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Glycosylation of the envelope glycoprotein gp130 of simian immunodeficiency virus from sooty mangabey (*Cercocebus atys*)

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The envelope glycoprotein 130 ('130' referring to an M_r of 130000) of simian immunodeficiency virus from sooty mangabey (*Cercocebus atys*) (SIV_{SM}) was isolated from the cell-free supernatant of the SIV_{SM}-infected human T-cell line H9, metabolically labelled with D-[6-^{3}H]glucosamine. After digestion with *Staphylococcus aureus* V8 proteinase, radiolabelled *N*-glycans were liberated from resulting glycopeptides by sequential treatment with endo-β-*N*-acetylglucosaminidase H and peptide:*N*-glycosidase F and fractionated by h.p.l.c. and gel filtration. Individual oligosaccharide species were characterized by enzymic microsequencing, chromatographic analyses and, in part, by acetolysis. The oligosaccharide structures thus established include oligomannosidic glycans with five to nine mannose residues as well as fucosylated and partially sialylated bi-, tri- and tetra-antennary *N*-acetyl-lactosaminic oligosaccharide species, the latter of which carry, in part, additional galactose residues or *N*-acetyl-lactosamine repeats. In comparison with the corresponding envelope glycoprotein 120 from human immunodeficiency virus type 1 (HIV-1), propagated in the same cell line [Geyer, Holschbach, Hunsmann and Schneider (1988) J. Biol. Chem. **263**, 11760–11767], carbohydrates of the simian glycoprotein were found to consist of decreased amounts of oligomannosidic glycans and increased quantities of higher-branched *N*-acetyl-lactosaminic species.

INTRODUCTION

The identification of human immunodeficiency virus type 1 (HIV-1) as a causative agent for the acquired immunodeficiency syndrome (AIDS) [1,2] has led to the search for, and the characterization of, related simian retroviruses [3–5]. These simian immunodeficiency viruses (SIVs) have several properties in common with HIV-1: characteristic virion morphology, antigenicity and genome organization, as well as tropism and a cytopathic effect on human T4 lymphocytes in culture (for review, see [6]). It has been shown that HIV and SIV isolates interact with the same cellular receptor, the CD4 molecule, utilizing closely related epitopes [7]. In both cases, virus binding is mediated by their external envelope glycoproteins (gp120/gp130) (the 120 and 130 referring to their M_r values of 120000 and 130000) [8,9], suggesting common structural features of these viral constituents, although they display only a little sequence similarity and serological cross-reactivity [10].

HIV-1 gp120 and SIV gp130 represent highly glycosylated molecules with more than 20 potential *N*-glycosylation sites [11,12]. In the case of HIV-1 gp120, there is now strong evidence that proper glycosylation is critical for its binding to the cellular receptor. Expression of gp120 in bacteria or complete deglycosylation of the molecule results in proteins unable to bind to the CD4 receptor [13–15]. Furthermore, it has been reported that the infectivity of HIV-1 seems to be influenced by the state of glycosylation, since blocking of the initial events in *N*-linked oligosaccharide trimming results in a diminished infectivity of the virus particles, although their binding to the CD4 receptor is not affected [15–19]. The precise role of the oligosaccharide substituents, however, remains to be investigated.

In a previous study [20] we have, therefore, analysed the structures of the oligosaccharide side chains of gp120 from HIV-1 propagated in the human T-cell line H9. Here we describe the characterization of the oligosaccharide species attached to gp130 from SIV_{SM}, an isolate from sooty mangabey (*Cercocebus atys*) [21]. In order to compare the glycosylation of HIV-1 gp120 and SIV_{SM} gp130, SIV_{SM} was propagated in the same cell line, and the glycoprotein was similarly isolated from the culture supernatant. Oligosaccharide side chains were enzymically released, fractionated by h.p.l.c. and gel filtration, and characterized by enzymic microsequencing, chromatographic procedures and, in part, by acetolysis.

MATERIALS AND METHODS

Materials

α1-2-specific mannosidase from *Aspergillus oryzae* was generously provided by Dr. H. Yamaguchi (University of Osaka, Osaka, Japan). Newcastle-disease virus was generously given by Dr. H. D. Klenk (University of Marburg, Marburg, Germany). The structure and origin of oligosaccharide standards used for chromatographic comparison and calibration were given in detail previously [20]. Glucose oligomers with 1–25 glucose units were prepared by partial hydrolysis of dextran as described by Yamashita *et al.* [22].

Abbreviations used: endo H, endo-β-*N*-acetylglucosaminidase H from *Streptomyces griseus* (EC 3.2.1.96); GlcNAcOH, *N*-acetylglucosaminitol; gp130, (virus envelope) glycoprotein 130, i.e. one with an M_r of 130000 (etc.); HIV-1, human immunodeficiency virus type 1; H9-cells, human lymphoblastoid T-cell line, clone 9; PNGase F, peptide:*N*-glycosidase F from *Flavobacterium meningosepticum* (EC 3.2.2.18); SIV_{SM}, simian immunodeficiency virus originally isolated from sooty mangabey (*Cercocebus atys*); NeuAc, *N*-acetylneuraminic acid; GlcNAc, *N*-acetylglucosamine.

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Preparation of radiolabelled SIV_{SM} gp130

Human lymphoblastoid T-cell line H9, chronically infected with the SIV_{SM} isolate, was kindly supplied by Dr. L. Lowenstine [21]. Virus-infected cells were maintained in RPMI-1640 medium (Selectamine; Gibco, Eggenstein, Germany) containing 10% (v/v) fetal-bovine serum and 2 mM-glutamine. For metabolic labelling, 4.5×10^7 cells in the exponential growth phase were 'starved' for 30 min in glucose-free medium, then incubated with 5 mCi of D-[6-^{3}H]glucosamine hydrochloride (33 mCi/mmol; Amersham, Braunschweig, Germany) for 2 h in glucose-free medium, for 4 h in glucose-deficient medium (0.13 mg of glucose/ml) and for another 20 h in normal RPMI medium [20]. After centrifugation, radiolabelled glycoprotein was isolated from the cell-free supernatant by indirect immunoprecipitation using a cross-reacting high-titred serum of a naturally infected African green monkey (*Cercopithecus aethiops*) and Protein A–Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) [10,23,24]. Glycoprotein bound in immunocomplexes was dissolved by heating for 3 min at 96 °C in 0.15 M-Tris/HCl, pH 8.8, containing 0.24% SDS and 1% dithiothreitol. Insoluble material was removed by centrifugation, and the supernatant was checked for homogeneity of the radiolabelled viral glycoprotein by analytical SDS/PAGE [25] and subsequent fluorography. In order to remove salts and detergents the glycoprotein was precipitated with acetone and redissolved in buffer for proteolytic digestion.

Identification of radiolabelled monosaccharides

For determination of labelled sialic acid components, a sample (5×10^3 c.p.m.) of SIV_{SM} gp130 was treated with 0.1 ml of 25 mM-H_2SO_4 at 80 °C for 30 min. After neutralization with NaOH and addition of 1.5 ml of acetone, the sample was kept at −20 °C for 2 h. After centrifugation the supernatant was dried, redissolved in water, and the ^{3}H radioactivity released was determined. After desalting, radiolabelled sialic acids were identified by h.p.l.c. on an Aminex HPX-87H column [20,26]. For the release of neutral and amino-sugar components, the acetone precipitate was further hydrolysed with 500 µl of 250 mM-H_2SO_4 in aq. 90% acetic acid at 80 °C for 6 h. After cooling, 550 µl of 0.5 M-NaOH was added, and samples were rotary-evaporated and freeze-dried. Samples were re-*N*- and de-*O*-acetylated and freeze-dried [26]. The radiolabelled monosaccharides obtained were again identified by h.p.l.c. on an Aminex HPX-87H column.

Isolation and fractionation of oligosaccharides

Proteolytic digestion of gp130, release of radiolabelled sugar side chains by treatment with endo-β-*N*-acetylglucosaminidase H (endo H) and peptide:*N*-glycosidase F (PNGase F), separation of oligosaccharides from residual (glyco)peptides by gel filtration or reversed-phase h.p.l.c., reduction of oligosaccharides as well as fractionation of oligosaccharide alditols by h.p.l.c. using columns of LiChrosorb Diol, LiChrosorb NH_2 (Merck, Darmstadt, Germany) or Mikropak AX-5 (Varian, Walnut Creek, CA, U.S.A.) and desalting of oligosaccharide alditols were carried out as described previously [20,26].

Analytical methods

Acetolysis of oligomannosidic glycans as well as de-*O*-acetylation, reduction and chromatographic identification of the reaction products were as detailed elsewhere [27,28]. For size determination of oligosaccharide alditols and fragments derived therefrom, a column (1.6 cm × 120 cm) of Bio-Gel P-4 (−400 mesh) was used at 55 °C with aq. 0.02% NaN_3 as eluant. Radiolabelled glycans were combined with a mixture of glucose oligomers, applied to the column, and fractions (1.8 ml each) were collected at 0.3 ml/min. The elution position of glucose oligomers was determined by continuous-flow refractive-index measurement, whereas radiolabelled compounds were detected by monitoring the concentrated fractions for radioactivity with a model-4450 liquid-scintillation counter (Packard, Downers Grove, IL, U.S.A.) after addition of scintillation cocktail (Roth, Karlsruhe, Germany).

Enzymic digestions

Treatment with neuraminidase from *Vibrio cholerae*, α-mannosidase, β-*N*-acetylhexosaminidase and β-galactosidase from jack beans (*Canavalia ensiformis*), α-galactosidase from green coffee (*Coffea*) beans, α-fucosidase from ox kidney and endo-β-galactosidase from *Escherichia freundii* were performed as described previously [20,29]. For digestion with neuraminidase from Newcastle-disease virus [30], a viral suspension was used as enzyme preparation. The radiolabelled oligosaccharides (about 200 c.p.m.) were dissolved in 20 µl of 10 mM-

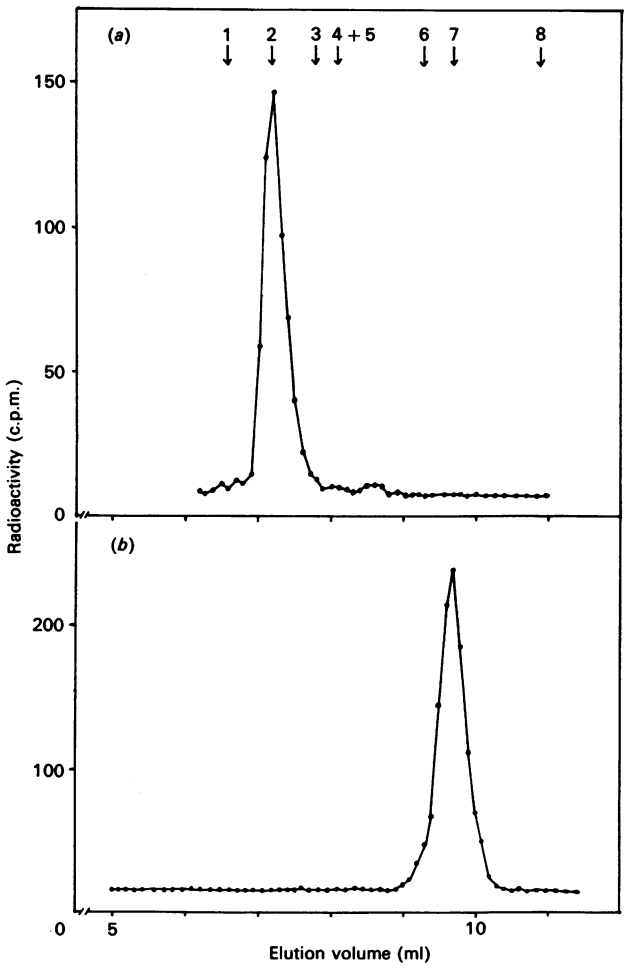


Fig. 1. Identification of radiolabelled sugar constituents in SIV_{SM} gp130

Partially radiolabelled monosaccharides obtained from gp130, metabolically labelled with [6-^{3}H]glucosamine, by mild acid hydrolysis with 25 mM-H_2SO_4 at 80 °C (*a*) and subsequent hydrolysis with 250 mM-H_2SO_4 in aq. 90% acetic acid at 80 °C (*b*) were separated by h.p.l.c. using an Aminex HPX-87H column (0.78 cm × 30 cm) at 50 °C and 5 mM-H_2SO_4 as eluant. Fractions (0.1 ml) were collected at 0.5 ml/min and monitored for radioactivity. Numbers with arrows indicate the elution volumes of monosaccharide standards: 1, *N*-glycolylneuraminate; 2, NeuAc; 3, Glc; 4, Man; 5, Gal; 6, Fuc; 7, GlcNAc; 8, GalNAc.

sodium phosphate buffer, pH 6.0, and incubated for 2 h at 37 °C with 2 nkat of enzyme (determined with fetuin as substrate). Degradation of oligosaccharides (200–2000 c.p.m.) with β-galactosidase or β-*N*-acetylhexosaminidase from *Diplococcus pneumoniae* (both from Boehringer, Mannheim, Germany) [31,32] was carried out at 37 °C in 50 μl of 50 mM-sodium acetate buffer, pH 6.0, for 24 h or 50 mM-sodium citrate buffer, pH 5.0, for 8 h respectively, with 0.17 nkat of enzyme. Digestion with α1-2-mannosidase from *A. oryzae* [33] was performed with 8 pkat of enzyme in 50 μl of 0.1 M-sodium acetate buffer, pH 5.0, at 37 °C for 48 h.

RESULTS

Radiolabelled SIV_{SM} gp130

H9-cells producing simian immunodeficiency virus, originally isolated from sooty mangabey (SIV_{SM} [21]), were metabolically labelled with D-[6-^{3}H]glucosamine. Viral glycoprotein was isolated from the cell-free supernatant by immunoprecipitation and analysed by SDS/PAGE. Subsequent fluorographic detection revealed only one radiolabelled protein band with an apparent molecular mass of about 130 kDa (results not shown). Since our structural analyses were based only on monitoring of ^{3}H radioactivity, this glycoprotein preparation could be used without further purification. Starting from three cell-culture flasks (containing 4.5×10^7 cells and 5 mCi of [^{3}H]glucosamine each), viral glycoprotein with 6×10^5 c.p.m. was obtained. For determination of the corresponding radioactive sugar constituents, an aliquot was hydrolysed, and released monosaccharides were identified by h.p.l.c. (Fig. 1). The results demonstrated that *N*-acetylneuraminic acid (NeuAc) and *N*-acetylglucosamine (GlcNAc) were the only radiolabelled sugar components. Since about one-third of the ^{3}H radioactivity was released by mild acid hydrolysis and identified as NeuAc, it can be concluded that [6-^{3}H]glucosamine-labelled gp130 contained radiolabelled GlcNAc and NeuAc in the ratio 2:1.

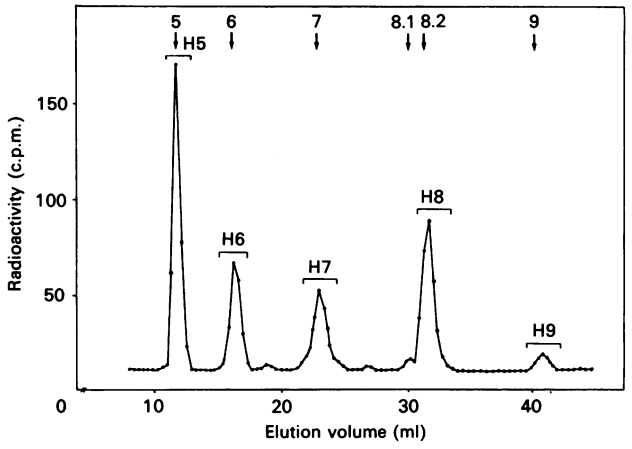


Fig. 2. Separation of endo H-sensitive glycans from SIV_{SM} gp130

Oligosaccharide alditols obtained from gp130 glycopeptides, metabolically labelled with [6-^{3}H]glucosamine, after endo H treatment and reduction were fractionated on a LiChrosorb Diol column (5 μm particle size; 4 mm × 150 mm) using acetonitrile/water (37:13, v/v) as eluant. Fractions (0.4 ml) were collected at 0.5 ml/min and monitored for radioactivity. Numbers (5–9) with arrows indicate elution volumes of oligomannosidic oligosaccharide alditol standards ($Man_{5-9}GlcNAcOH$) (comprising two isomers of $Man_8GlcNAcOH$). H5–H9 (with brackets), endo H-sensitive oligosaccharide alditol fractions obtained from SIV_{SM} gp130.

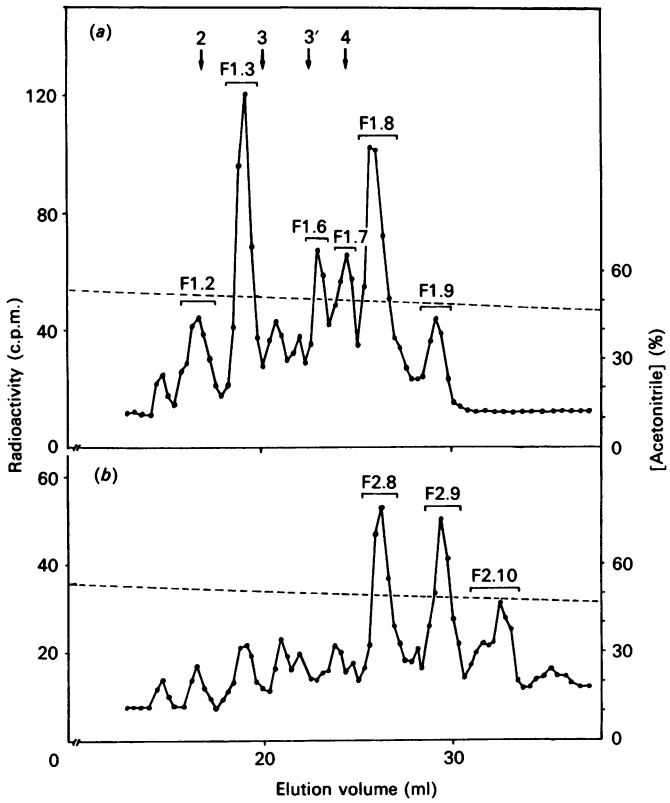


Fig. 3. Separation of *N*-acetyl-lactosaminic asialo-oligosaccharides from SIV_{SM} gp130

Asialo-oligosaccharide alditols, obtained from endo H-resistant gp130 glycopeptides, metabolically labelled with [6-^{3}H]glucosamine, after treatment with PNGase F, fractionation by reversed-phase h.p.l.c., reduction and desialylation, were separated by h.p.l.c. using a LiChrosorb NH_2 column (5 μm particle size; 4.6 mm × 250 mm) and a linear gradient of acetonitrile (55–45%, v/v; 45 min) in 15 mM-potassium phosphate buffer, pH 5.3. Fractions (0.4 ml) were collected at 1 ml/min and monitored for radioactivity. (*a*) Separation of fraction F1 glycans; (*b*) separation of fraction F2 species. Numbers (2–4) with arrows indicate elution volumes of bi-, 2,4- and 2,6-branched isomers (3 and 3') of tri-, and tetra-antennary asialo-oligosaccharide alditol standards respectively. F1.2–F1.9 in (*a*) and F2.8–F2.10 in (*b*), oligosaccharide alditol fractions obtained from SIV_{SM} gp130.

Fractionation of oligosaccharides

After digestion with V8 proteinase from *S. aureus*, gp130 glycopeptides were treated with endo-β-*N*-acetylglucosaminidase H (endo H). Resulting oligosaccharides (4.2% of total radioactivity) were separated from residual glycopeptides by Bio-Gel P-4 chromatography and reduced with $NaBH_4$. Oligosaccharide alditols thus obtained were fractionated by h.p.l.c., yielding fractions H5–H9 (Fig. 2). Endo H-resistant glycans were liberated by peptide:*N*-glycosidase F (PNGase F) and separated from peptide material by reversed-phase h.p.l.c. Two oligosaccharide fractions, F1 and F2, comprising 55.4 and 40.4% of the total radioactivity respectively, were obtained (results not shown). After reduction, aliquots of these fractions were set apart for determination of their sialylation patterns (see below). The remaining glycans were treated with neuraminidase from *V. cholerae*, and desialylated oligosaccharide alditols of both fractions were further fractionated by h.p.l.c., yielding, as major oligosaccharide alditol subfractions, F1.2, F1.3, F1.6–F1.9 and F2.8–F2.10 (Fig. 3).

Table 1. Degradation of oligomannosidic oligosaccharide alditols from SIV_{SM} gp130

Oligosaccharide alditol fractions obtained from SIV_{SM} gp130, metabolically labelled with [6-^{3}H]glucosamine, after treatment with endo H were subjected to digestion with α1-2-mannosidase from *A. oryzae* or jack-bean α-mannosidase and to mild acetolysis. Products were characterized by gel filtration and their co-elution with oligosaccharide standard alditols. Corresponding elution volumes are expressed in glucose units [22].

Degradation by:	No. of glucose units of fragments obtained from: H5	H6	H7	H8	H9	Co-eluted standard oligosaccharide alditols
(Before treatment)	6.7	7.5	8.5	9.5	10.3	$Man_{5-9}GlcNAcOH$
α1–2-Mannosidase	6.7	6.7	6.7	6.7	6.7	$Man_5GlcNAcOH$
α-Mannosidase	3.4	3.4	3.4	3.4	3.4	$ManGlcNAcOH$
Mild acetolysis	4.3	(4.3)*			–†	$Man_2GlcNAcOH$
		5.2	5.2			$Man_3GlcNAcOH$
	5.7	(5.7)*		6.0		$Man_4GlcNAcOH$
		6.7	6.8			$Man_5GlcNAcOH$
				7.8		$Man_6GlcNAcOH$

* Minor component.
† Not done.

Characterization of oligomannosidic oligosaccharides

The number, anomeric configurations and linkage positions of mannose residues present in fraction-H5–H9 glycans (see Fig. 2) were determined by degradation with α-mannosidase from jack beans [34] and with α1-2-mannosidase from *A. oryzae* [33]. Untreated oligosaccharides and reaction products were analysed by gel filtration (see Table 1). Upon degradation with the jack-bean enzyme, which cleaves all α-linked mannose residues irrespective of their linkage positions, in all cases a radioactive fragment was generated which was co-eluted with the authentic Manβ1-4GlcNAcOH standard at an elution volume equivalent to 3.4 glucose units [22], indicating that all endo H-released glycans represented oligomannosidic and no hybrid-type species. After incubation with the α1-2-mannosidase, zero (H5), one (H6), two (H7), three (H8) and four (H9) mannose residues were released with concurrent formation of a radioactive fragment eluted at a position equivalent to 6.7 glucose units, i.e. the elution position of $Man_5GlcNAcOH$ [22].

For isomer identification, oligomannosidic alditol fractions H5–H8 were partially degraded by acetolysis [27]. By using very mild hydrolysis conditions, α1-6 linkages of peracetylated oligomannosidic glycans are preferentially cleaved. Thus the isomeric structure of a given oligosaccharide can be deduced from the number and sizes of fragments produced. The fragments obtained after de-*O*-acetylation and reduction were, therefore, analysed by gel filtration (cf. Table 1). Owing to metabolic labelling of glycans with [^{3}H]glucosamine, only fragments containing *N*-acetylglucosaminitol could be detected in the present study. Fraction H9 glycans were not further analysed.

As evidenced by acetolysis fragments $Man_2GlcNAcOH$ and $Man_4GlcNAcOH$, fraction H5 represented oligosaccharide species comprising mannose residues 3, 4, 4′, A and B (for designation of sugar residues in oligomannosidic glycans, see Table 2 and [35]). Fraction H6 was found to represent a mixture of two isomers: a major component containing Man-C (leading to fragments $Man_3GlcNAcOH$ and $Man_5GlcNAcOH$ upon acetolysis) and, to a minor extent, species with Man-D_3 (generating $Man_2GlcNAcOH$ and $Man_4GlcNAcOH$). In the case of fractions H7 and H8, acetolysis fragments $Man_3GlcNAcOH$ and $Man_5GlcNAcOH$ or $Man_4GlcNAcOH$ and $Man_6GlcNAcOH$ indicated the presence of oligosaccharides containing outer Man residues D_3 or D_3 and D_1, respectively (see Table 2). Taken together, the results allow the identification of the H5–H9 glycans as a series of oligomannosidic oligosaccharide alditols containing one β- and four to eight α-linked mannose residues, zero to four of which could be shown to be α1-2-linked. Obviously these alditols originate from the common oligomannosidic $Man_{5-9}GlcNAc_2$ species generally functioning as intermediates during glycoprotein-*N*-glycan biosynthesis in animal cells [36,37].

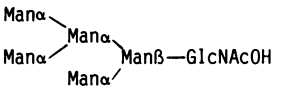
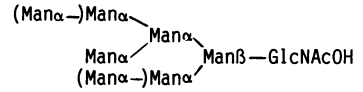
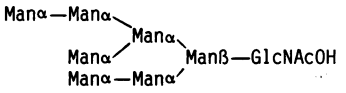
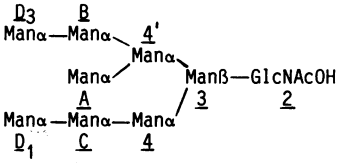
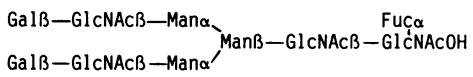
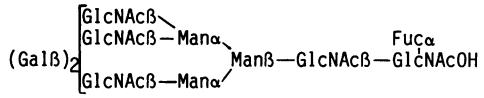
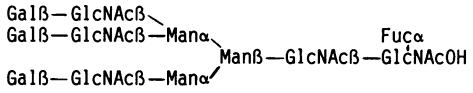
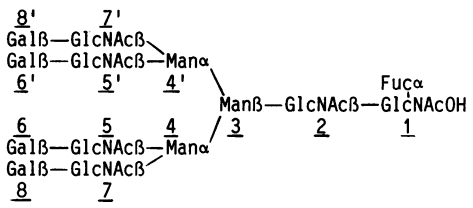
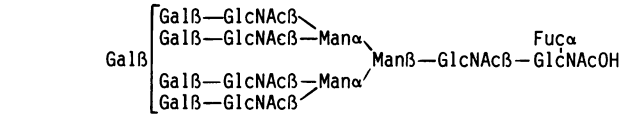
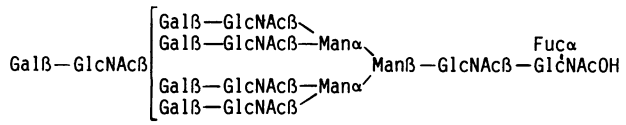
Characterization of *N*-acetyl-lactosaminic asialo-oligosaccharides

The major asialo-oligosaccharide alditol fractions obtained by h.p.l.c. subfractionation (F1.2, F1.3, F1.6–F1.9, F2.8–F2.10, pooled as indicated by horizontal brackets in Fig. 3) were characterized by gel filtration and h.p.l.c. in conjunction with authentic oligosaccharide standards. Enzymic sequencing was carried out by digestion with β-galactosidase, β-*N*-acetylhexosaminidase, α-mannosidase, α-fucosidase and, if necessary, with α-galactosidase and endo-β-galactosidase [34,38]. Reaction products were analysed by Bio-Gel P-4 chromatography (see, for example, Fig. 4) and their sizes were determined by co-chromatography with glucose oligomers [22,39]. The results obtained are summarized in Table 3, and the structures deduced therefrom are included in Table 2.

The oligosaccharide alditols of fraction F1.2 were identified as biantennary complex-type glycans with an α-linked fucose residue in the core region of the oligosaccharide from the following evidence. They are co-eluted with the authentic standard oligosaccharide alditol Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcOH in both h.p.l.c. and gel filtration. When the glycans were incubated with jack-bean β-galactosidase, the elution volume of the truncated product revealed the loss of two galactose residues. Further digestion with jack-bean β-*N*-acetylhexosaminidase resulted in the removal of two *N*-acetylglucosamine residues. Since the *D. pneumoniae* enzyme, specifically cleaving β1-2-linked *N*-acetylglucosamine residues [32], caused the same shift of the elution volume, the two *N*-acetylglucosamine residues are thought to be β1-2-linked. The oligosaccharide fragment produced thereby was eluted at about 8.5 glucose units, a position similar to that at which the authentic standard alditol Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcOH was eluted [22]. Further degradation with α-mannosidase and α-fucosidase resulted in the loss of two mannose and one fucose residues respectively, yielding a fragment which was co-eluted with Manβ1-4GlcNAcβ1-4GlcNAcOH and comprised about 5.5–6 glucose units [22].

Table 2. Structures proposed for the main oligomannosidic and desialylated complex-type oligosaccharide alditol fractions of SIV_{SM} gp130 from H9 cells, metabolically labelled with [6-^{3}H]glucosamine.

The amount (mol %) of each glycan was roughly estimated from the distribution of [^{3}H]glucosamine radioactivity in the different structures (see the text). The general structure of the glycans and the anomeric configurations of Man-3 and GlcNAc-2 are proposed in accordance with the common structures of glycoprotein-*N*-glycans [36, 37]. In the structures of H8 and F1.7 the designation of sugar residues in oligomannosidic [35] and *N*-acetyl-lactosaminic [40] glycans respectively is shown.

Fraction	Structure	Mol %
H5		7.5
H6		4
H7		4
H8		7
F1.2		7.5
F1.3		13
F1.6		7
F1.7		7.5
F1.8/2.8		19
F1.9/2.9		10

Glycans of fractions F1.3 and F1.6 are concluded to represent the triantennary species with two or three galactose residues shown in Table 2. In gel filtration they were eluted at about 16 or 17 glucose units (see Table 3). Incubation with jack-bean β-galactosidase led to a shift of their elution volumes that corresponded to the release of two or three galactose residues. Further digestion with β-*N*-acetylhexosaminidase from jack beans resulted in the loss of three *N*-acetylglucosamine residues and a radioactive fragment that was co-eluted with the core oligosaccharide, $Man_3GlcNAc(Fuc)GlcNAcOH$ (see above), which could be digested by α-mannosidase and α-fucosidase in the same manner as described for fraction F1.2. Fraction F1.3 was also digested with *D. pneumoniae* enzymes [31,32] (see Table 3). The results demonstrated that diplococcal β-galactosidase released two β1-4-linked galactose residues, producing the same fragment as the jack-bean enzyme, whereas only one *N*-acetylglucosamine residue was split off by diplococcal β-*N*-acetylhexosaminidase. Since the latter enzyme is known to be

Table 3. Degradation of *N*-acetyl-lactosaminic oligosaccharide alditols from SIV_{SM} gp130 with exoglycosidases

Main subfractions of asialo-oligosaccharide alditols isolated from SIV_{SM} gp130, metabolically labelled with [6-^{3}H]glucosamine, were sequentially digested with the enzymes indicated, and the products were analysed by gel filtration using a calibrated Bio-Gel P-4 column. The results are expressed in glucose units [22,39] of the oligosaccharide fragments obtained.

Enzyme used	No. of glucose units of fragments obtained from:						
	F1.2	F1.3	F1.6	F1.7	F1.8/2.8	F1.9/2.9	F2.10
None	14.5	16.2	17.3	19.3	20.3	22.5	26.8*
Endo-β-galactosidase	—	—	—	—	20.3	18.5/4.5	18.3
α-Galactosidase	—	—	—	—	20.3	—	—
β-Galactosidase†	12.5	13.5‡	13.7	15.3	15.5	15.5/18.5§	
β-*N*-acetylhexosaminidase†	8.5‡	8.5/11.8‖			8.5		—
α-Mannosidase	6.7	6.7			6.7		—
α-Fucosidase	5.9	5.7			5.7		—

* Main component of this fraction; contained smaller species as well.
† From jack beans.
‡ Treatment with the enzyme from *D. pneumoniae* yielded the same fragment.
§ Treatment with β-galactosidase without prior digestion with endo-β-galactosidase.
‖ Treatment with diplococcal β-*N*-acetylhexosaminidase in the case of F1.3.

sterically hindered in hydrolysing GlcNAcβ1-2Man-linkages if the corresponding mannose residue is also substituted by β1-6-linked GlcNAc, 2,6- and 2,4-branched triantennary oligosaccharides can be distinguished [32]. From the results described above and from their chromatographic properties on h.p.l.c. (fraction F1.6 is co-eluted with the 2,6-branched triantennary oligosaccharide standard; see Fig. 3*a*), fraction F1.3 and F1.6 glycans are thought to represent the 2,6-branched species shown in Table 2.

Fraction-F1.7, -F1.8, -F1.9, -F2.8 and -F2.9 glycans all represent tetra-antennary structures, as could be deduced from gel-filtration data and studies using exoglycosidases, which also revealed that F1.8 and F2.8, as well as F1.9 and F2.9, were identical. Upon digestion with jack-bean β-galactosidase, four or five galactose residues were released in the case of F1.7 or F1.8/2.8 respectively, giving rise to a radioactive fragment which was eluted in gel filtration at about 15.5 glucose units, i.e. the position typical for the degalactosylated tetra-antennary basic structure [39]. On incubation with jack-bean β-*N*-acetylhexosaminidase, four *N*-acetylglucosamine residues were liberated and the fragment was converted into the trimannosyl core oligosaccharide mentioned above (see Table 3). Thus fraction F1.7 is supposed to contain a tetra-antennary oligosaccharide with four galactose residues and one fucose residue, presumably located at the innermost *N*-acetylglucosamine. Since F1.8/2.8 species were insensitive towards α-galactosidase from green coffee (*Coffea*) beans and endo-β-galactosidase from *Escherichia freundii* (see Table 3 and Fig. 4), it is assumed that they comprise tetra-antennary glycans substituted by a fifth β-linked galactose residue. Fraction-F1.9/2.9 oligosaccharides, however, which were eluted on the Bio-Gel column at about 22.5 glucose units, could be degraded by endo-β-galactosidase yielding two fragments of about 18.5 and 4.5 glucose units (cf. Fig. 4), which is consistent with the liberation of a Gal-GlcNAc-Gal trisaccharide. From the known specificity of the enzyme [41], the lactosamine repeat is concluded to be linked to C-3 of galactose. Further digestion of the 18.5-glucose-units-fragment with β-galactosidase resulted again in the formation of a reaction product with 15.5 glucose units, demonstrating the loss of three galactose residues, whereas direct incubation of F1.9/2.9 glycans with β-galactosidase removed four galactose residues. Therefore F1.9/2.9 glycans are assumed to represent tetra-antennary oligosaccharides with one *N*-acetyl-lactosamine repeat (see Table 2).

As already indicated by the respective h.p.l.c. profile (cf. Fig. 3*b*), fraction F2.10 turned out to be a mixture of two species, the major compound of which had an elution volume corresponding to about 27 glucose units in gel filtration. After incubation with endo-β-galactosidase, the elution volume of the resulting oligosaccharide fragment shifted to about 18.3 glucose units, indicating the presence of two *N*-acetyl-lactosamine repeats. Owing to the small amounts available, F2.10 glycans could not be further characterized.

Analysis of sialic acid substitution

Monosaccharide constituent analysis and enzymic desialylation showed NeuAc to be the only acidic component in gp130 oligosaccharides. In order to determine their sialylation pattern, the two fractions of *N*-acetyl-lactosaminic glycans obtained by reversed-phase h.p.l.c. after cleavage with PNGase F (fractions F1 and F2, see above) were analysed by anion-exchange h.p.l.c. (Fig. 5). On the basis of total radioactivity, fraction F1 contained neutral, mono-, di-, tri- and tetra-sialylated oligosaccharides in a ratio of about 1:1.7:2.2:1.4:1, whereas fraction-F2 glycans represented only neutral, mono- and di-sialylated species (1.0:1.3:0.53). Differences in elution volumes of monosialylated species as well as peak heterogeneities observed in the case of fraction-F2 glycans are possibly attributable to different linkage positions of sialic acid substituents. When α2-3-specific neuraminidase from Newcastle-disease virus [30] was used for desialylation, tri- and tetra-sialylated species of fraction F1 completely disappeared (results not shown), indicating that at least one or two sialic acid residues were α2-3-linked in these glycans. In the same manner, mono- and di-sialylated glycans of fraction F1 could be shown to be predominantly substituted by α2-6-linked NeuAc, whereas fraction F2 glycans seemed to contain both α2-3- and α2-6-linked NeuAc substituents. Since fractions F1 and F2 were shown to lead, in part, to the same asialo-oligosaccharides (e.g. F1.8/F2.8 and F1.9/F2.9), fractionation by reversed-phase h.p.l.c. was obviously influenced by the sialylation of these species. Therefore it may be assumed that the tetra-antennary oligosaccharides of F1.8 and F2.8, as well as F1.9 and F2.9, differed in the number of sialic acid substituents present. Owing to the small amounts of material available, the

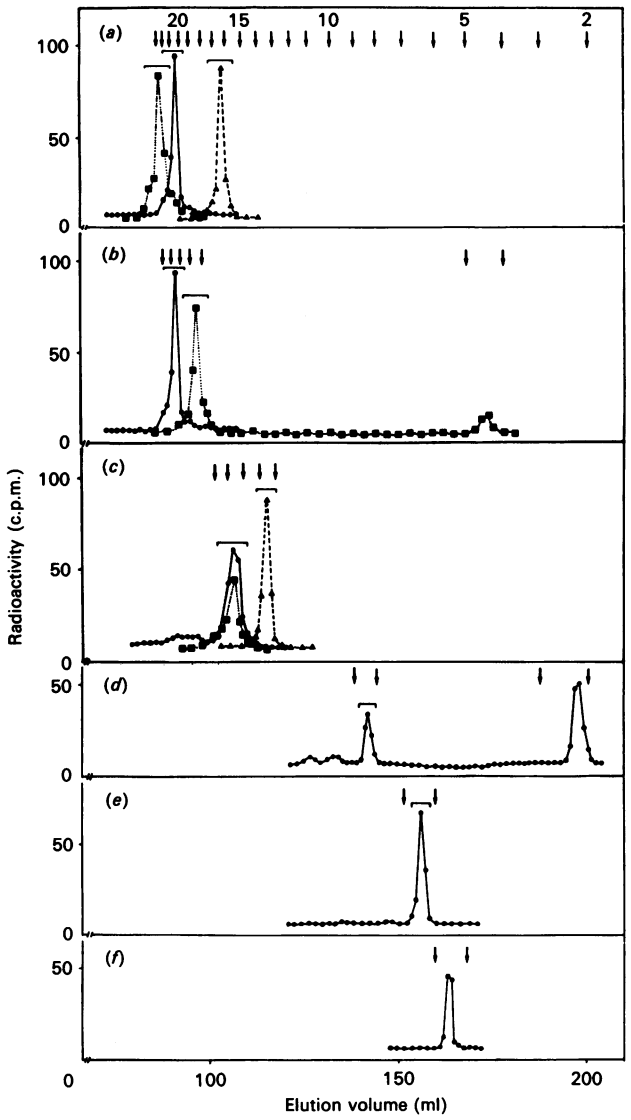


Fig. 4. Sequential degradation of SIV_{SM} gp130 asialo-oligosaccharide alditol fractions F1.3, F1.8 and F1.9 with exoglycosidases

After analysis of the starting material by gel filtration (*a*), fractions were successively digested with endo-β-galactosidase (except fraction F1.3) (*b*), β-galactosidase (*c*), β-*N*-acetylhexosaminidase (*d*), α-mannosidase (*e*) and α-fucosidase (*f*). Reaction products were chromatographed on the same Bio-Gel P-4 column (−400 mesh; 1.6 cm × 120 cm) at 55 °C with aq. 0.02 % NaN_3 as eluant. Fractions (1.8 ml) were collected at 0.3 ml/min and monitored for radioactivity. Column calibration with glucose oligomers (with 2–23 glucose residues) is shown by arrows. Closed triangles, circles and squares correspond to F1.3, F1.8 and F1.9 respectively. For simplification of the Figure, only the results obtained from F1.8 are included in (*d*)–(*f*). Horizontal brackets indicate fractions pooled for further digestion.

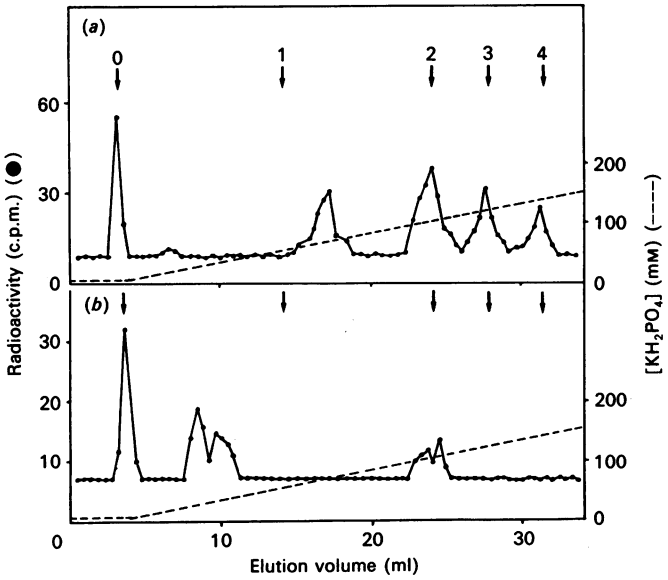


Fig. 5. Analysis of *N*-acetyl-lactosaminic glycans from SIV_{SM} gp130 by ion-exchange h.p.l.c.

Aliquots of the oligosaccharide alditol fractions obtained after treatment of metabolically labelled gp130 glycopeptides with PNGase F, reversed-phase h.p.l.c. and reduction were subjected to ion-exchange h.p.l.c. using a Mikropak AX-5 column (4.6 mm × 250 mm) and a linear gradient of 5–300 mM-potassium phosphate buffer, pH 4.4, in 60 min. Fractions (0.4 ml) were collected at 1 ml/min and monitored for radioactivity. (*a*) and (*b*) are analyses of glycans from fractions F1 and F2 respectively; 0–4 with arrows, elution volumes of oligosaccharide standards with zero to four sialic acid residues.

sialylation pattern of individual glycans could not be determined. On average, glycans of fraction F1 and F2 were substituted by 2 and 0.8 sialic acid residues respectively (compare Fig. 5). On the assumption that all GlcNAc- and NeuAc-residues were equally labelled, molar proportions of the individual species could be roughly estimated (see Table 2).

DISCUSSION

In the present study, SIV_{SM} was propagated on the human lymphoblastoid cell line H9 in the presence of D-[6-^{3}H]-glucosamine. gp130, isolated from the culture supernatant, solely contained GlcNAc and NeuAc as radioactive sugar constituents, thus allowing a specific detection of its oligosaccharide substituents by monitoring of ^{3}H radioactivity.

Oligosaccharide species obtained comprised about 24 mol % of oligomannosidic glycans carrying predominantly five or eight mannose residues, whereas *N*-acetyl-lactosaminic glycans represented a highly diverse mixture of species with two, three (partially incomplete) or four lactosamine antennae with or without additional β-linked galactose or lactosamine repeats. As a common feature, complex-type glycans carry an α-linked fucose residue in the $Man_3GlcNAc_2$ core region. Oligomannosidic *N*-acetyl-lactosaminic as well as carbohydrate substituents of SIV_{SM} gp130 are typical of glycoprotein-*N*-glycans [36,37,40].

Comparison of the glycosylation pattern of SIV_{SM} gp130 with that of HIV-1 gp120, propagated in the same host cell line [20], reveals quantitative and qualitative differences insofar as the envelope glycoprotein of SIV_{SM} contains only half the oligomannosidic glycans found in HIV-1 gp120, no hybrid-type species and increased amounts of *N*-acetyl-lactosaminic substituents. In general, carbohydrate side chains of SIV_{SM} gp130 appear to be further processed. Whereas oligomannosidic glycans with eight and nine mannose residues predominate in the case of HIV-1 gp120, SIV_{SM} gp130 contains high amounts of Man_5-glycans and only traces of Man_9 species. Similarly, bi- and tri-antennary complex-type glycans prevail in HIV-1 gp120, whereas SIV_{SM} gp130 contains high amounts of tetra-antennary species. SIV_{SM} gp130 glycans were found to contain up to four sialic acid residues, whereas HIV-1 gp120 oligosaccharides carry two of them at most. Furthermore, it is noteworthy that HIV-1 gp120, propagated in H9-cells, does not carry any *N*-acetyl-lactosamine repeats. Likewise, recombinant gp120, produced in Chinese-

hamster ovary cells, which are known to contain high activities of the glycosyltransferases involved in polylactosaminoglycan formation [42], possesses only small amounts of such repeating units [43,44]. Possibly, this observation refers to influences of the polypeptide backbone on the type of oligosaccharide synthesized.

As stated in the Introduction, infectivity of HIV-1 and binding of the virus to its cellular receptor seem to be dependent on proper glycosylation of its envelope glycoproteins. Since simian viruses utilize the same cell-surface molecule during infection, the carbohydrates of SIV glycoproteins might be similarly important. The glycosylation patterns of immunodeficiency-virus glycoproteins reported in this and other studies [20,43,44], however, reveal a great structural variety of the carbohydrate substituents present. Therefore, it may be suggested that the glycosylation of the molecules *per se* is essential for their biological activity, e.g. by stabilizing the overall conformation of the proteins. On the other hand, studies using inhibitors of oligosaccharide trimming seem to indicate that high-mannose-type glycans might be important for correct interaction of HIV-1 with its host-cell receptor [15–19]. This notion is supported by the observation that mannose-specific lectins like concanavalin A [45,46] or the one from *Gerardia savaglia* (Epizoanthidae), which preferentially binds to α1-2-linked mannoses [47], interfere with the virus–receptor interaction, thus blocking glycoprotein-induced cell fusion and syncytium formation *in vitro*. All immunodeficiency-virus glycoproteins studied so far have been shown to contain considerable amounts of high-mannose-type glycans. Thus oligomannosidic oligosaccharides represent a common structural feature of these molecules. Since deglycosylation experiments demonstrated that the presence of some, but not all, carbohydrate side chains is required for biological activity [15], the observed structural heterogeneity of total glycans does not rule out the involvement of distinct, possibly oligomannosidic, oligosaccharides in receptor binding and/or virus infectivity.

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