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Carbohydrate binding properties of the envelope glycoproteins of human immunodeficiency virus type 1

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Here, we confirm and extend our previous findings on human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein *N*-acetylglucosaminyl binding properties. We show the occurrence of saturable, temperature, pH, and calcium dependent carbohydrate-specific interactions between recombinant precursor gp160 (rgp160) and two affinity matrices: D-mannose-divinylsulfone-agarose, and natural glycoprotein, fetuin, also coupled to agarose. Binding of rgp160 to the matrices was inhibited by soluble mannosyl derivatives, α-D-Man_{17}-BSA and mannan, by β-D-$GlcNAc_{47}$-BSA and by glycopeptides from Pronase-treated porcine thyroglobulin, which produces oligomannose and complex N-linked glycans. Glycopeptides from Endoglycosidase H-treated thyroglobulin partially inhibited rgp160 binding, as did the asialo-agalacto-tetraantennary precursor oligosaccharide of human α_1-acid glycoprotein for binding to fetuin-agarose. β-D-Glucan and β-D-Gal_{17}-BSA had no or only limited effect. Also, surface unit rgp120 specifically interacted with fetuin-agarose and soluble fetuin, but in the latter case with a twofold reduced affinity relative to rgp160. After affinity chromatography, rgp160 was specifically retained by the two matrices and eluted by mannan in both cases, while rgp120 was not retained by fetuin-agarose but only eluted as a significantly retarded peak, which confirms its specific but weak interaction. Thus, rgp160 interacts with both oligomannose type, and the mannosyl core of complex type N-linked glycans, and its gp120 region plays a role in this interaction. Because fetuin and asialofetuin inhibit to nearly the same extent, the binding of rgp160 or rgp120 to fetuin-agarose, interaction with sialic acid or β-D-galactosyl structures of complex N- or O-linked glycans can be ruled out. Specific rgp160 and rgp120 binding to a *p*-aminophenyl-β-D-GlcNAc-agarose matrix, which was inhibited by β-D-$GlcNAc_{47}$-BSA and by fetuin, confirms that HIV-1 envelope glycoproteins can also specifically interact with the *N*-acetylglucosaminyl core of oligosaccharide structures.

Keywords: *N*-acetylglucosaminyl binding, human immunodeficiency virus type 1 (HIV-1)

Introduction

Human immunodeficiency virus type 1 (HIV-1) presents two envelope glycoproteins, outer-membrane gp120 and transmembrane gp41, which are derived from precursor polyprotein gp160 by proteolytic cleavage [1]. Infection of CD4+ cells by HIV-1 occurs via the binding of gp120 to membrane CD4 [2], and this is followed by gp41-triggered fusion between the virus envelope and target cell membrane [3]. Inasmuch as gp120 is highly glycosylated, it has long been thought that at least some of its carbohydrate side chains might be involved in virus infectivity, all the more so since lectin–carbohydrate interactions are known to mediate target cell attachment and pathogenicity of several microorganisms [4, 5]. However, there is now indication that for HIV-1 N-linked glycans are not directly involved in gp120 binding to its CD4 receptor [6, 7], nor do they appear as major determinants of the virus *in vitro* infectivity for CD4+ lymphoid cells [8].

We have recently reported that gp120 exposed on viral particles as well as recombinant gp160 (rgp160) display *N*-acetyl-β-D-glucosaminyl-binding properties for *N*-acetylglucosamine residues presented at high density on protein carriers [7]. In the light of current hypotheses [9] that HIV-1 entry into target cells necessitates additional binding of gp120 to membrane component(s) different from CD4 at post-CD4-binding steps, and that under some circumstances HIV-1 can even infect CD4-cells [10, 11], it is then possible that such a lectin property could be used by the virus to interact with the carbohydrate moieties of the corresponding molecules.

The present study was undertaken to confirm our first findings and to further analyse the carbohydrate-binding properties of rgp160 at the molecular level. The results we found are similar to those reported for several other carbohydrate binding proteins which have been described to present both mannosyl and *N*-acetylglucosaminyl binding properties [12, 13] that can be specific for terminal nonreducing *N*-acetylglucosaminyl, mannosyl, fucosyl and

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glucosyl residues, or for the core of oligosaccharide ligands, or for both the core and terminal residues of complex N-linked oligosaccharides [13]. Indeed, we demonstrate here that, as well as an *N*-acetylglucosaminyl-binding capacity, rgp160 also specifically interacts with mannosyl residues which can present as oligomannose structures or as core mannosyl residues of natural glycoprotein N-linked glycans.

Materials and methods

Recombinant gp160 and gp120

Soluble rgp160 of HIV-1_{LAI} [14] purified to 90% homogeneity (gift from Pasteur Mérieux Sérums et Vaccins, Lyon, France) was obtained from BHK-21 cells infected with a recombinant gp160/vaccinia virus as described [15]. Soluble rgp120, >90% pure, was a gift from the MRC AIDS Directed Program (South Mimms, UK).

Radiolabelling was performed by the iodogen method, as described by Fenouillet *et al.* [6]. Iodinated glycoproteins were separated from $Na^{125}I$ by filtration through a Sephadex G-25 (PD10) column (Pharmacia, Uppsala, Sweden). Specific activity of both [^{125}I]rgp160 and [^{125}I]rgp120 was 1.1 MBq μg^{-1}. Sodium dodecyl sulfate polyacrylamide (7.5%) gel electrophoresis was performed to assess homogeneity of the preparations. Gel filtration of [^{125}I]rgp160 was performed at 20 °C on a Bio-Gel A-1.5 m column (7 cm × 1 cm; Bio-Rad, Richmond, CA, USA) pre-equilibrated in buffer: 0.02 M Tris, 0.15 M NaCl, 0.01 M $CaCl_2$, 0.05% BSA, pH 7.4. Flow rate was 0.8 ml min^{-1}. The column was calibrated by Dextran blue 2000 (2000 kDa) (Pharmacia LKB, Uppsala, Sweden), thyroglobulin (660 kDa), bovine IgG (155 kDa) and albumin (67 kDa) (Sigma Chemicals, St. Louis, MO, USA) (see Fig. 5).

Using human or rabbit antisera (the latter prepared in our laboratory) and soluble CD4 (sCD4; a gift from D. Klatzmann), we verified that labelled glycoproteins were still immunoreactive and bound to CD4. Antisera (2 µl diluted 1:10 or 1:100) or sCD4 (1 µg) were dotted onto nitrocellulose filters; after 30 min at 20 °C, filter strips were saturated for 30 min at 37 °C with 2 ml phosphate-buffered saline (PBS) supplemented with 5% bovine serum albumin (BSA; Sigma) to prevent nonspecific binding; after washing with PBS–BSA 0.5%, the strips were incubated for 1 h at 37 °C with [^{125}I]rgp120 or [^{125}I]rgp160, washed twice with PBS-BSA 0.5%, Tween 0.02% (Sigma) and then autoradiographed.

Binding of recombinant envelope glycoproteins to carbohydrate substituted affinity matrices

The binding capacity of [^{125}I]rgp160 to D-mannose-divinylsulfone-agarose (mannose-agarose) or to *p*-aminophenyl - β - D - *N* - acetylglucosaminyl - agarose (GlcNAc - agarose), both from E. Y. Laboratories (San Mateo, CA, USA) or to fetuin-agarose (Sigma) was investigated as follows: 20 µl affinity matrix were suspended in an equal volume of buffer: 0.02 M Tris, 0.15 M NaCl, 0.01 M $CaCl_2$, 0.05% BSA, pH 7.4. After incubation with various concentrations of [^{125}I]rgp160 for 1 h at 37 °C, and washing twice in 500 µl buffer, solid phase-bound radioactivity was counted in a gamma counter (Packard). Results were expressed as duplicate mean values. Binding of [^{125}I]rgp120 to fetuin-agarose was analysed in the same manner.

Physico-chemical characteristics of the interactions were analysed by performing the assays under different conditons: pH ranging from 6 to 8.2; buffer without or with different $CaCl_2$ concentrations; or at three temperatures: 4 °C, 19 °C and 37 °C. Buffers were prepared with double distilled, deionized, sterile water. Ca^{2+} concentrations were assessed with a Dimension autoanalyzer (Dupont de Nemours, France). It was verified that no rgp120 or rgp160 binding to the matrices was detected in Ca^{2+} free or in EDTA (disodium salt; Sigma) buffer.

Carbohydrate specificity of rgp160/rgp120 binding to affinity matrices

To determine carbohydrate specificity of the interactions, radiolabelled glycoproteins were preincubated for 45 min at 37 °C with the following carbohydrates or carbohydrate derivatives (all from Sigma) diluted in buffer: β-D-glucan, D-mannan at 0.05–2 mg ml^{-1}; fetuin and asialofetuin, porcine thyroglobulin at µM concentrations; methyl α-D-mannopyranoside (Me-α-Man), methyl α-D-glucopyranoside (Me-α-Glc) and methyl α-D-galactopyranoside (Me-α-Gal) at mM concentrations. In some experiments, µM concentrations of the following compounds were used: fucose-substituted complex-type asialo-agalacto or galactosylated biantennary core of porcine thyroglobulin; complex-type asialo-galactosylated triantennary oligosaccharides from bovine fetuin; oligomannose 9 from porcine thyroglobulin; complex-type asialo-agalacto-tetraantennary precursor oligosaccharide prepared by exo-glycosidase digestion of human α_1-acid glycoprotein. All the latter compounds (Oxford Glycosystems, UK) were prepared using anhydrous hydrazine, separated from the peptide material by adsorption chromatography, and further purified by a combination of anion exchange and gel filtration chromatography. Their purity was >90% as determined by HPAE-PAD and ^{1}H-NMR.

Additional synthetic neoglycoproteins (a gift of D. Bladier), tested up to 60 µM, were obtained by conjugating BSA with various 4-nitrophenylglycosides (Sigma) as described by Westphal and Schmidt [16]. The sugar/BSA substitution ratio was 17 for α-D-Man-BSA and β-D-Gal-BSA and 47 for β-D-GlcNAc-BSA [7].

Enzymatic digestion of porcine thyroglobulin

In some experiments, 10 mg porcine thyroglobulin (Sigma) were treated for 2 h at 37 °C with 1 mg Pronase (Sigma) in

0.02 M Tris, 0.01 M $CaCl_2$, and 0.15 M NaCl. Pronase was then inactivated by heating the solution at 100 °C for 10 min.

In other experiments, 5 mg thyroglobulin were treated twice for 18 h at 37 °C, as described in [17], in 1 ml 100 mM sodium phosphate buffer, pH 7.4, with 150 mU endoglycosidase H (Endo H) from Boehringer (Mannheim, Germany), and then submitted to gel filtration on a PD10 column in PBS, pH 7.4. Thereafter, endoglycosidase-treated thyroglobulin was digested by Pronase.

To control the efficiency of deglycosylation, aliquots of intact or of glycosidase-treated thyroglobulin (5, 0.5, 0.05 µg) were dotted onto nitrocellulose filters. After 30 min at 20 °C, the filter strips were saturated for 1 h at 20 °C with 2 ml Hank's balanced salt solution (HBS; Flow Laboratories, Irving, UK) supplemented with 1 mM $CaCl_2$ (HBS-Ca), 5% BSA, to prevent nonspecific binding of ConA. Excess BSA was washed out with HBS-Ca, 0.5% BSA (HBS-Ca-B), and the strips were incubated in HBS-Ca-B with or without peroxidase-labelled 50 nM ConA (Sigma). After 1 h at 37 °C, strips were washed twice in HBS-Ca-B, 0.02% Tween. Diaminobenzidine (Sigma) in HBS, 1% H_2O_2, was used for staining.

The effect of Pronase (GP-thyroglobulin) or endoglycosidase/Pronase treated thyroglobulin (GP-Endo H-thyroglobulin) on [^{125}I]rgp160 or on [^{125}I]rgp120 interaction with the affinity matrices was then evaluated. As control, we verified that the enzymes incubated under our experimental conditions without thyroglobulin-derived products had no effect on rgp160 or on rgp120 interaction with the matrices.

Envelope glycoprotein affinity chromatography on mannose-agarose or fetuin-agarose

Fifty µl (100 000–150 000 counts min^{-1}) [^{125}I]rgp160 were incubated for 1 h at 37 °C with 500 µl mannose-agarose in 500 µl HBS, 0.01 M $CaCl_2$, and 0.05% BSA. The elution volume of complexes formed by preincubating radiolabelled rgp160 with 2 mg ml^{-1} mannan for 45 min at 37 °C was determined as control. Bound glycoproteins were eluted with 16 mg ml^{-1} mannan in buffer. Gel filtration of the eluted glycoprotein was determined as described above.

The same conditions were used to incubate radiolabelled rgp160 or rgp120 with fetuin-agarose. In this case, the control elution volume was determined by preincubating the glycoprotein with 20 µM fetuin, and elution of bound glycoprotein was performed with 20 mg ml^{-1} mannan or 20 µm fetuin. As control, it was verified that 20 µM BSA applied to the column did not induce any elution.

Results

Rgp160 Interaction with mannose-agarose and fetuin-agarose matrices

We have previously shown that HIV-1 envelope glycoprotein presents specific β-D-*N*-acetylglucosamine binding properties [7]. To extend these findings, we examined here [^{125}I]rgp160 interactions with two different affinity matrices: mannose-agarose, in which mannose residues are presented via a divinylsulfone bridge, and fetuin-agarose, which presents the natural bovine fetuin glycoprotein.

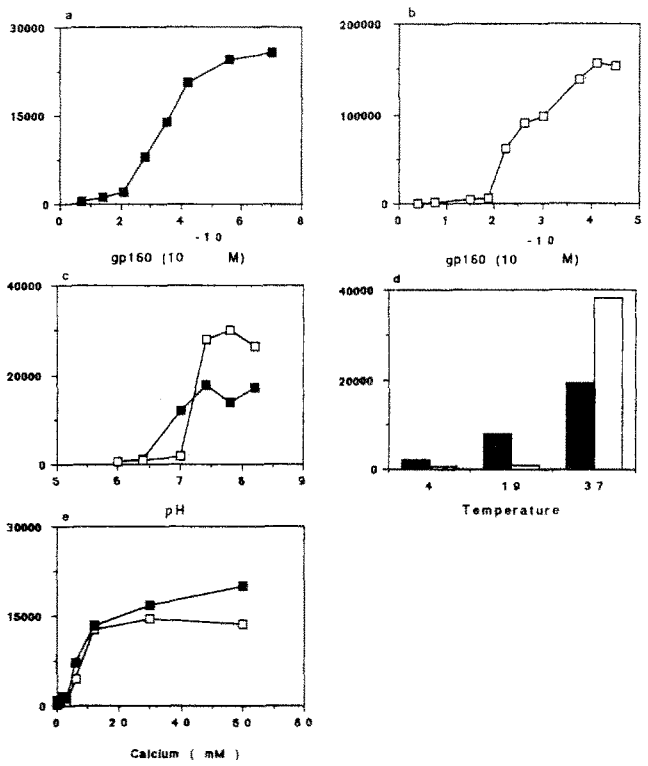

Figure 1. Influence of physico-chemical conditions on [^{125}I]rgp160 binding to ■, mannose-agarose and to □, fetuin-agarose. (a, b) rgp160 concentration: different amounts of [^{125}I]rgp160 were incubated for 1 h at 37 °C with 20 µl of matrix; (c) effect of pH; (d) temperature: [^{125}I]rgp160 (10^{-10} M) was incubated with the matrix at 4 °C, 19 °C or 37 °C; (e) effect of Ca^{2+} concentration. Representative results of at least three independent experiments.

[^{125}I]rgp160 bound to both matrices in a dose dependent and saturable manner (Fig. 1a, b). Binding was influenced by the pH: undetectable below pH 6.5 for mannose-agarose, or below pH 7.1 for fetuin-agarose, it plateaued from pH 7.4 upwards (Fig. 1c). Binding depended highly on the temperature: inefficient at 4 °C, and intermediate (with mannose-agarose) or borderline (with fetuin-agarose) at 19 °C, it was strongest at 37 °C in both cases (Fig. 1d). With respect to Ca^{2+}, significant binding was already noted at 2.5 mM Ca^{2+} with mannose-agarose but not with fetuin-agarose, where it occurred significantly at 6.6 mM; in both cases the plateau began at 13 mM Ca^{2+} (Fig. 1e), which indicates specific calcium dependency of binding.

In Table 1 are presented the mean 50% inhibiting concentrations (C_{50}) and maximum inhibition percentages noted when different soluble carbohydrate structures were preincubated with [^{125}I]rgp160 before addition to

Table 1. Inhibition of [^{125}I]rgp160 binding to mannose-agarose by preincubation with different carbohydrate compounds.

Carbohydrate compound	C_{50}	*Maximum inhibition noted with*	
		Concentration	*% inhibition*
Mannan	0.5 mg ml^{-1}	2 mg ml^{-1}	71 ± 12
	Simple carbohydrates		
Me-α-Man		80 mM	13 ± 13
Me-α-Glc		80 mM	27 ± 24
Me-α-Gal		80 mM	8 ± 8
	Neoglycoproteins		
α-D-Man$_{17}$-BSA	6 μM	60 μM	81 ± 21
β-D-GlcNAc$_{47}$-BSA	60 μM	60 μM	54 ± 9
β-D-Gal$_{17}$-BSA		60 μM	32 ± 10
	Natural glycopeptides		
GP-thyroglobulin	1 mg ml^{-1}	2 mg ml^{-1}	80 ± 15
GP-Endo H-thyroglobulin		2 mg ml^{-1}	45 ± 31

Results are expressed as mean % inhibition ± SE of 3 to 7 independent experiments.

mannose-agarose in order to test the carbohydrate specificity of the interaction. Binding of rgp160 was inhibited to >50% by mannan, soluble α-D-Man$_{17}$-BSA, β-D-GlcNAc$_{47}$-BSA or GP-thyroglobulin glycopeptides. GP-Endo-H-thyroglobulin glycopeptides gave only partial inhibition. β-D-Gal$_{17}$-BSA had a more limited effect. β-D-glucan had no effect (data not shown).

It was verified that the Endo H-treated thyroglobulin did not interact anymore in a dot-blot assay with ConA (data not shown). Therefore, it can be assumed that the oligomannose chains of this preparation had been removed appropriately.

Binding of radiolabelled rgp160 to fetuin-agarose was similarly inhibited (Table 2) by mannan, α-D-Man$_{17}$-BSA, β-D-GlcNAc$_{47}$-BSA and GP-thyroglobulin, in addition to fetuin as well as asialofetuin. GP-Endo H-thyroglobulin had limited inhibitory effect. β-D-Gal$_{17}$-BSA had no significant effect.

In addition to evaluating C_{50}, we also attempted in these experiments to estimate the intrinsic dissociation constant (K_d) of soluble rgp160/carbohydrate complexes by the procedure of Horejsi *et al.* [18]. The K_d, however, could not be determined in this manner, because of the lack of linearity of the plot determining the free soluble (S)/bound (B) rgp160 ratio versus the concentration (C) of soluble

Table 2. Inhibition of [^{125}I]rgp160 or of [^{125}I]rgp120 binding to fetuin-agarose by different carbohydrate compounds.

Carbohydrate compound	C_{50}		*Maximum % inhibition*	
	rgp160	*rgp120*	*rgp160*	*rgp120*
Mannan	0.05 mg ml^{-1}	0.25 mg ml^{-1}	72 ± 26	77 ± 14
		Simple carbohydrates		
N-acetylglucosamine			39 ± 39	26 ± 26
N-acetylgalactosamine			29 ± 29	0
		Neoglycoproteins		
α-D-Man$_{17}$-BSA	13 μM	9 μM	84 ± 16	74 ± 26
β-D-GlcNAc$_{47}$-BSA	4 μM	12 μM	72 ± 18	60 ± 23
β-D-Gal$_{17}$-BSA			28 ± 28	17 ± 17
		Natural glycoproteins		
Fetuin	5 μM	2 μM	96 ± 4	79 ± 24
Asialofetuin	6 μM	6 μM	94 ± 5	75 ± 30
Thyroglobulin		0.9 μM		74 ± 15
		Natural glycopeptides		
GP-thyroglobulin	1 mg ml^{-1}	1 mg ml^{-1}	86 ± 9	86 ± 5
GP-Endo H-thyroglobulin			42 ± 27	54 ± 24

Results are expressed as mean % inhibition ± SE of 3 to 7 independent experiments. Concentrations for maximum % inhibition were 0.5 mg ml^{-1} for mannan, 2 mg ml^{-1} for the glycopeptides, 75 mM for *N*-acetylglucosamine and *N*-acetylgalactosamine, 60 μM for the neoglycoproteins, 20 μM for fetuin and asialofetuin, 2.75 μM for thyroglobulin.

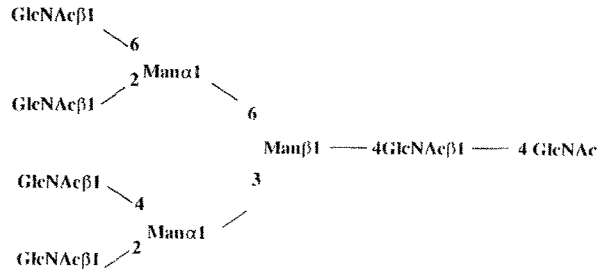

Figure 2. Structure of the complex type asialo-agalacto-tetra-antennary precursor oligosaccharide isolated from human α_1-acid glycoprotein.

carbohydrate added, which did not allow us to apply the relevant calculations.

With both matrices simple derivatives such as Me-α-Man, Me-α-Glc or Me-α-Gal had no significant inhibitory effects, even at concentrations up to 80 mM (Table 1, and data not shown). *N*-Acetylglucosamine and *N*-acetylgalactosamine presented an irreproducible and limited effect at 80 mM only (Table 2), but not at 30 mM (data not shown). Of note, 20 μM complex type asialo-agalacto-tetraantennary precursor oligosaccharide of human α_1-acid glycoprotein (see Fig. 2) inhibited rgp160 binding to fetuin-agarose by a mean of 50% ($n = 2$). In contrast, 20 μM oligomannose 9 from porcine thyroglobulin, complex fucose-substituted asialo-agalacto- or galacto-biantennary core of porcine thyroglobulin and complex-type asialo-galactosylated triantennary oligosaccharides from bovine fetuin did not show any significant effect. For logistical reasons, these structures were not tested at concentrations > 20 μM. Therefore, considering the C_{50} values reported in Tables 1 and 2, it cannot be excluded that higher concentrations would not have induced inhibitory effects.

Taken together, these data indicate that rgp160 specifically interacts, presumably in a complex manner, both with mannosyl and β-D-*N*-acetyl-glucosaminyl derivatives, when the latter are presented either on a carrier or at high density, but not with sialic acid or β-D-galactosyl derivatives.

Interaction of rgp120 with fetuin-agarose

Because our previous results suggested that the *N*-acetylglucosaminyl binding site of HIV-1 envelope glycoprotein was presumably located on gp120 [7], we also examined whether [^{125}I]rgp120 specifically bound to fetuin-agarose. Again, we observed specific, saturable, pH, temperature and calcium dependent rgp120 binding to the matrix (data not shown), the latter being similar to that observed with rgp160. Binding was inhibited, though to a lesser extent than that of [^{125}I]rgp160, by fetuin and asialofetuin, as well as by β-D-GlcNAc$_{47}$-BSA, α-D-Man$_{17}$-BSA, thyroglobulin, GP-thyroglobulin and mannan, but not by β-D-Gal$_{17}$-BSA (Fig. 3; Table 2). Interestingly, however, low asialofetuin concentratons (2–3 μM) could enhance rgp120 binding to the matrix, suggesting possible

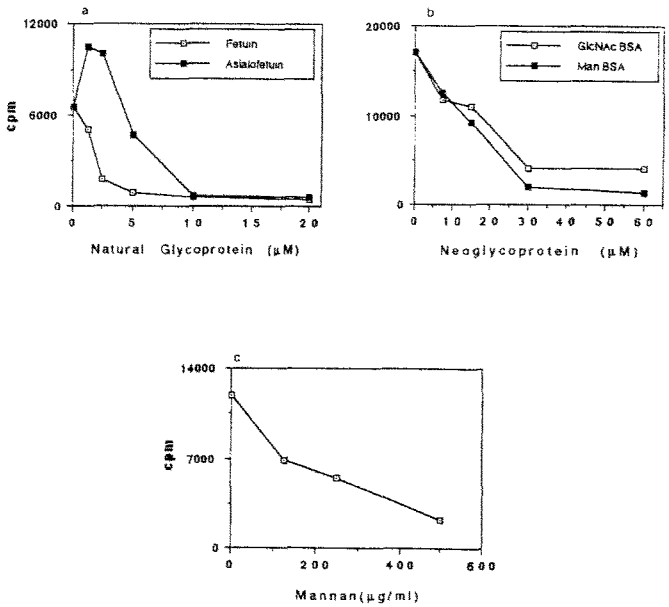

Figure 3. Inhibition of [^{125}I]rgp120 binding to fetuin-agarose by: (a) fetuin or asialofetuin; (b) β-D-GlcNAc$_{47}$-BSA or α-D-Man$_{17}$-BSA; (c) mannan.

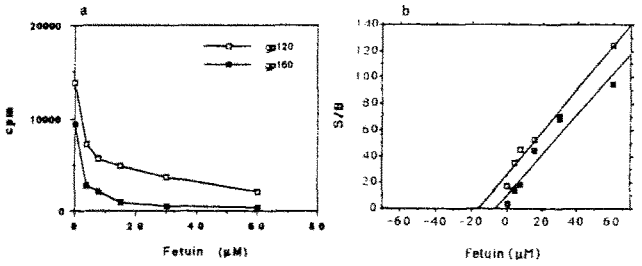

Figure 4. Inhibition by fetuin of (a) [^{125}I]rgp160 or [^{125}I]rgp120 binding to GlcNAc-agarose; (b) K_d of the interaction between rgp160 or gp120 and fetuin. S/B: soluble/bound rgp160 or rgp120.

conformational changes in the same manner as when sCD4 binds to gp120 [19].

Thus, in addition to intereacting with *N*-acetylglucosamine residues presented on neoglycoproteins, gp120 can also interact with these carbohydrate moieties present on natural complex type glycans, such as bovine fetuin and porcine thyroglobulin, as well as with mannosyl core residues or oligomannose structures.

Binding of rgp160 and of rgp120 to GlcNAc-agarose

Because soluble β-D-GlcNAc$_{47}$-BSA inhibited the binding of rgp160 to both mannose-agarose and fetuin-agarose matrices, we examined in addition rgp160 binding to GlcNAc-agarose. The results obtained (Fig. 4) confirmed our previous findings obtained with three other matrices: β-D-GlcNAc$_{47}$-BSA-sepharose, asialo-agalactothyroglobulin-agarose and GlcNAc-divinylsulfone-agarose [7].

Again (data not shown), rgp160 binding to the matrix

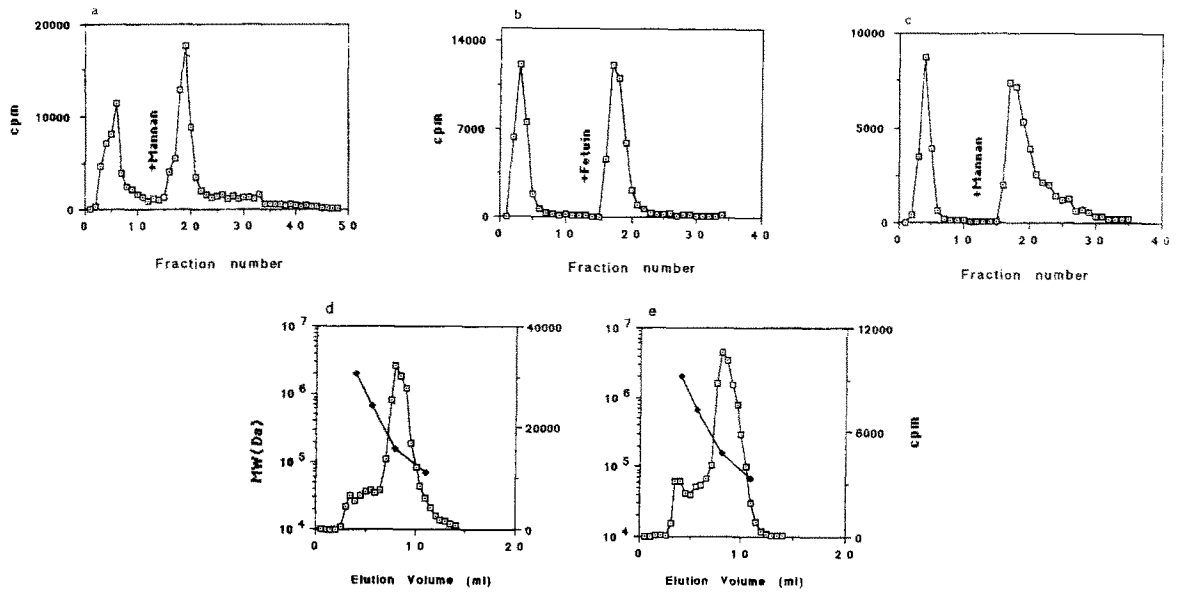

Figure 5. [^{125}I]rgp160 affinity chromatography on (a) mannose-agarose, and (b) fetuin-agarose; specific elution by fetuin or by (c) mannan. Gel filtration chromatography on a Bio-Gel A-1.5 m column of (d) rgp160 which had not been submitted to affinity chromatography and of (e) rgp160 specifically eluted by mannan from mannose-agarose; rgp160 specifically eluted from fetuin-agarose had the same elution profile. Molecular mass markers were Dextran blue 2000 (2000 kDa), thyroglobulin (660 kDa) bovine IgG (155 kDa) and albumin (67 kDa).

was saturable, temperature dependent (most efficient at 37 °C), calcium dependent (optimum at 2.5 mM $CaCl_2$) and pH dependent (maximum at pH 7.4). The interaction was carbohydrate-specific inasmuch as it was significantly inhibited by β-D-$GlcNAc_{47}$-BSA (maximum inhibition of $51.5 \pm 8\%$ being attained at 60 μM; $n = 8$) and by fetuin ($95 \pm 1.5\%$ of inhibition with 60 μM; $n = 3$) (Fig. 4(a)).

Here, the physico-chemical conditions of [^{125}I]rgp160 binding to GlcNAc-agarose allowed us to calculate the K_d of soluble rgp160–fetuin complexes by Horejsi's procedure. Bound (B) and free soluble (S) rgp160 fractions were measured after addition of soluble fetuin (concentration C). This resulted in the inhibition of [^{125}I]rgp160 binding to the matrix so that the S/B ratio increased with C, the linear plot of S/B versus C yielding K_d as its intercept with the x axis. Using the data of a representative experiment gave a K_d of 8 μM (Fig. 4b), a value of the same order of magnitude as the C_{50} observed for soluble fetuin when the binding of rgp160 to fetuin-agarose was examined, and in the same range as the K_d previously reported for rgp160/β-D-$GlcNAc_{47}$-BSA complexes [7].

When the same experiment was performed with [^{125}I]rgp120, 60 μM fetuin inhibited by 95% its binding to GlcNAc-agarose (Fig. 4a), the K_d of rgp120–fetuin complexes being then 16 μM (Fig. 4b). Thus, gp120 is involved in the binding of *N*-acetylglucosamine but its affinity for soluble fetuin appears lower than that of rgp160.

Affinity chromatography of envelope glycoproteins on mannose-agarose and fetuin-agarose

To confirm the specificity of these interactions by a different experimental approach, [^{125}I]rgp160 was submitted to affinity chromatography on mannose-agarose. This resulted in 72% of the molecules being retained on the column, most of which (62% of the total amount) being specifically eluted with 16 mg ml^{-1} mannan while the rest remained still bound to the matrix (Fig. 5a). The 28% of molecules that had not been retained by the matrix were, nonetheless, eluted with mannan as a significantly retarded peak relative to the elution volume of precomplexed rgp160, but they were not retained but only eluted as a significantly retarded peak upon repeated chromatography of the matrix. This suggests the heterogeneity of rgp160 species and/or that some degree of molecular alteration possibly occurred during the radiolabelling procedure.

Comparable results were obtained using fetuin-agarose: 70% of rgp160 molecules were retained on the matrix, and eluted either by 20 μM fetuin (Fig. 5b) or by 20 mg ml^{-1} mannan (Fig. 5c), but not by 20 μM BSA, which demonstrated the specificity of the elution. As a control, it was verified that the gel filtration chromatography elution profiles or rgp160 specifically eluted fractions were similar to that of original rgp160 (which had not been submitted to affinity chromatography): in all cases, there existed a minor peak eluted with the void volume of the column and which could correspond to oligomeric rgp160 [20] and

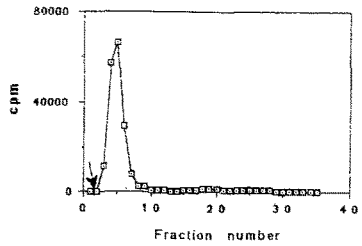

Figure 6. [^{125}I]rgp120 affinity chromatography on fetuin-agarose. The arrow indicates the elution volume of [^{125}I]rgp120–fetuin preformed complexes obtained after 45 min incubation at 37 °C.

a major peak of 155 kDa apparent molecular mass (Fig. 5d,e) which indicates the presence of monomeric rgp160. Therefore, the specifically eluted fractions were indeed rgp160.

As to radiolabelled rgp120, it was not retained on the matrix but eluted only as a significantly retarded fraction compared with the elution volume of rgp120–fetuin preformed complexes (Fig. 6).

These data confirm that rgp160 specifically interacts with the two matrices via mannosyl core residues of N-linked complex carbohydrates in the case of fetuin and with oligomannose structures such as those of mannan. That rgp120 was not retained by fetuin-agarose but only eluted as a retarded fraction suggests the involvement of gp41 in addition to that of gp120, some sites of which might possibly be more readily accessible on precursor gp160 than on mature gp120 [21].

Discussion

HIV-1 envelope glycoprotein has been shown to interact specifically with different carbohydrate derivatives: polyanionic compounds such as dextran sulfate or heparin [20, 22, 23]; *N*-acetylglucosamine structures presented at high density on protein carriers [7]; galactose or galactosyl sulfatides presented on a lipid carrier as galactosyl sulfatide ceramide or galactosyl ceramide [11, 24], the linkage between galactose and ceramide appearing then essential for gp120 binding [24].

Here, we confirm and extend our own previous results on the GlcNAc-binding properties of rgp160 and viral gp120 [7], by showing that rgp160 and rgp120 can also interact with mannosyl structures in the same manner as already reported for other *N*-acetylglucosamine-binding proteins [12, 13]. Glycoprotein binding to carbohydrate-substituted matrices was highly temperature dependent, which implies enthalpically driven interactions such as that recently reported for CD4 binding to gp120 [25], for example.

The interaction of rgp160 with mannose-agarose affinity matrix was specifically inhibited by mannosyl residues presented by mannan, which comprises a high mannose type oligosaccharide structure based on a backbone of repeating (1-6)-linked α-mannose residues with short 1-2 and (1-3)-linked α-mannose side chains [26], and by the α-D-Man_{17}-BSA neoglycoprotein. An *N*-acetylglucosaminyl neoglycoprotein, β-D-$GlcNAc_{47}$-BSA, and glycopeptides derived by Pronase digestion from natural porcine thyroglobulin (which contains 31% of oligo mannose and 69% of complex N-linked glycans [27, 28]) also inhibited rgp160 binding to mannose-agarose. Glycopeptides obtained from thyroglobulin treated with Endo-H, which specifically cleaves N-linked oligosaccharides with oligomannose cores but not complex type N-linked glycans, had limited effect.

These results indicate that rgp160 can indeed bind (i) to mannosyl residues immobilized on a divinylsulfone matrix, or presented at high density in solution by a neoglycoprotein or as N-linked glycans from a natural glycoprotein, or presented by a peptidic carrier as oligomannose or core mannosyl structures of complex N-linked glycans; and (ii) to soluble *N*-acetylglucosaminyl residues presented on a neoglycoprotein or on glycans from natural glycoproteins, or immobilized on *p*-aminophenyl-agarose. Such findings are compatible with a mixed mannosyl/*N*-acetylglucosaminyl binding protein. This was confirmed by showing mannosyl/*N*-acetylglucosaminyl specific binding of rgp160 with an affinity matrix substituted by a natural glycoprotein, bovine fetuin, the carbohydrate structure of which consists for 20% of O-linked glycans and for 80% of N-linked complex type glycans [29–31]. Here, in addition to soluble fetuin and asialofetuin, binding of rgp160 to the matrix was inhibited also by α-D-Man_{17}-BSA, β-D-$GlcNAc_{47}$-BSA, mannan, GP-thyroglobulin, and complex type asialo-agalacto-tetraantennary precursor oligosaccharide of human α_1-acid glycoprotein, but not by β-D-Gal_{17}-BSA.

Mannosyl specificity of rgp160 interactions with the matrices was further indicated by affinity chromatography experiments, which showed that the majority (approximately 70%) of radiolabelled molecules were specifically retained on both mannose-agarose and fetuin-agarose matrices, and eluted in both cases by mannan. That not all the labelled molecules were retained might be due to the complexity of the interaction, to the heterogeneity of rgp160 species population, or to their alterated conformation and/or degradation during radiolabelling or during the purification procedure which, nonetheless, resulted in a 90% homogeneous preparation as determined by SDS-PAGE.

Binding of rgp160 to the matrices was not inhibited by Me-α-Man but by mannan and by α-D-Man_{17}-BSA, which indicates that this binding property depends on mannosyl residue presentation. Similarly, envelope glycoprotein binding was noted for *N*-acetylglucosaminyl structures presented in neoglycoprotein [7] or glycoprotein carriers, or at high density on free complex type asialo-agalacto-tetraantennary chains, which could inhibit rgp160 binding to fetuin-agarose. However, because the 8 μM K_d noted here for rgp160 binding to fetuin is close to that previously shown for rgp160 binding to β-D-$GlcNAc_{47}$-BSA (12 μM) [7], this

suggests that the carrier's molecular constitution does not play a major role in the affinity of such binding.

The interaction of rgp160 with carbohydrates is probably of a complex nature inasmuch as the maximum level of binding inhibition to the matrices by some of the soluble compounds was only partial. For example, maximum inhibition of rgp160 binding to mannose-agarose could be ordered as follows: α-D-Man_{17}-BSA (81%) > GP-thyroglobulin > mannan > β-D-$GlcNAc_{47}$-BSA > β-D-Gal_{17}-BSA (32%). Such possible complexity of interaction is also exemplified by the lack of linearity for the S/B versus *C* plot that is observed in all cases investigated here, but for rgp160 binding to GlcNAc-agarose, when attempting to estimate the soluble complexes K_d by Horesji's procedure [18]. Therefore, in such cases only C_{50} could be estimated which, nonetheless, were of the same order of magnitude than those reported for some, but not all, other mannose-binding proteins [12, 13].

Because glycopeptides derived from Endo H-treated thyroglobulin had limited effect on rgp160 binding to mannose-agarose and to fetuin-agarose, it may be further suggested that rgp160 can bind to high mannose as well as to core N-linked mannosyl and/or *N*-acetyl-β-D-glucosaminyl structures of complex type glycans such as present on fetuin or on thyroglobulin, [27–31]. Fetuin and asialofetuin had similar inhibitory effects on the binding of rgp160 to fetuin-agarose, whereas β-D-Gal_{17}-BSA had no effect, and one may then exclude the role of sialic acid or galactosyl structures of N- or O-linked glycans in these interactions.

As to the region of rgp160 involved in carbohydrate binding, we observed that rgp120 specifically interacted with fetuin-agarose as well as with GlcNAc-agarose, but its affinity for soluble fetuin was twice as low as that of rgp160. Moreover, rgp120 eluted as a significantly retarded fraction after affinity chromatography on fetuin-agarose, whereas rgp160 was retained by the matrix and specifically eluted, which further confirms rgp120 weaker affinity for fetuin. These data suggest that, in addition to mature gp120, either transmembrane gp41 or gp120 structures, which may be more readily accessible when the molecule is presented as precursor gp160 [21], participate in the interaction.

Nevertheless, we examined three gp120 critical regions for their possible involvement in carbohydrate binding: the CD4-binding region [32], the third variable loop (V3) known as the principal neutralization determinant [33], and gp120 C-terminus [34]. Neither rgp160 preincubation with soluble recombinant CD4 [2] nor with monoclonal antibodies to these two defined gp120 domains (the use of which is described in [35]) modified its binding to mannose-agarose or to fetuin-agarose matrices (data not shown).

Taken together, the present results indicate that as well as the *N*-acetyl-β-D-glucosaminyl binding property previously described [7], HIV-1 envelope glycoproteins also present mannosyl binding properties for core mannosyl residues of complex type N-linked or for oligomannose glycans. Such properties could be used by HIV-1 to attach 'accessory' membrane molecules besides CD4, as an additional step necessary for virus–cell or for cell–cell fusion which is needed for infectivity [9, 10], and/or they could possibly be used to infect target cells by CD4-independent mechanisms [10, 11, 36].

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