas*, α-fucosidase Fraction VI was eluted 1 min later, in a position identical to the authentic $Man_3GlcNAc_2$-ol glycan (Figure 2d). A summary of the structures and the molar percentages of oligosaccharides derived from gp120 expressed in Sf9 cells is presented in Table 1.

The only other data that provide a structural analysis of gp120 expressed in Sf9 cells [28] used N-glycanase (PNGase F) release of oligosaccharides from tryptic peptides. The authors used both HPAEC and ^{1}H-NMR spectroscopy to characterize an oligomannosidic series of glycans, none of which contained fucose residues. Reasons for this difference are unknown but may be related to differences in expression vectors, level of protein expression, culture conditions, time of baculovirus infection or even clonal differences in the lepidopteran cells. As shown previously [4–8], the presence of fucose does indicate that processing reactions similar to those found in higher eukaryotes are possible even if, as the present data show, no further processing to complex-type sialylated glycans takes place. The differences seen in the type of glycosylation may depend on the conformation of the expressed protein, yet other conformationally dependent properties such as the binding to CD4 appear normal [29]. Infection with baculovirus results in cell death and

Table 1. Summary of the Composition and Structures of Recombinant gp120. The molar composition of each species was calculated from HPAEC separation of Fractions I–VII (see text for details). The nomenclature for mannose branching is shown in the structure for Fraction I

P4 fraction	*Structure*	*Composition (%)*
Fraction I	single Man_9 isomer	11
		
Fraction II	single Man_8 isomer (either)	17
		
Fraction III	majority is Man_7 isomer	12
		
Fraction IV	single Man_6 isomer	8
		
Fraction V	single Man_5 isomer	8
		
Fraction VI	single Man_3 core fucosylated species	30
		
Fraction VII	single Man_3 isomer	13
		

cells are usually harvested just prior to total cell destruction to maximize the yield of expressed protein. These times vary from 48 to 66 h; the later times may correlate with increased complex-type glycan synthetic potential [32].

No evidence for O-linked glycans was found by the absence of species smaller than $Man_3GlcNAc_2$ observed after P-4 chromatography and confirms the results from the previous study by Cumming's group [28].

Fucosyltransferase specificity and N-linked processing in insect cells

Measurement of fucosyltransferase activity in Sf9 cells and comparison with a human T-cell line revealed a similar substrate specificity. Only those oligosaccharide acceptors with terminal GlcNAc residues accommodated transfer. $Man_3GlcNAc_2$ and $Man_5GlcNAc_2$ were not acceptors

for this reaction in either insect or human cell extracts. The transferase activity in lepidopteran cells was 1.7 pmol min^{-1} per mg protein, 500-fold less than in the human cell line (740 pmol min^{-1} per mg protein). To confirm that the *in vitro* activity was in α1,6 linkage, fucosylated products were digested with *C. lampas* α-fucosidase using linkage-specific concentrations. In both cell lines used, all the radiolabelled fucose incorporated was released with α-fucosidase. The substrate specificity of lepidopteran α-fucosyltransferase appears very similar to the human α1,6-fucosyltransferase [33], which depends on the prior action of GlcNAc-transferase I on $Man_5GlcNAc_2$. However, in CHO parental cells, endogenous proteins bearing $Man_3(Fuc)GlcNAc_2$ have been described [34] and indicates that either mannosylated oligosaccharides are acceptors for CHO cell transferase (unlike the human enzyme) or GlcNAc-transferase I has added a GlcNAc residue that is subsequently cleaved. The present data suggest that lepidopteran cells do not fucosylate oligomannose structures *in vitro*, and are therefore dependent on GlcNAc addition to generate a substrate for core fucosyltransferase. Cultured insect cells have levels of GlcNAc-transferase I [35] and β4-GalNAc transferase [36] sufficient to modify oligomannosidic N-linked glycans. Unfortunately, the presence of either specific membrane β-N-acetylglucosaminidases [37, 38] and/or secreted β-hexosaminidases [36] has precluded a precise analysis of N-linked glycans from mature glycoproteins from this source. Further work will be needed to prove that oligosaccharides are differently processed in insect cells via constitutive alternate pathways that include a β-N-acetylglucosaminidase activity. The potential of cultured insect cells to perform glycosylation reactions (core fucosylation, outer arm N-acetylhexosamine addition) more analogous to vertebrates indicates that the N-linked oligosaccharide processing apparatus in the late ER/*cis* and *medial* Golgi elements is conserved throughout evolution [39].

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