ROLE OF *N*-LINKED GLYCANS IN THE INTERACTION BETWEEN THE ENVELOPE GLYCOPROTEIN OF HUMAN IMMUNODEFICIENCY VIRUS AND ITS CD4 CELLULAR RECEPTOR

Structural Enzymatic Analysis

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The interaction of two glycoproteins, gp120, the major envelope protein of the human immunodeficiency virus (HIV), and CD4, a 55-kD cell membrane molecule, accounts for HIV's tropism and cytopathic effect (1). CD4, predominantly expressed on helper T lymphocytes but also on other cells, including macrophages and monocytes, is considered as the main cellular receptor for HIV (2, 3). The envelope glycoprotein of HIV is first synthesized as a 160-kD precursor that is later cleaved into external gp120 and transmembrane gp41 (4). The binding site to CD4 is located on gp120, while the region responsible for membrane fusion appears to be located at the NH_2 terminus of gp41 (5).

Carbohydrate moieties of glycoproteins are known to play different roles in intracellular routing (6), protection of the molecule against proteolytic degradation (7), governing correct folding and stabilizing bioactive conformation of the molecule (7), or as epitopes for antibody immune responses (8, 9). Carbohydrates are also involved in the regulation of molecular metabolism (10–12), growth and differentiation (11, 13), and in cell-to-cell adhesion (14, 15).

In viruses, carbohydrates have been implicated directly in the virus biological activity (16), or indirectly in the maintenance of its tridimensional and quaternary structure (16–18). They are also used by viruses as well as by parasites to escape the immune defenses of the infected host (19).

Few, and sometimes contradictory, results have been reported regarding the carbohydrate composition of gp120 and its role in binding to CD4 (20–22), and even less have been reported with respect to CD4.

Using different glycosidases, we have approached the carbohydrate composition and optimal deglycosylation conditions of soluble recombinant gp160 and gp120,

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and of CD4. Binding assays of gp120 or of gp160 to radiolabeled soluble CD4 at the molecular level and to CD4 receptor expressed at the cell membrane have allowed us to examine whether deglycosylation of either molecule was of importance for their interaction.

Materials and Methods

Soluble Recombinant gp120 and gp160. gp120 and gp160 were gifts from Transgène S. A. (Strasbourg, France). Briefly, the *env*-coding sequence of gp120, in which a chain-terminating codon was introduced to prevent the synthesis of gp41, was inserted in the thymidine-kinase gene of vaccinia virus (VV)[1]. Recombinant VV-infected BHK-21 cells produced gp120 that was then easily released from the cell membrane. The molecule was purified by ammonium sulfate and ethanol precipitation, followed by reverse phase HPLC. The gp160 precursor was produced using a similar protocol. To prevent the subsequent cleavage of the precursor gp160 into gp120 and gp41, the potential coding sequence of the cleavage site was deleted (23).

Soluble CD4. Soluble recombinant CD4 (sCD4) was a gift from Dr. R. Axel (Columbia University, New York, NY) (24–27).

Purification of Anti-gp120 Murine Monoclonal (110-4). mAb 110-4 (Genetic Systems, Seattle, WA) (28, 29) recognizes an epitope located at position 308–328 on gp120 of HIV-1 LAV_{BRU}. IgG were purified in one step by filtration of 1 ml of ascites fluid through a Sephadex G 200 column (Pharmacia Fine Chemicals, Uppsala, Sweden) (2 × 100 cm). Thereafter, 10 mg of IgG was coupled to 4 g of CNBr Sepharose CL4B (Pharmacia Fine Chemicals) according to the manufacturer's instructions.

Iodination of sCD4 and of gp160. 10 μg of sCD4 in 50 μl of PBS pH 7.4, and 0.25 mCi of Na ^{125}I (13–17 mCi/μg) (Amersham International, Amersham, UK) were added to 5 nmol of an iodogen-coated tube and incubated for 10 min at room temperature. The reaction was stopped by adding 10 μl of tyrosine at 9 mg/ml. The iodinated glycoprotein was desalted from Na ^{125}I by filtration through a Sephadex G25 (PD 10) column (Pharmacia Fine Chemicals). Specific radioactivity of radiolabeled sCD4 (^{125}I-sCD4) was ~5.5 μCi/μg.

5 μg of soluble gp160 were radiolabeled according to the same protocol. Specific radioactivity of radiolabeled gp160 (^{125}I-gp160) was there ~36 μCi/μg.

Analytical Deglycosylation of ^{125}I-gp160. This treatment was carried out under different conditions: either 1 ng of ^{125}Igp160 (50,000 cpm) was incubated with different amounts of Endo F commercial mixture (Endo F N-glycanase) (Boehringer Mannheim Biochemicals, FRG) (2×10^{2}–2×10^{-2} mU) at 37°C for 24 h in 30 μl of 50 mM PBS, pH 7.4, with or without 1% (vol/vol) Triton X-100 (Sigma Chemical Co., St. Louis, MO) and 0.02% (wt/vol) SDS (Bio-Rad Laboratories, Richmond, CA) or it was treated with different amounts of Endo F *N*-glycanase free (Boehringer Mannheim Biochemicals) (2×10^{2}–2×10^{-2} mU) at pH 5.0 in 30 μl of 100 mM sodium acetate buffer with or without 1% Triton X-100 and 0.02% SDS. Treatment with Clostridium perfringens neuraminidase (Sigma Chemical Co.) (50 mU), or with Endo H (Boehringer Mannheim Biochemicals) (2×10^{-4}–2 mU) was performed at pH 5.0 under similar conditions. Native or deglycosylated proteins were heated at 100°C for 3 min in the presence of 3% β-ME (Sigma Chemical Co.), and then submitted to 10% SDS-PAGE according to Laemmli (30). Gels were exposed to Kodak x OMat film for 20 h.

Deglycosylation of gp120 and gp160 Used for Binding Assays. Deglycosylation of gp160 and of gp120 was performed as follows. 20 μg of the molecule was incubated in 120 μl of PBS, pH 7.4. Endo F *N*-glycanase (3 U) was sequentially added four times at 3-h intervals. After digestion, aliquots of 1 μg of gp120 and of gp160 were analyzed by SDS-PAGE and Coomassie staining. Moreover, radiolabeled ^{125}I-gp160 was used in one experiment in order to determine by analytical electrophoresis the degree of deglycosylation obtained for this molecule under these conditions (data not shown).

Analytical Deglycosylation of ^{125}I-sCD4. Increasing amounts of endoglycosidase (2×10^{-4}–2 mU of Endo H; 2×10^{-2}–2×10^{2} mU of Endo F *N*-glycanase or Endo F) were added to

[1] *Abbreviation used in this paper:* VV, vaccinia virus.

7 ng of ^{125}I-sCD4 (50,000 cpm) in a final volume of 30 μl of previously described buffer in the presence of Triton X-100 and 0.02% SDS. SDS-PAGE was then performed.

Preparative Deglycosylation of ^{125}I-sCD4 for Binding Assays. Deglycosylated ^{125}I-sCD4 was prepared as follows. 100 μl of ^{125}I-sCD4 (10^6 cpm) were incubated with 500 mU of Endo F *N*-glycanase at pH 7.4 under the same conditions as described above, with the exception that SDS was omitted.

Molecular Binding Assays. To test the role of CD4 carbohydrate moieties in the interaction of this molecule with gp120 or gp160 (thereafter referred to as gp120/160), we compared the binding capacity of glycosylated and deglycosylated ^{125}I-sCD4 in the following test. 50 μl of mAb 110-4 coupled to Sepharose CL4B (Pharmacia Fine Chemicals) (vol/vol) were incubated with different amounts of gp 120/160 (0.5 ng to 500 ng) for 2 h at 4°C. Unbound soluble gp120/160 was eliminated by two washes with 1 ml of PBS buffer, and a fixed amount of glycosylated or deglycosylated ^{125}I-sCD4 (60,000 cpm) was added. The mixture was incubated for 3 h at 4°C in order to prevent further deglycosylation by glycosidases. After two washes, solid phase–bound radioactivity was counted in a gamma counter (Kontron Analytical, Zurich, Switzerland). The role of carbohydrate moieties of gp120/160 in its interaction with CD4 was analyzed in the same assay but with deglycosylated gp120/160 bound to mAb 110-4 coupled to Sepharose CL4B.

CD4$^+$ Lymphoid Cells. Cells of the CEM line (American Type Culture Collection, Rockville, MD) were cultured at 37°C in RPMI 1640 medium (Flow Laboratories, Inc., Irvine, Scotland) supplemented with 10% FCS, 1% glutamine, and 1% antibiotics (Gibco Laboratories, Paisley, Scotland) in a humidified atmosphere with 5% CO_2.

Binding of Native or Deglycosylated gp120/160 to the CD4$^+$ Cells. Buffer solutions used in this assay were those described by McDougal et al. (31). Different amounts of the native or deglycosylated gp120/160 (0.25–250 ng) were incubated for 1 h at 37°C with 10^6 CEM cells in 25 μl of buffer. The cells were washed and resuspended in 25 μl of mAb 110-4 diluted in buffer (1:100). After 30 min at 4°C, they were washed, and incubated again for 30 min at 4°C with 1:25 sheep anti-mouse IgG coupled to biotin. After washing, cells were resuspended in 25 μl of streptavidin-phycoerythrin (Becton Dickinson & Co., Mountain View, CA) (1:25) for 30 min at 4°C. After two more washes, cells were resuspended in 500 μl of buffer containing 1% paraformaldehyde. Membrane fluorescence intensity was measured by the FACS analyzer (Becton Dickinson & Co.).

Assay for the Inhibition of Syncitium Formation between HIV-infected CEM Cells and Molt-T4 Cells. 10^4 CEM cells that were chronically infected with HIV-1 LAV_{BRU} or HIV-2_{ROD} were cocultured with 4×10^4 uninfected Molt T4 cells in wells (100 μl) of 96-microtiter plates. Cells were incubated for 18 h at 37°C in the presence of different concentrations (6.25–6,250 ng/ml) of native or deglycosylated gp120. The number of syncitia was then counted. As a positive control for the inhibition of cell fusion, the effect of 2.5 μg/ml of anti-Leu-3a (Becton Dickinson & Co.), an HIV-neutralizing anti-CD4 mAb (1), was determined in parallel in each experiment.

Capacity of Native or Deglycosylated gp120/160 to Inhibit ^{125}I-gp160 Binding to CD4$^+$ Cells. 5×10^6 CEM cells were incubated with 50 μl (200,000 cpm) of ^{125}I-gp160 (2×10^{-9} M) and 50 μl of different concentrations (1.6×10^{-7}–3.1×10^{-10} M) of unlabeled native or deglycosylated gp120/160. After 3 h at 37°C, cells were washed with 2×1 ml of PBS buffer and radioactivity of the pellet was determined in a gamma counter.

Results

Endoglycosidases are specific enzymes that cleave carbohydrates at their attachment point on the glycoprotein amino acid skeleton, dependent on parameters such as the accessibility of the internal N acetyl glucosamine, the nature of the glycans, the enzyme concentration, and pH (32).

Deglycosylation of ^{125}I-gp160. To determine whether the accessibility of glycan moieties of gp160 was important for the efficiency of deglycosylation, we tested different conditions of enzymatic treatment. Treatment of gp160 with 0.02% SDS and 1%

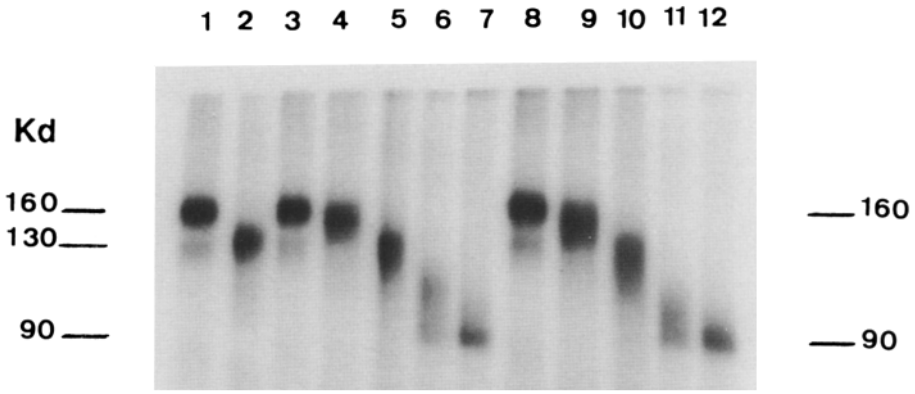


FIGURE 1. SDS PAGE and autoradiography of ^{125}I-gp160 after neuraminidase or Endo F *N*-glycanase treatment. Lane *1*, Overnight incubation at pH 7.4 as control. Lane *2*, 50 mU of neuraminidase. Lanes *3–7*, 2×10^{-2}–2×10^{2} mU of Endo F *N*-glycanase in the absence of SDS and Triton X-100. Lanes *8–12*, 2×10^{-2}–2×10^{2} mU of Endo F *N*-glycanase in the presence of 0.02% SDS and 1% Triton X-100.

Triton X-100, or by heating at 100°C for 3 min before incubation (data not shown), did not change the end result of the procedure; accessibility of glycans to the enzymes was identical whether the molecule had been denatured or not (Figs. 1 and 2).

Endo F *N*-glycanase, which comprises Endo F and contaminant *N*-glycanase (33), cleaves all glycan species from the molecule (34, 35); complete deglycosylation was obtained with 200 mU of enzymes, leading to the decrease of the molecular mass of the deglycosylated molecule from 160 to 90 kD (Fig. 1).

Endo F cleaves most of the high mannose, hybrid type glycans, and biantennary species (32, 34). Deglycosylation of gp160 by 200 mU of Endo F decreased its molecular mass to 130 kD (Fig. 2).

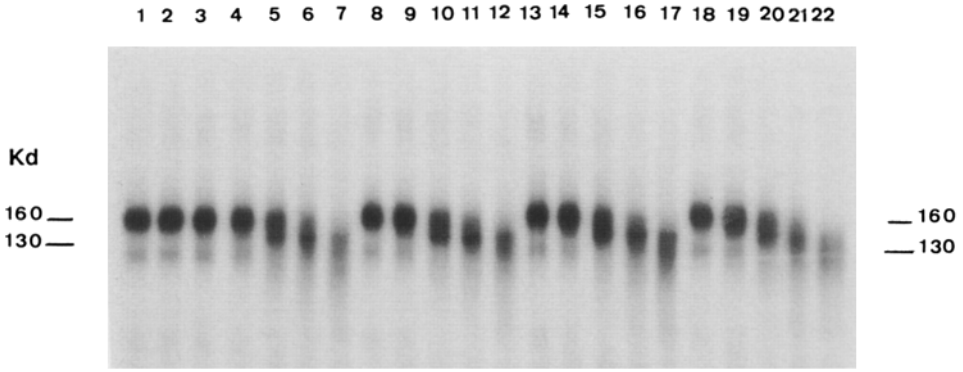


FIGURE 2. SDS-PAGE and autoradiography of ^{125}I-gp160 after Endo H or Endo F treatment. Lanes *1* and *2*, incubation at pH 7.4 (*1*) or 5 (*2*) as controls. Lanes *3–7*, 2×10^{-4}–2 mU of Endo H in the absence of SDS and Triton X-100. Lanes *8–12*, 2×10^{-4}–2 mU of Endo H in the presence of 0.02% SDS and 1% Triton X-100. Lanes *13–18*, 2×10^{-2}–2×10^{2} mU of Endo F in the absence of SDS and Triton X-100. Lanes *19–22*, 2×10^{-2}–2×10^{2} mU of Endo F in the presence of 0.02% SDS and 1% Triton X-100.

Endo H cleaves most of the high mannose species (32). Here again, deglycosylation of gp160 by this enzyme (2 mU) decreased its molecular mass to 130 kD (Fig. 2).

Desialylation by neuraminidase, which removes sialic acid molecules, decreased the molecular mass of gp 160 to 130 kD (Fig. 1).

To compare and characterize the efficiency of the deglycosylation of gp160 and gp120 used for the binding assays, SDS-PAGE analysis of these molecules was performed before and after enzymatic treatment. Native gp160 and its radioactive counterpart had similar characteristics (data not shown). Treatment of gp120 with Endo H, Endo F *N*-glycanase, and Endo F resulted in decreases of molecular mass in the same proportions as for gp160 (Fig. 3). Lower molecular mass bands are enzymes (65 and 72 kD) (Fig. 3, lane *2*, for example).

Deglycosylation of ^{125}I-sCD4. To rule out the hypothetical influence of glycan ac-

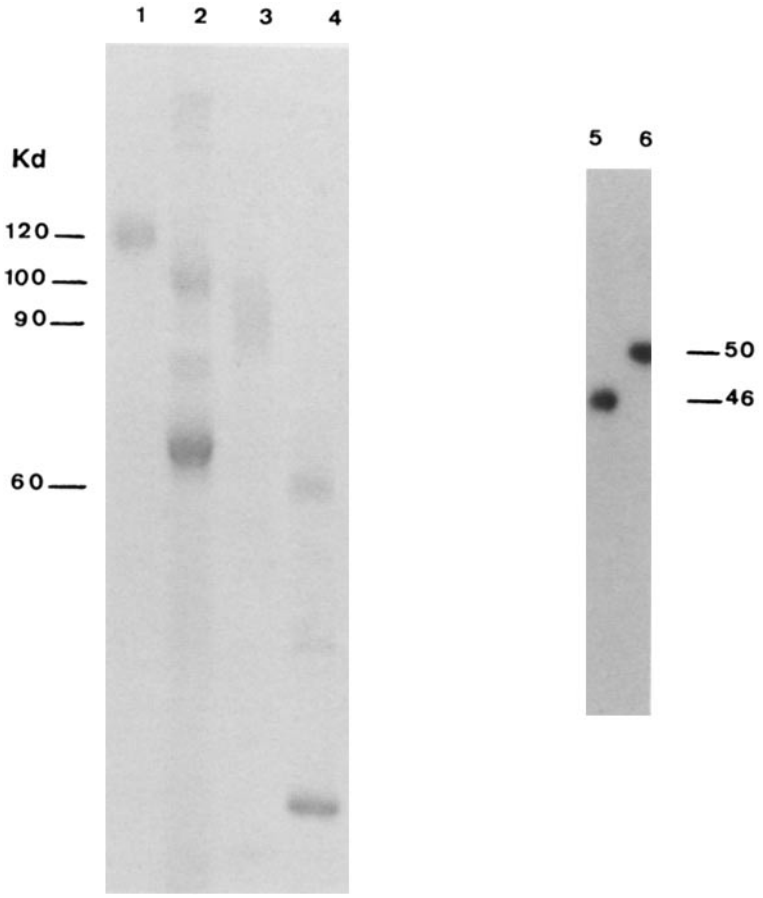


FIGURE 3. SDS-PAGE of gp120 and of ^{125}I-sCD4 used in the binding assays after treatment with different glycosidases. Lane *1*, gp120 incubated overnight at pH 7.4 as control. Lane *2*, 1 μg of gp120 treated with neuraminidase (500 mU). Lane *3*, 1 μg of gp120 treated with Endo H (20 mU) in the absence of SDS and Triton X-100. Lane *4*, 20 μg of gp120 incubated with Endo F *N*-glycanase as used in binding assays (1 μg aliquot). Lane *5*, deglycosylated ^{125}I-sCD4 used in the binding assay (absence of SDS). Lane *6*, ^{125}I-sCD4 incubated overnight at pH 7.4 as control.

cessibility to the enzymes, we first compared the degree of deglycosylation obtained under denaturing in the presence of SDS or nondenaturing conditions (data not shown). Results obtained under both conditions were similar.

5 mU of Endo H, as well as 200 mU of Endo F, failed to deglycosylate 7 ng of sCD4 (Fig. 4), whereas Endo F *N*-glycanase at pH 7.4 deglycosylated sCD4 to the same extent whether in the presence (Fig. 4) or in the absence (Fig. 3) of SDS.

Three forms of the molecule appeared when using 2×10^{-1} mU of Endo F *N*-glycanase: glycosylated CD4 (50 kD); CD4 with one glycan chain (48 kD); and totally deglycosylated CD4 (46 kD) (Fig. 4). The use of 2 mU of Endo F *N*-glycanase at pH 7.4 led to a unique homogeneous deglycosylated form of the molecule of 46 kD (Fig. 4), a shift in molecular mass that could be accounted for by the removal of two glycan chains. When evaluated in the presence of Triton X-100 and in the absence of SDS, deglycosylation of sCD4 used for binding assays was also found to be complete (Fig. 3).

Binding of ^{125}I-sCD4 to gp120/160. mAb 110-4 recognizes an epitope located between amino acid 308 and 328 of gp120 of the LAV_{BRU} isolate of HIV-1. Although this mAb significantly inhibits the binding of HIV-1 to $CD4^+$ cells and even neutralizes virus infectivity, it recognizes soluble gp120 already complexed to $CD4^+$ cells and only reduces by twofold the avidity of gp120 binding to CD4 (28, 29). This indicates that the epitope recognized by mAb 110-4 is not involved in the binding site of gp120 to CD4. We took advantage of this characteristic to set up a quantitative assay to measure binding of sCD4 to the beads. mAb 110-4 was coupled to Sepharose CL4B beads and then complexed with native gp120/160; ^{125}I-sCD4 was added and allowed to bind to gp120/160. ^{125}I-sCD4 fixed to gp120/160-mAb 110-4-Sepharose

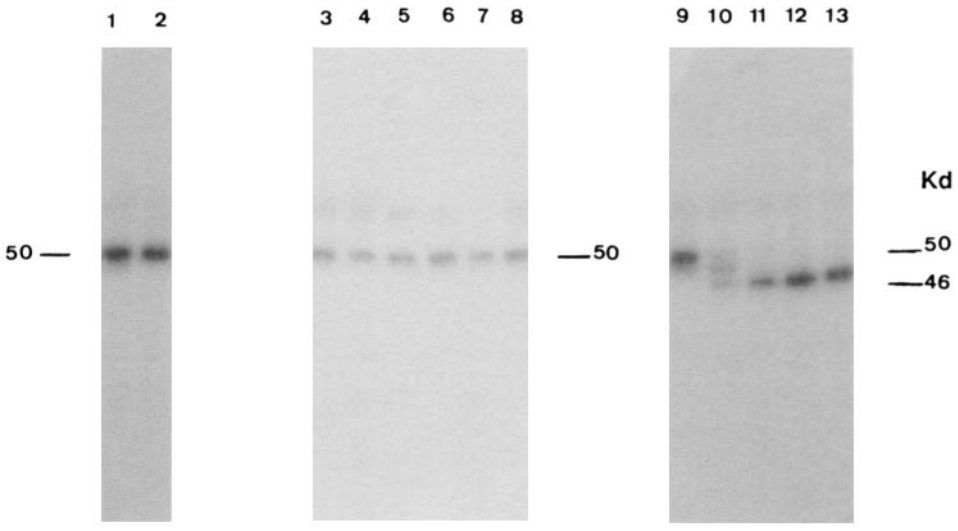


FIGURE 4. SDS PAGE and autoradiography of ^{125}I-sCD4 after Endo F *N*-glycanase, or Endo F or Endo H treatment. Lanes *1* and *3*, overnight incubation at pH 5 (*1*) or 7.4 (*3*) as controls. In all other lanes, ^{125}I-sCD4 was treated in the presence of 0.02% SDS and 1% Triton X-100. Lane *2*, Endo H (5 mU); Lanes *4* to *8*, pure Endo F (2×10^{-2}–2×10^{2} mU); Lanes *9–13*, Endo F N-glycanase (2×10^{-2}–2×10^{2} mU).

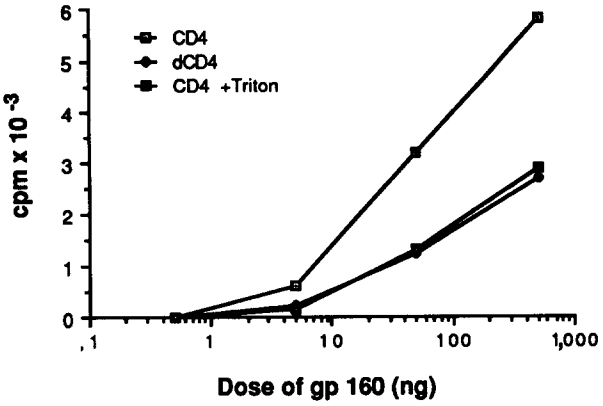


FIGURE 5. Effect of deglycosylation of ^{125}I-sCD4 on the binding to gp160. After incubation of mAb 110-4-Sepharose CL4B with gp160 (0.5–500 ng), 50,000 cpm of native (CD4) or deglycosylated (dCD4), or Triton X-100-treated native ^{125}I-sCD4 (CD4 + Triton) were added for 2 h. Bound radioactivity was counted.

complexes was separated from the unbound molecule by centrifugation. Fig. 5 shows that binding of ^{125}I-sCD4 increased with the amount of native gp160 that was used in this assay, and similar results were obtained when gp120 was used in the assay (Fig. 6). ^{125}I-sCD4 binding was specific because it was proportional to the concentration of gp120/160 used, and no binding was obtained when the same amount of gp160 was incubated with *N*-acetyl glucosamine coupled to Sepharose instead of mAb 110-4 (data not shown).

Carbohydrates of sCD4 Are not Necessary for Binding to Native gp120/160. Preliminary experiments had shown that 0.02% SDS abolished the specific binding of ^{125}I-sCD4 to gp120/160 (results not shown). Therefore, because total deglycosylation of sCD4 with Endo F *N*-glycanase at pH 7.4 could also be obtained in the absence of SDS, we used only 1% Triton X-100 in all the experiments. Results of Fig. 5 clearly show that deglycosylated (see Fig. 3) and native ^{125}I-sCD4 bound to native gp160 with a similar avidity; the apparent decrease of the binding capacity of deglycosylated ^{125}I-sCD4 is due only to the effect of Triton X-100 since the same decrease of binding of glycosylated ^{125}I-sCD4 was obtained in the presence of a similar concentration of Triton X-100.

Role of the Carbohydrate Moieties of gp120/160 in the Binding to sCD4. Preliminary experiments had shown that gp120/160 fixed to mAb-110-4-Sepharose complexes could

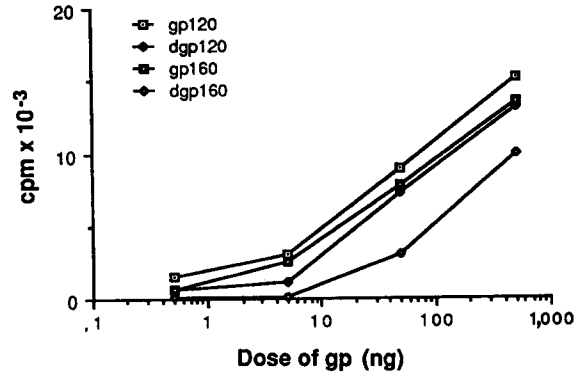


FIGURE 6. Effect of deglycosylation of gp120/160 on the binding of sCD4. After incubation of mAb 110-4-Sepharose CL4B with native (gp120- gp160) or deglycosylated (dgp120–dgp160) glycoproteins (0.5–500 ng), 50,000 cpm of ^{125}I-sCD4 were added for 2 h. Bound radiography was counted.

still bind to sCD4. It was thus important to demonstrate that deglycosylated gp120/160 continued to bind to 110-4 mAb-Sepharose to the same extent as native gp120/160. As expected, complete deglycosylation of ^{125}I-gp160 did not change its immunoreactivity to mAb 110-4 (Table I).

In the molecular assay used, ^{125}I-sCD4 could still bind to deglycosylated gp120/160 in a dose-dependent manner that was proportional to the amount of gp120/160 used (0.5–500 ng) (Fig. 6). However, binding capacity of ^{125}I-sCD4 to deglycosylated gp120 and gp160 was reduced by two- to fivefold as compared with the respectively native molecules.

Binding of Native and Deglycosylated gp120/160 to the Membrane CD4 of CEM Lymphoid Cells. To further investigate the influence of carbohydrate moieties of gp120/160 in the interaction with CD4, we compared the capacity of native and deglycosylated gp120/160 to bind to CD4$^+$ cells of the CEM line in an indirect immunofluorescence assay. Different amounts of native or deglycosylated gp120/160 (0.25–250 ng) were incubated with 10^6 cells and the amount of gp120/160 bound to the cells was determined by the fluorescence intensity obtained after labeling with mAb 110-4. Results of these experiments (Fig. 7) clearly show that deglycosylation of gp120 or gp160 did not abolish their binding capacity to CD4$^+$ cells, but here again, the binding capacity of the deglycosylated gp120 and gp160 was reduced four to six times, as compared with the respectively native molecules.

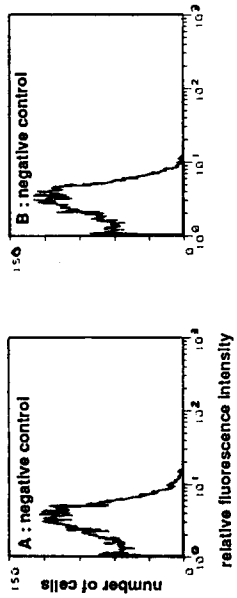
Native and Deglycosylated gp120 Inhibit to the Same Extent Syncytium Formation between HIV-infected and Uninfected CEM Cells. Native and deglycosylated gp120 were tested for their capacity to inhibit formation of syncitia between HIV1- or HIV2-infected CEM cells and fresh uninfected Molt T4 cells. Complete inhibition of syncitium formation by native gp120 was noted with 0.62 μg/ml for HIV1-infected cells, with 6.25 μg/ml for HIV2-infected cells (Fig. 8) and when cells were incubated with Leu-3a (2.5 μg/ml), an anti-CD4 antibody. Results in the same range were obtained with deglycosylated gp120. Thus, despite the absence of carbohydrate chains, the deglycosylated molecule neutralizes cell fusion between HIV-infected and uninfected CD4$^+$ cells with the same efficiency as the native molecule.

Affinity of the Binding of Native and Deglycosylated gp120/160 to CEM Cells. The affinity for the CD4 receptor of native gp120 and gp160 and of deglycosylated gp120 was measured in competition experiments of native vs. ^{125}I-gp160 molecules for the binding to CD4$^+$ cells. Fig. 9 shows the ^{125}I-gp160 binding curve to CEM cells in the presence of increased concentrations of native and deglycosylated gp120 (1.6×10^{-7}–3.1×10^{-10} M); the $K_{0.5}$, i.e., the concentration of native molecule required to reduced by 50% ^{125}I-gp160 binding, was four times higher for deglycosylated ($5 \times$

TABLE I

Fixation of Native (gp) or of Deglycosylated (dgp) gp160 (50,000 cpm) to 110-4 Sepharose CL4B (50 μl)

Exp.	Bound gp	Bound dgp
	cpm	
1	13,344	14,015
2	14,816	13,531



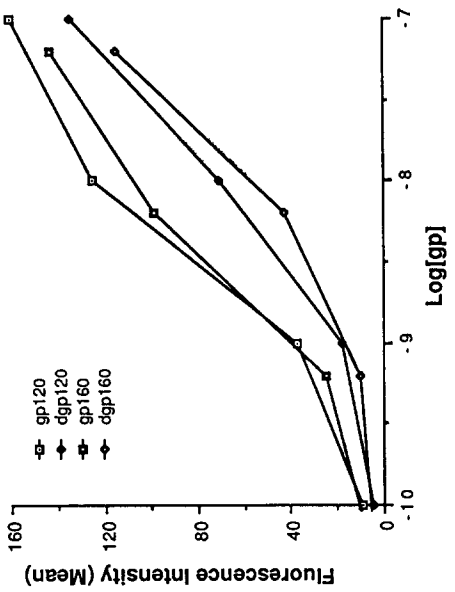


FIGURE 7. Binding of native and deglycosylated gp120 or gp160 to $CD4^+$ CEM cells. Cells were incubated with different amounts of glycoproteins. Attachment was determined by indirect immunofluorescence with mAb 110-4. Fluorescence intensity was then measured by FACS analyzer. (*Left*) Autofluorescence of the cells (*A*). Fluorescence of the cells incubated with the mAb but in the absence of glycoprotein (*B*). Incubation with 25 ng of gp120 (gpl20) (*C*), or with 25 ng of gpl60 (gpl60) (*D*). Incubation with 25 ng of deglycosylated gp 120 (dgpl20) (*E*), or with 25 ng of deglycosylated gpl60 (dgpl60) (*F*). (*Right*) Mean fluorescence intensity obtained with CEM cells incubated with increasing concentrations of gpl20 (gpl20), deglycosylated gpl20 (dgpl20) gpl60 (gpl60), and deglycosylated gpl60 (dgpl60).

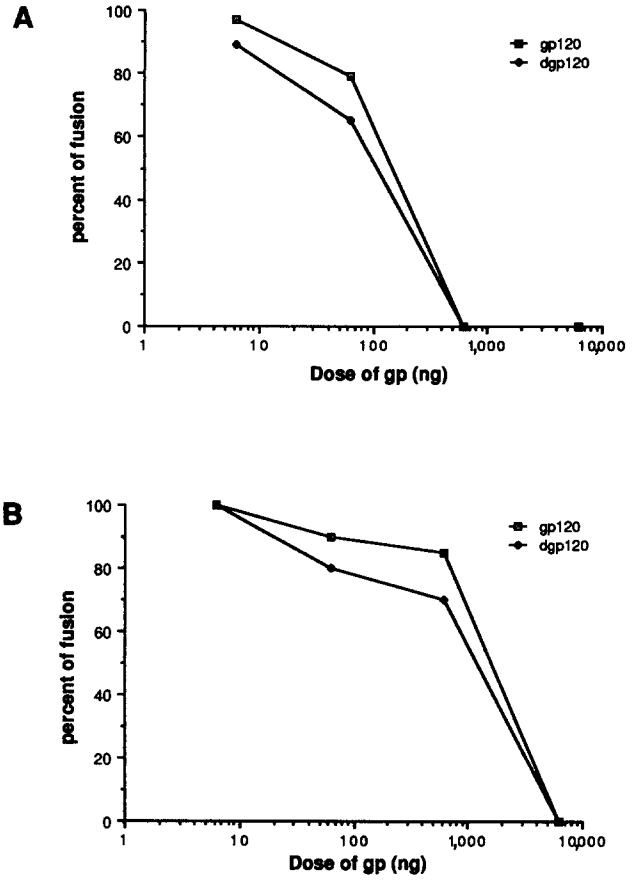


FIGURE 8. Inhibition of syncitium formation between HIV-infected and noninfected CD4+ cells. Chronically infected CEM cells with HIV1 (*A*) or HIV2 (*B*) were cocultured with fresh Molt T4 cells. Syncitia were counted after 18 h. Inhibition of syncitium formation by increasing amounts of native (gp120) or deglycosylated (dgp120) glycoprotein in culture medium was observed and expressed by reference to 100%; i.e., the number of syncitia in the absence of gp120 in the medium.

10^{-8} M) than for native gp120 (1.25×10^{-8} M). The method of Muller (36) enables us to calculate a dissociation constant by using the formula $K_d = 3/8$ (I-T), where T = (^{125}I-gp160) = 2.10^{-9} M; I = $K_{0.5}$ = 1.25×10^{-8} M for native gp120 and 5×10^{-8} M for deglycosylated gp120). Applying this formula generated $K_d = 4.7 \times 10^{-9}$ M for native gp120 and 18×10^{-9} M for deglycosylated gp120. These K_d

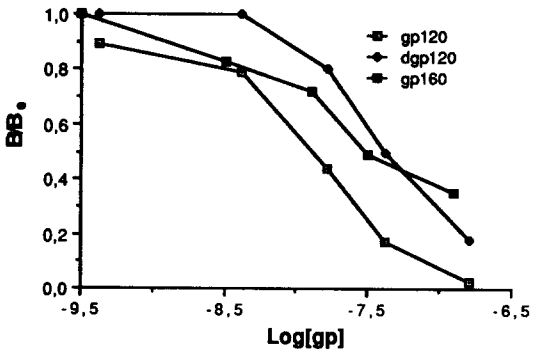


FIGURE 9. Affinity of the binding of native gp120 and gp160 and of deglycosylated gp120 to CEM cells. 10^6 CEM cells were incubated overnight with ^{125}I-gp160 and different concentrations of gp120 (gp120), gp160 (gp160), or deglycosylated gp120 (dgp120). ^{125}I-gp160 bound to cells was then counted.

values demonstrate that the presence of carbohydrate chains is not a major factor for gp120 binding with high affinity to CD4 molecules expressed at the cell membrane.

Discussion

The objective of this study was to analyze the glycan moieties of gp120/160 and of sCD4 and to further examine whether carbohydrates of either molecule played a significant role in the interaction between HIV and $CD4^+$ cells since, up to now, contradictory results have been reported at least with respect to gp120/160 (20–22). To evaluate this role at the molecular level, we needed efficient and reliable methods that totally deglycosylated the two molecules involved without denaturing or proteolyzing their peptide backbones. This led us first to examine, with different glycosidases, the accessibility of carbohydrate chains and their composition owing to enzyme specificity.

sCD4, which possesses two glycosylation sites (37), was produced in mammalian cells, and so its glycosylation was supposedly close to what it is normally. Deglycosylation of sCD4 resulted in a 4-kD shift of the initial molecular mass that corresponded well with the removal of two glycans of 2 kD each. 2 kD is indeed the approximate molecular mass of a triantennary chain composed of 10–12 residues of ~200 daltons each, or of a high mannose type that also comprises 10 residues (38). These two carbohydrate moieties were easily removed only by Endo F *N*-glycanase at pH 7.4, a condition under which *N*-glycanase is also active in addition to Endo F (33–35). Thus, glycans of CD4 are probably either of triantennary structure, or they may represent some rare fucosylated hybrid or high mannose structures that are usually resistant to pure Endo F or Endo H activity (32–35). These results also suggest the homogeneity of the glycosylation of the sCD4 used here, because all the molecules were similarly sensitive to *N*-glycanase activity. This is surprising in regard to the well known glycan heterogeneity (38). That removal of *N*-linked glycans was possible in the absence of SDS can be interpreted as resulting from the good accessibility to the enzymes of glycan moieties on the native folded CD4 molecule; moreover, these conditions of deglycosylation (in the absence of SDS) allowed us to obtain a deglycosylated form of sCD4 that was active in binding assays to gp120/160.

Deglycosylation of sCD4 did not reduce its ability to bind to gp120/160. This clearly demonstrates that carbohydrate moieties of CD4 are not essential for the binding to gp120 and confirms other observations that cells expressing CD4 that lacks *N*-linked glycosylation sites can be infected by HIV (37). This finding is in line with the recent observation that glycosylation sites are located on the third and fourth domains of CD4, while only the first Ig-like domain of CD4, which lacks *N*-linked glycans, appears to be directly involved in the receptor function for HIV (27, 39). This, however, cannot rule out the possible role of CD4 carbohydrate moieties in the interaction with its natural ligands such as HLA class II molecules for example (40).

Treatment of gp120/160 by Endo F and by Endo H yielded a partial degree of deglycosylation that was similar with both enzymes, indicating that approximately half of the glycans of the molecule are sensitive to these enzymes. The carbohydrate moieties cleaved may then be of the high mannose type possibly associated with some hybrid or biantennary species (32, 33). Other glycans, cleaved only by *N*-glycanase activity at pH 7.4, are probably of particular high mannose or biantennary chains that are not sensitive to Endo H and Endo F activity, or they may be triantennary

species (33–35). This confirms and expands recent findings reported by others (41). As for CD4, the total removal of carbohydrates from gp120/160 in the absence of SDS indicates that their attachment point is accessible on the molecule in its native conformation.

Sialic acid content of gp160 appears to be quite important, since treatment with neuraminidase resulted in the loss of ~30 kD of its apparent molecular mass. This is in agreement with results previously reported by McDougal et al. (42), and it may correspond to high sialylation of carbohydrate chains; however, this shift is in apparent contradiction with the elevated number of high mannose type species that are removed by Endo F or Endo H. The maximum sialylation of triantennary chains is normally associated with a sialic acid content of ~20% of the carbohydrates (37). However, as determined by enzymatic deglycosylation, no more than half of glycan moieties of gp120/160 are able to be sialylated (biantennary or triantennary chains). Therefore, desialylation of gp 120/160 should not result in a shift more important than 10% of the molecular mass, i.e., 10–15 kD. The shift obtained here may then be due to polysialic series branched at the periphery of the chains.

There has been considerable speculation (20–22, 43) as to the role of carbohydrates moieties of gp120, whose glycosylation sites are highly conserved (44), in the interaction with CD4. The molecules used in the present study were completely deglycosylated as indicated by the reduction in molecular mass, observed with autoradiography or Coomassie staining after SDS-PAGE, which was in the same range as what has been previously reported by other authors (20). Additional proof demonstrating that the gp120 and gp160 used in the binding assays were fully deglycosylated was obtained by HPLC analysis on a Biosyl TSK 250 column (Bio-Rad Laboratories) that confirmed the lack of detectable glycosylated contaminant in the preparations used and the exclusive presence of homogeneous deglycosylated form of the molecules. Moreover, gas liquid chromatography of the deglycosylated preparations was performed after purification on a C1 column; here again, contamination by glycosylated molecule was undetectable (<3–5%) (B. Fournet, O. Koll, and J. Montreuil, personal communication).

Thus, our results clearly indicate that completely deglycosylated gp120 or gp160 can still bind to the CD4 receptor, either in a soluble form or expressed at the cell membrane, with a capacity that is only slightly reduced by two- to sixfold. In addition, fixation of ^{125}I-gp160 (2×10^{-9} M) to CD4$^+$ cells is totally inhibited by native as well as by deglycosylated gp120, although the 50% inhibition concentration ($K_{0.5}$) is four times higher with the deglycosylated form ($K_{0.5} = 5 \times 10^{-8}$ M) than with the native form of the molecule ($K_{0.5} = 1.25 \times 10^{-8}$ M).

Taken together, the results presented here demonstrate that deglycosylated recombinant soluble gp120/160 retains the structural and biological properties of the native viral glycoprotein, binds with high affinity to CD4$^+$ cells (K_d, 18 nM) and inhibits syncytium formation mediated by HIV1- or HIV2-infected cells within the same range of efficiency as the native molecule. This indicates that the carbohydrate chains of gp120 do not play a significant role in its interaction with CD4. However, one cannot exclude that such limited reduction in the affinity of deglycosylated gp120 to bind to its CD4 cellular receptor does not significantly impair the infectivity and cytopathology of nonglycosylated viral particles. Moreover, it appears that antibodies can neutralize HIV infectivity in vitro by interfering only to such extent with gp120-

CD4 interaction. Nevertheless, our findings are in apparent contradiction with recent reports from other authors (20–22). The drastic decrease of the binding capacity to CD4 of completely deglycosylated gp120, obtained by Matthews et al. (20) might then not be due only to the removal of carbohydrates per se, as suggested, but it may be rather related with conformational factors due to changes within the structure of the molecule in relation with the use of denaturing agents such as SDS. In our hands, for example, the use of 0.02% SDS in deglycosylation procedures of gp160 diminished its binding to CD4 by 20 times (data not shown).

On the other hand, the fact that native and deglycosylated gp120 inhibit syncitium formation between HIV-infected and noninfected $CD4^+$ cells in the same range argues against the interpretation brought by Gruters et al. (22) that it is the abnormal glycosylation of viral glycoproteins generated by the treatment of cultured HIV-infected cells with castanospermine and deoxynojirimycin (which both inhibit oligosaccharide processing) that renders the virus unable to efficiently infect these cells and to induce syncitium formation because of the lack of binding of abnormally glycosylated gp to CD4. Similarly, results of syncitium inhibition by lectin (Con A) reported by Lifson et al. (43) could be explained by steric hindrance, as suggested by the authors rather than by the direct involvement of carbohydrate moieties in gp120-CD4 interaction.

Alternatively, it cannot be excluded that carbohydrates of gp120/160 play a role in a hypothetical secondary interaction of gp120/160 with the membrane. The function of carbohydrates has been investigated in the case of glycoprotein hormones. For example, specific deglycosylation of luteinizing hormone leads to a molecule that has an increased capacity to bind to its cellular receptor but that has a significantly diminished ability to stimulate adenylate cyclase activity. It has been proposed in this case that sugars are required for transduction of the hormone signal into the cells (45).

Based on these facts (20–22), one can hypothesize that *N*-linked carbohydrates of HIV gp120/160 may play a role in the folding of the protein or in its stabilisation. However, a major and direct role of glycan moieties in gp120/160-CD4 interaction can be excluded. Experiments with gp120/160 obtained by cloning in procaryote cells will be necessary, as will be the design of a synthetic conformational peptide including the binding site to CD4 receptor that could be made for the development of a synthetic vaccine in order to test this hypothesis.

Summary

gp120 and CD4 are two glycoproteins that are considered to interact together to allow the binding of HIV to $CD4^+$ cells. We have utilized enzymatic digestion by endoglycosidases in order to analyze *N*-linked carbohydrate chains of these proteins and their possible role in the interaction of gp120 or gp160 with CD4. SDS denaturation was not necessary to obtain optimal deglycosylation of either molecule, but deglycosylation of CD4, nonetheless, depended on the presence of 1% Triton X-100. Endo H and Endo F that cleave high mannose type and biantennary glycans diminish the molecular mass of the glycoproteins from 120 or 160 Kd to 90 or 130 Kd, respectively; but these enzymes had no action on CD4 glycans. Endo F *N*-glycanase mixture, which acts on all glycan species, including triantennary chains, led to complete deglycosylation of gp120/160 and of CD4. Therefore, probably half of the glycan

moieties of gp120/160 are composed of high mannose and biantennary chains, the other half being triantennary species. The carbohydrate structures of CD4 seems to be triantennary chains. To analyze the binding of gp120/160 to CD4, we used a molecular assay in which an mAb (110-4) coupled to Sepharose CL4B allowed the attachment of soluble gp120/160 to the beads; ^{125}I-sCD4 was then added to measure the binding of CD4 to different amounts of gp120/160. Binding to gp160 was not modified when using completely deglycosylated ^{125}I-sCD4, while deglycosylation of gp120 or of gp160 resulted in the decrease of the binding to native CD4 by two- and fivefold, respectively. Native and deglycosylated gp120/160 bound to CD4$^+$ cells with comparable affinities. In addition, deglycosylated gp120 displaced ^{125}I-gp160 binding to CD4$^+$ cells and inhibited fusion of fresh Molt-T4 cells with CEM HIV1- or HIV2-infected cells to the same extent. Taken together, these results indicate that carbohydrates of CD4 and of gp120/160 do not play a significant role in the in vitro interaction between these two molecules.

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