Glycoconjugate Journal (1994) **11**:73–79

The role of calcium and *N*-linked glycans in the oligomerization and carbohydrate binding properties of human immunodeficiency virus external envelope glycoprotein

MHENIA HAIDAR[1], NABILA SEDDIKI[2], JEAN CLAUDE GLUCKMAN[2] and LILIANE GATTEGNO[1]*

[1] *Laboratoire de Biologie Cellulaire, Faculté de Médecine Paris-Nord, 93012 Bobigny, France*

[2] *CNRS URA 1463, CERVI, Hôpital de la Pitié-Salpêtrière, 75651 Paris Cedex 13, France*

Received 19 July 1993, revised 16 February 1994

Envelope glycoproteins of human immunodeficiency virus (gp120 and gp41) occur as oligomers. Here, we show by gel filtration analysis that gp120 oligomerization *in vitro* is calcium- and temperature-dependent. Recombinant gp120 (rgp120) species were recovered as monomers at 20 °C in the absence of calcium, but as tetramers at 37 °C in 10 mM $CaCl_2$. Under the latter condition, *N*-glycanase-deglycosylated rgp120 formed hexamers. Relative to intact rgp120, which has been reported to display carbohydrate-binding properties for *N*-acetyl-β-D-glucosaminyl and mannosyl residues, deglycosylation enhanced rgp120 specific binding to mannose-divinylsulfone-agarose, para-aminophenyl-β-D-GlcNAc-agarose and fetuin-agarose matrices. Taken together, these results rule out the role of homologous lectin-carbohydrate interactions via *N*-linked glycans in the rgp120 oligomerization, even though its lectin properties may also be calcium-dependent. Deglycosylation may unmask domains of rgp120 polypeptide backbone that independently play a role either in rgp120 lectin activity or in calcium-dependent oligomerization.

Keywords: HIV; env glycoprotein; gp120; oligomerization; glycosylation; calcium

Introduction

Envelope glycoproteins of human immunodeficiency virus (HIV), external gp120 and transmembrane gp41, are expressed as di-, tri- or tetrahetero-oligomers at the surface of virions or on infected cells [1–3]. The gp120 subunit is highly glycosylated [4–9], and *N*-linked glycans represent approximately 50% of its apparent molecular weight (MW) in addition to a minor fraction of *O*-linked structures [10].

We have recently shown that gp120, whether in native or recombinant (rgp120) form, and recombinant precursor rgp160, display calcium-dependent carbohydrate-binding properties for *N*-acetyl-β-D-glucosaminyl residues of oligosaccharides, oligomannose chains and the mannosyl core of complex type *N*-linked glycans [11, 12]. Therefore, the hypothesis that different gp120 molecules may interact with each other by lectin-carbohydrate interactions to form oligomers may be proposed, since this mechanism has already been demonstrated for some other glycoproteins [13, 14].

The aim of the present report is to examine whether *N*-linked glycans are involved in the carbohydrate binding properties of rgp120 and possibly also in its oligomerization, which we have recently reported to be calcium-dependent [15]. Therefore, using gel filtration chromatography, we comparatively analysed the oligomerization of intact and *N*-glycanase-treated rgp120 and its ability to bind to different carbohydrate derivatives.

Materials and methods

Recombinant gp120

Rgp120, >90% pure, was a gift from the MRC AIDS Directed Program (South Mimms, UK). Radiolabelling was performed by the iodogen method, as described [5]. The iodinated glycoprotein was separated from $Na^{125}I$ by filtration through a Sephadex G-25 (PD 10) column (Pharmacia, Uppsala, Sweden). The specific activity was 1.1 MBq μg^{-1}.

Homogeneity of the rgp120 preparation was assessed by

* To whom correspondence should be addressed.

sodium dodecyl sulfate-polyacrylamide gel (10%) electrophoresis (SDS-PAGE) [4, 5].

Using human, or rabbit antisera prepared in our laboratory, and soluble CD4 (sCD4: a gift from D. Klatzmann, CERVI, Hôpital Pitié Salpêtrière, Paris, France), we verified that labelled rgp120 was normally immunoreactive and bound to CD4, as previously described [12].

Enzymatic digestion of rgp120

^{125}I-rgp120 ($\simeq$11 ng) in 94 µl PBS, pH 7.2, was incubated at 37 °C with 6 µl of endoglycosidase F/*N*-glycanase (Boehringer Mannheim, Germany; 300 mU µl^{-1}). After 12 h incubation, the same amount of enzyme was again added for 12 h. The efficiency of deglycosylation was controlled by SDS-PAGE (10%), by gel filtraton of intact or *N*-glycanase-treated ^{125}I-rgp120, and by the binding to ConA dotted on to nitrocellulose. Aliquots (2 µl; 2µg) of ConA (Sigma, Saint-Louis, MO) or of anti-gp160 rabbit serum (2 µl; diluted 1:10 or 1:100; a gift from A. Benjouad, CERVI, Hôpital Pitié-Salpêtrière, Paris, France) were dotted on to nitrocellulose filters. After 30 min at 20 °C, the filter strips were saturated for 1 h at 37 °C with 2 ml PBS supplemented with 5% BSA (PBS-BSA) to prevent nonspecific binding. Excess BSA was washed out with PBS-BSA-Tween 20, 0.01% (Sigma), and the strips were incubated for 1 h at 37 °C with intact or *N*-glycanase-treated radiolabelled rgp120 (10 000 cpm); after six washes with PBS-BSA, the strips were exposed at 20 °C for 19 h.

Binding of rgp120 to carbohydrate substituted affinity matrices

The binding of radiolabelled rgp120 to fetuin-agarose (Sigma) was investigated as follows: 20 µl of the 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 (Tris-Φ Ca-BSA). After incubation with various concentrations of rgp120 (0.4–6 × 10^{-10} M) for 1 h at 37 °C, unbound rgp120 was washed out twice in 500 µl of buffer. Solid phase-bound radioactivity was counted in a gamma counter (LKB) and the results were expressed as means of duplicates. The physicochemical characteristics of the interaction were analysed by performing the assays under different conditions: buffer without $CaCl_2$ (Tris-BSA) or with different $CaCl_2$ concentrations; pH ranging from 6 to 8.2; and different temperatures, 4, 19 and 37 °C.

Binding of intact or *N*-glycanase-treated ^{125}I-rgp120 (0.4–0.6 × 10^{-9} M) to D-mannose-divinyl-sulfone-agarose (mannose-agarose), *p*-aminophenyl-β-D-GlcNAc-agarose (GlcNAc-agarose), both from E. Y. Laboratories (San Mateo, CA, USA), or to fetuin-agarose, was also investigated at 37 °C in Tris-Φ-Ca-BSA as described [12]. Results were expressed as means of duplicates.

To determine the carbohydrate specificity of the interactions, prior to the binding test radiolabelled rgp120 (0.4–0.6 × 10^{-9} M) was incubated for 45 min at 37 °C with µM concentrations of fetuin or β-D-GlcNAc-BSA (gift from D. Bladier, Laboratoire de Biotechnologie des Proteines, Bobigny, France), or with mM concentrations of mannose 6-phosphate (M6P) (Sigma) [12]. The carbohydrate derivatives were then present in the assay mixture during the whole experiment. Reversal of rgp120 binding to fetuin-agarose was investigated as follows: 250 µl of radiolabelled rgp120 (5 × 10^{-10} M) was incubated for 1 h at 37 °C with 50 µl of affinity matrix in Tris-Φ-Ca-BSA. Unbound glycoprotein was washed out twice in 500 µl of buffer; the matrix was then incubated at 20 °C for 15 h with gentle shaking in 500 µl of buffer supplemented or not with 30 mM of M6P. The rgp120 molecules which were specifically eluted from the fetuin-agarose matrix were analysed by SDS-PAGE (4–20%).

Gel filtration chromatography

Gel filtration of ^{125}I-rgp120 was performed at 20 °C or at 37 °C on Sephadex G-200 columns (8 × 1 cm or 30 × 0.9 cm) (Pharmacia) pre-equilibrated in either of the following buffers: 0.02 M Tris, 0.15 M NaCl, 0.01 M $CaCl_2$, pH 7.4 without (Tris-Φ-Ca) or with 0.05% BSA (Tris-Φ-Ca-BSA); or phosphate buffered saline (PBS, 0.15 M NaCl, 0.008 M Na_2HPO_4, 0.002 M NaH_2PO_4), pH 7.4, 0.05% BSA (PBS-BSA). In some experiments ^{125}I-rgp120 was pre-incubated for 1 h at 37 °C in Tris-Φ-Ca or in PBS. Chromatography was performed at 10 ml h^{-1} flow rate in buffer with BSA. Under these conditions, no radioactivity nonspecifically bound to the columns.

The columns were calibrated by Dextran blue 2000 (2000 kDa), thyroglobulin (669 kDa), catalase (232 kDa), aldalose (158 kDa), albumin (67 kDa) and ovalbumin (43 kDa), all from Pharmacia.

Intact and *N*-glycanase treated rgp120 molecules were also analysed by PAGE (4%) under non-denaturating conditions, i.e. without SDS and mercaptoethanol, after 1 h incubation at 37 °C in Tris-Φ-Ca buffer.

Results and discussion

As previously reported [12], we observed specific, saturable, calcium-, pH- and temperature-dependent rgp120 binding to fetuin-agarose (Fig. 1a–d), mannose-agarose and GlcNAc-agarose (data not shown), the maximum of which corresponded to 20% of the radioactivity added. Binding of rgp120 to the matrices was specifically reversed by carbohydrate derivative ligands: 84% of rgp120 binding to fetuin-agarose was reversed by M6P, a specific ligand of rgp120 [16]. Specifically eluted molecules were characterized as rgp120 according to their MW after SDS-PAGE (Fig. 1e).

We evaluated, then, whether *N*-glycanase, which cleaves both oligomannose and complex-type *N*-linked glycans [17], efficiently deglycosylated rgp120. After enzyme treatment, intact and *N*-glycanase-treated radiolabelled rgp120

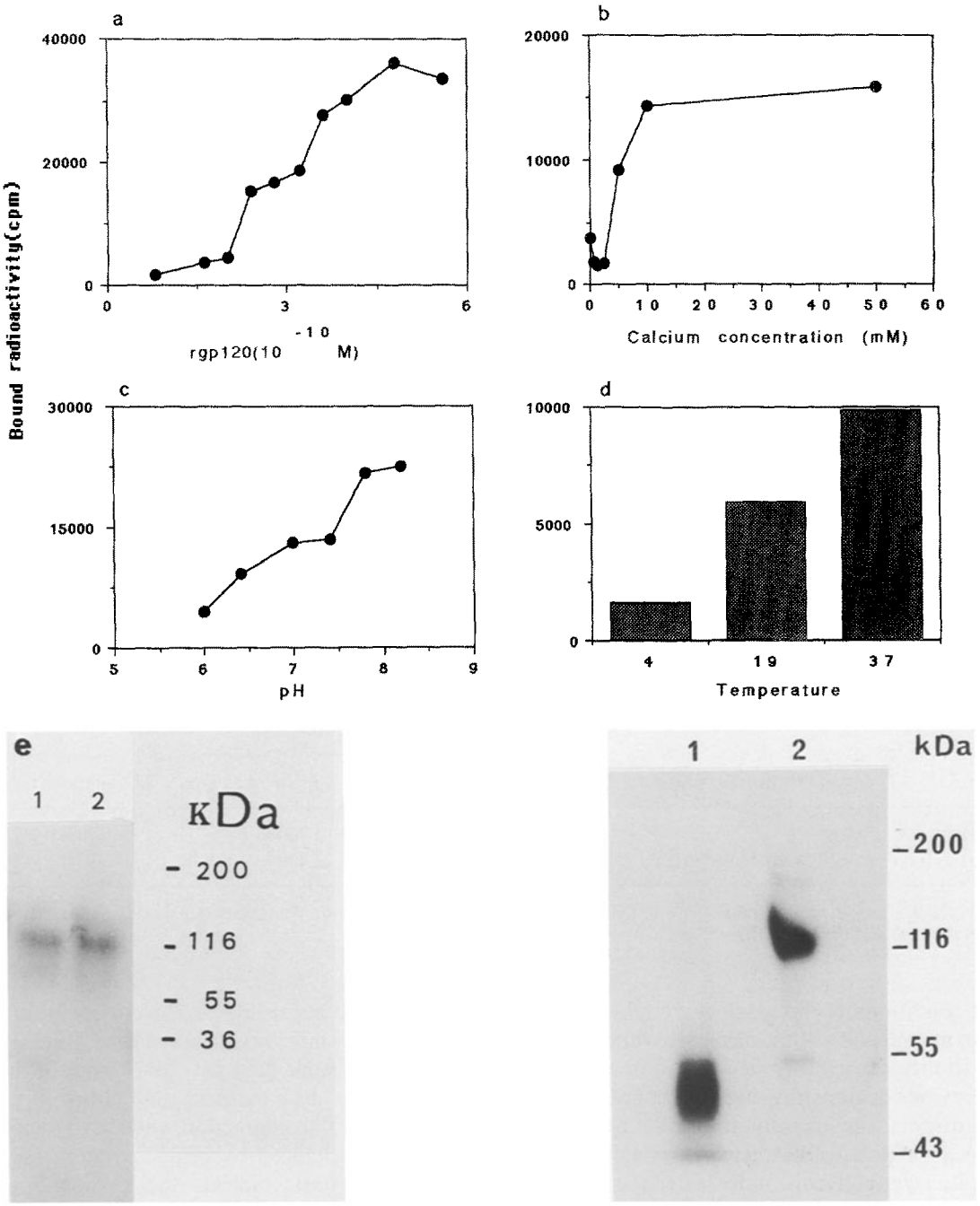

Figure 1. Binding of rgp120 to fetuin-agarose. (a) Different amounts of rgp120 were incubated for 1 h at 37 °C with 20 µl of matrix; (b) effect of Ca^{2+} concentration; (c) effect of pH; (d) temperature: rgp120 was incubated with the matrix at 4 °C, 19 °C or 37 °C; (e) SDS-PAGE and autoradiography of rgp120 (1) after and (2) before specific elution from fetuin-agarose by M6P.

migrated as single bands of 116 and 52 kDa MW, respectively, as determined by SDS-PAGE (Fig. 2a). As expected, intact rgp120, but not *N*-glycanase-treated rgp120, bound to ConA dotted on to nitrocellulose (Fig. 2b). In line with previous findings [4, 5], these data indicate the appropriate removal of *N*-linked glycans from rgp120. In addition, intact and deglycosylated rgp120 bound in a similar manner to rabbit anti-gp160 polyclonal antibodies (Fig. 2b), which indicates that deglycosylation did not modify rgp120 immunoreactivity.

The effect of *N*-linked glycans on gp120 carbohydrate-binding properties was then evaluated. Binding of deglycosylated rgp120 to mannose-agarose, GlcNAc-agarose and fetuin-agarose was significantly enhanced relative to that of intact rgp120 (Table 1). Under the

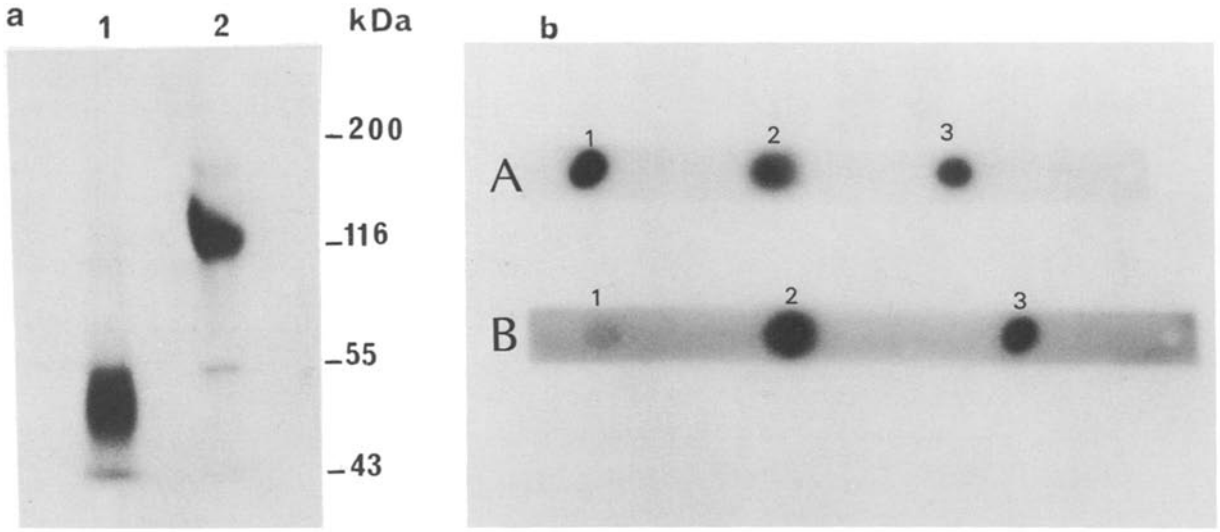

Figure 2. Characterization of intact and *N*-glycanase-treated rgp120. (a) SDS-PAGE and autoradiography of intact (lane 1) or *N*-glycanase-treated (lane 2) rgp120. (b) Binding of intact (A) or of *N*-glycanase-treated rgp120 (B) to ConA (1) or to polyclonal anti-gp160 rabbit antibodies; serum diluted 1:10 (2) or 1:100 (3).

Table 1. Binding of intact or *N*-glycanase-treated ^{125}I-rgp120 to carbohydrate-substituted affinity matrices.

	^{125}I-rgp120 binding (cpm) to:		
	Mannose-agarose	*GlcNAc-agarose*	*Fetuin-agarose*
(a) intact ^{125}I-rgp120	8 600 ± 2400	3 700 ± 600	7 400 ± 2800
(b) *N* glycanase-treated ^{125}I-rgp120	90 000 ± 8600**	23 400 ± 5000*	32 140 ± 5900*

Results are expressed as means ± SEM of five to seven independent experiments. Statistical significance of the differences between (a) and (b): * $p < 0.05$; ** $p < 0.01$.

experimental conditions of the assay, 4–7.5% of the intact rgp120 species and 40–47% of their deglycosylated counterparts bound to the matrices.

We have previous demonstrated [12] that gp120 binding to fetuin-agarose is significantly inhibited by mannan, α-D-Man-BSA, β-D-GlcNAc-BSA, fetuin, asialofetuin, thyroglobulin and its tryptic glycopeptides. Glycopeptides from endoglycosidase-H-treated thyroglobulin have limited effect, whereas β-D-Gal-BSA has no effect. Here also, preincubation of rgp120 with β-D-GlcNAc-BSA, followed by co-incubation with fetuin-agarose, led to the dose-dependent inhibition of its binding to the matrix, which presents *N*-acetylglucosaminyl residues [12], indicating the carbohydrate specificity of the interactions (Fig. 3 and data not shown). The C_{50} noted with deglycosylated rgp120 was two-fold higher than with intact rgp120 (20 vs 10 μM); therefore, such enhanced binding could not be related to increased rgp120 affinity for the carbohydrate derivatives. In addition, M6P also inhibited the binding of intact and of deglycosylated gp120 to fetuin-agarose (Fig. 4a,b), with a mean 72% ($n = 2$) and 92% ($n = 2$) inhibition, respectively, and a six-fold lower K_d (determined according to Horesji [18]) for deglycosylated rgp120 (0.4 mM vs 2 mM). These findings confirm our previous results [16] on gp120 specific interaction with M6P. They also indicate that *N*-linked glycans are not involved in this interaction, and they show on the contrary that it is enhanced by deglycosylation of gp120. Because previous reports indicate that *N*-linked glycans are not necessary to maintain the conformation of mature gp120, while they play a critical role in its folding and processing during synthesis [4, 5, 19, 20], increase of deglycosylated rgp120 binding to the carbohydrate-substituted matrices may rather result from the unmasking of region(s) of the polypeptide backbone able additionally to interact with specific carbohydrate ligands in a manner similar to that recently described for antibody binding to HIV envelope glycoproteins [21]. However, increased binding of deglycosylated gp120 to the affinity matrices may also be related to a reduced solubility of the deglycosylated molecule and to a better penetration of the agarose beads by these molecules, characterized by a smaller hydrodynamic volume.

We have previously shown [15] that rgp120 oligomerization

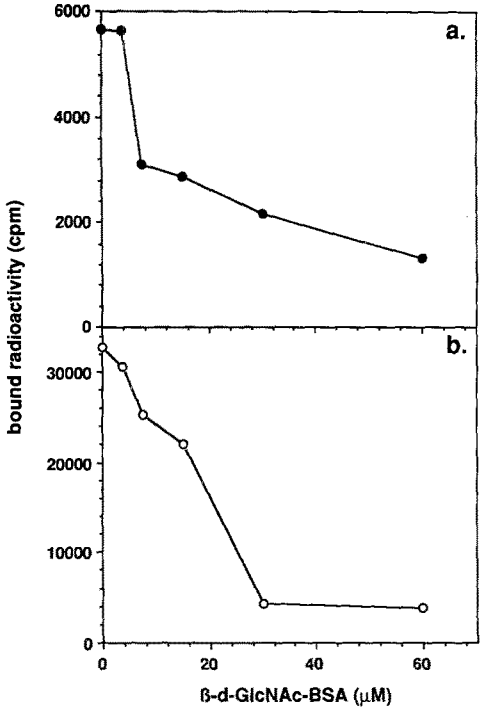

Figure 3. Inhibition by β-D-GlcNAc-BSA of the binding of intact (a) or *N*-glycanase-treated; (b) rgp120 to fetuin-agarose.

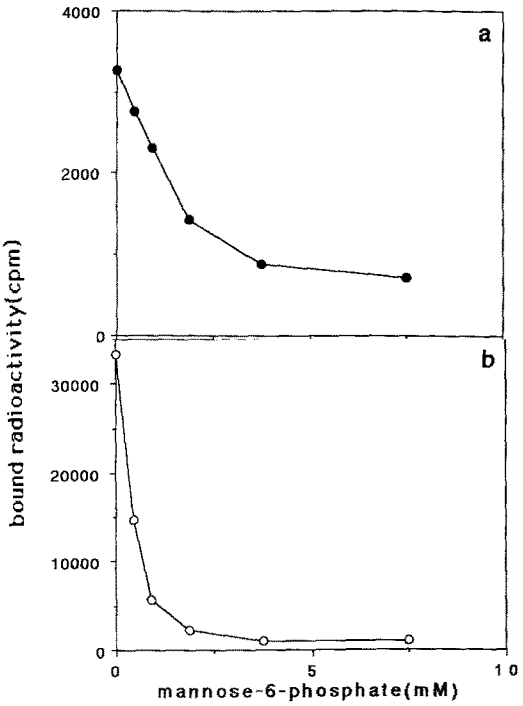

Figure 4. Inhibition by M6P of the binding of intact (a) or *N*-glycanase; (b) rgp120 to fetuin-agarose.

in vitro was calcium-dependent. This led us to examine the effects of calcium, temperature and gp120 *N*-linked glycans on the oligomerization of the molecule. After gel filtration chromatography at 20 °C in the absence of calcium and of SDS (Fig. 5a), intact and deglycosylated rgp120 were recovered especially as 116 and 52 kDa peaks, respectively, an indication that the majority of the molecules presented as monomers under these conditions. However, the multiple peaks observed with intact and deglycosylated rgp120 suggest that a minor proportion of the respective molecules were already oligomerized. After 1 h at 37 °C, rgp120 was recovered as 240 kDa species in calcium-free PBS (Fig. 5b), but as 440 kDa species in the presence of 10 mM of calcium (Fig. 5c). These findings are compatible with the occurrence of rgp120 dimers after preincubation at 37 °C in the absence of calcium and of tetramers in its presence. Under the latter conditions, *N*-glycanase-treated rgp120 was characterized as 300 kDa species (Fig. 5c). Considering the MW of each deglycosylated rgp120 monomer (circa 50 kDa), it may therefore be assumed that deglycosylated rgp120 presented as hexamers. The low MW compounds also noted after gel filtration chromatography of the rgp120 preparation represented less than 10% of the labelled molecules, and they may be either contaminants or degradation products. After incubation for 1 h at 37 °C in Tris-Φ-Ca buffer, intact and deglycosylated rgp120 was also analysed by PAGE (4%) under non-denaturating conditions: after 1 h migration, neither glycoprotein entered the gel; but after 3 h migration each presented the electrophoretic mobility corresponding to the MW of its monomeric form (data not shown). These results further demonstrate the oligomerization state of the intact and deglycosylated rgp120 in the presence of Ca^{2+} ions, and indicate the reversibility of this process.

The assembly of HIV envelope glycoproteins occurs in the endoplasmic reticulum (ER), where gp160 oligomerizes before it moves to the Golgi [1, 3, 22]. While assembly of oligomers likely involves the ectodomain of gp41 [1, 2, 22, 23], there is also indication that gp120 subunits are closely associated in the native envelope structure [24]. In this respect, it is of note that the ER is one of the major stores of cellular calcium, the high concentration of which distinguishes it from other compartments [22], and this might account for the calcium-dependence of gp120 oligomer formation noted here.

The involvement of lectin-carbohydrate interactions in gp120 oligomerization process – via their respective *N*-linked glycans – may be ruled out if only because deglycosylated rgp120 is still able to oligomerize and even to present as hexamers instead of tetramers as noted for the intact molecule under the same conditions. Such difference may be accounted for either by the unmasking of additional polypeptidic assembly domains or by modifications of the glycoproteins' physico-chemical properties (hydrophilicity, charge, etc) that would result in increasing their possible reciprocal interactions.

In conclusion, the present results indicate that gp120 *N*-linked glycans are not involved in its carbohydrate binding properties, inasmuch as they may interfere with such lectin activity, and that neither gp120 glycans nor its

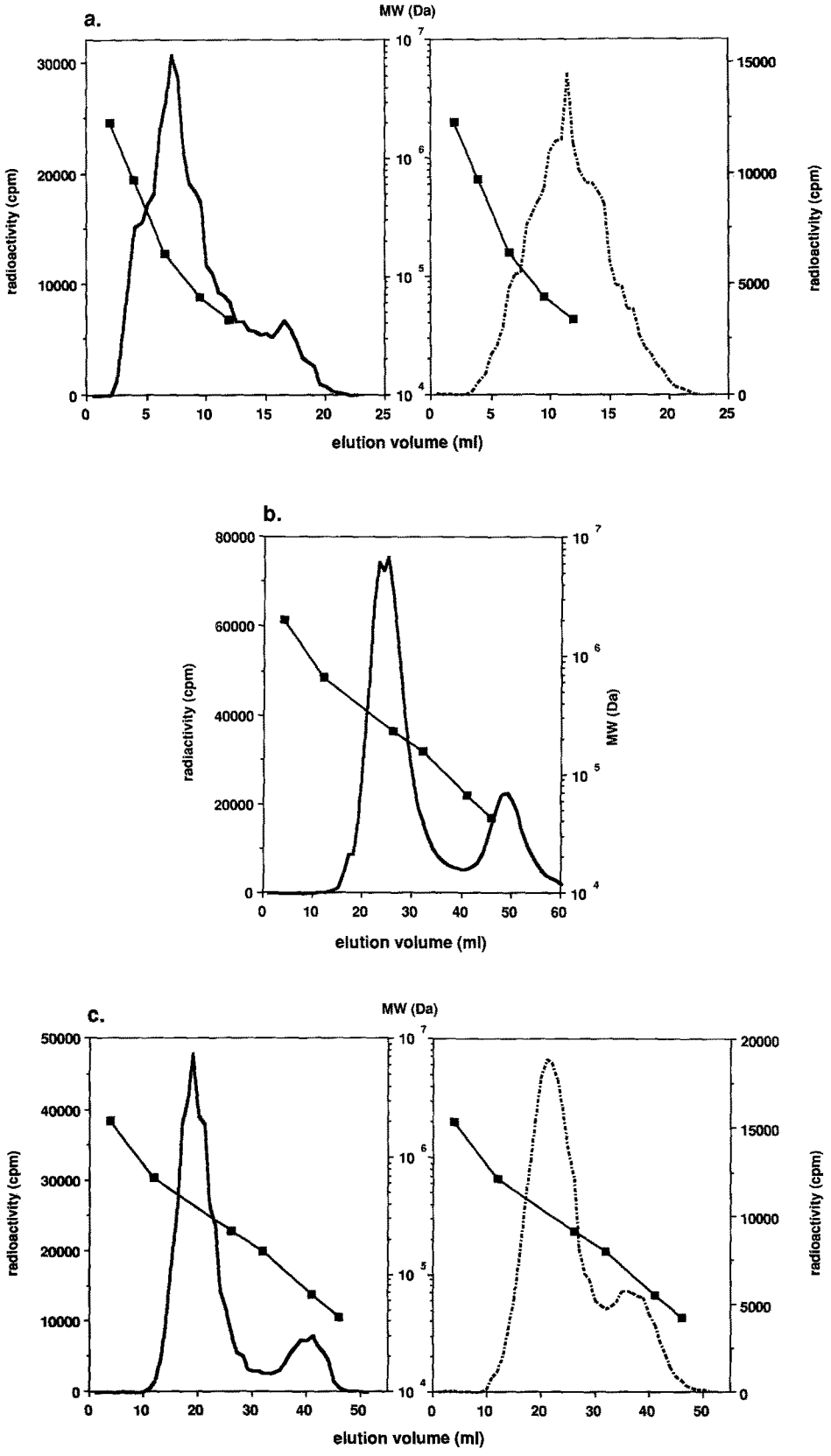

Figure 5. Gel filtration chromatography of intact (——) or of deglycosylated rgp120 (— · —. (a) PBS, 20 °C; Sephadex G-200 (8 × 1 cm column). Preincubation for 1 h at 37 °C in (b) PBS without calcium, or (c) in Tris-Φ-Ca; Sephadex G-200 (30 × 0.9 cm column). Molecular mass markers: (closed squares) dextran blue 2000 (2000 kDa), thyroglobulin (669 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa) and ovalbumin (43 kDa).

carbohydrate-binding properties play a role in the calcium- and temperature-dependent oligomerization of the molecule.

Acknowledgements

This work was supported by the Direction de la Recherche et des Enseignements Doctoraux (Ministère de l'Enseignement Supérieur et de la Recherche), Université Paris XIII and Université Paris VI, and by the Centre National de la Recherche Scientific (Paris, France).

References

1. Stein BS, Engleman EG (1990) *J Biol Chem* **265**:2640–49.
2. Earl PL, Dom RW, Moss B (1990) *Proc Natl Acad Sci USA* **87**:648–52.
3. Fenouillet E, Gluckman JC (1992) *Virology* **187**:825–28.
4. Fenouillet E, Gluckman JC, Bahraoui E (1990) *J Virol* **64**:2841–48.
5. Fenouillet R, Clerget-Raslain B, Gluckman JC, Guetard L, Montagnier L, Bahraoui E (1989) *J Exp Med* **169**:807–22.
6. Geyer H, Holschbach C, Hunsmann G (1988) *J Biol Chem* **263**:11760–67.
7. Mizuochi T, Spellman MW, Larkin M, Solomon J, Basa LJ, Feizi T (1988) *Biochem J* **254**:599–603.
8. Mizuochi T, Matthews TJ, Kato M, Hamako J, Titani K, Solomon J, Feizi T (1990) *J Biol Chem* **256**:8519–24.
9. Leonard CK, Spellman MW, Riddle L, Harris RJ, Thomas JN, Gregory TJ (1990) *J Biol Chem* **265**:10373–82.
10. Bolmstedt A, Olofsson S, Jansson ES, Jeansson S, Sjöblom I, Åkerblöm L, Hansen JES, Hu SL (1992) *J Gen Virol.* **73**: 3099–105.
11. Gattegno L, Sadeghi H, Saffar L, Bladier D, Clerget-Raslain B, Gluckman JC, Bahraoui E (1991) *Carbohydr Res* **213**:79–93.
12. Haidar M, Seddiki N, Gluckman JC, Gattegno L (1992) *Glycoconjugate J* **9**:315–23.
13. Barbaric S, Mrsa V, Mies B, Mildner P (1984) *Arch Biochem Biophys* **234**:567–75.
14. Bürgi W (1989) In *Alpharacid Glycoprotein: Genetics, Biochemistry, Physiological Functions, and Pharmacology* pp 723–45. (Alan M. Liss Inc. Ed.).
15. Mbemba E, Czyrski JA, Gattegno L (1992) *Biochim Biophys Acta* **118**:123–39.
16. Mbemba E, Gluckman JC, Gattegno L (1994) *Glycobiology*, in press.
17. Tarentino AL, Gomez CM, Plummer TH (1985) *Biochemistry* **24**:4665–71.
18. Horejsi V, Matousek M, Ticha T, Trnka T (1985) In *Lectins* (Bøg-Hansen TC, ed.) pp. 298–306. Berlin: Walter de Gruyter.
19. Fenouillet E, Gluckman JC (1991) *J Gen Virol* **72**:1919–26.
20. Fenouillet E, Jones I, Powell B, Schmitt D, Kieny MP, Gluckman JC (1993) *J Virol* **67**:150–60.
21. Benjouad A, Gluckman JC, Rochat H, Montagnier L, Bahraoui E (1992) *J Virol* **66**:2473–82.
22. Doms RW, Lamb RA, Rose JK, Helenius A (1993) *Virology* **193**:545–62.
23. Pinter A, Honnen WJ, Tilley SA, Bona C, Zaghouani H, Gorny MK, Zolla-Pazner S (1989) *J Virol* **63**:2674–79.
24. Weiss CD, Levy JA, White JM (1990) *J Virol* **64**:5674–77.