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Characterization of the Secreted, Native gp120 and gp160 of the Human Immunodeficiency Virus Type 1

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ABSTRACT

We have previously shown that the cell line $6D5_{451}$ chronically infected with the HIV-1 isolate HTLV-III_{451}, secretes the HIV-1 envelope glycoproteins gp120 and gp160 in the extracellular medium. The HTLV-III_{451} gp120 and gp160 were purified by sequential affinity chromatographic steps using a monoclonal antibody to HIV-1 gp41 and an anti-HIV-1-positive human serum. Amino acid sequence analysis of gp120 and gp160 showed the loss of the signal peptide. Digestion of the purified gp120 and gp160 with endoglycosidases revealed that both proteins are heavily glycosylated and contain complex carbohydrates, in contrast to the intracellular form of gp160 which has been shown to contain mannose-rich immature sugars. Competitive binding analysis showed that while both gp120 and gp160 bind CD4, the affinity of gp160 was five times lower than that of gp120. Both gp120 and gp160 inhibited syncytia formation by HIV-1-infected cells when mixed with $CD4^+$ cells. Furthermore, both gp120 and gp160 had strong mitogenic effects on the T cells from HIV-1-infected gibbons but not on cells from uninfected gibbons.

INTRODUCTION

THE *env* GENE OF THE HUMAN IMMUNODEFICIENCY VIRUS codes for gp160, which is intracellularly cleaved into the external viral envelope protein gp120 and the transmembrane protein gp41. Several studies using native and recombinant gp120 have clearly shown that gp120 binds strongly to the CD4 antigen on the surface of susceptible cells, and is responsible for viral attachment, the initial step in infection.[1–5] The highly hydrophobic gp41, which contains the putative fusogenic domain, is probably involved in the subsequent fusion with the cellular plasma membrane and internalization of the virus. In view of the external localization on the virus, gp120 and gp41 have also been the targets for viral neutralization by the host immune system.[6–8] Several studies, using native and recombinant gp120 as well as synthetic peptides representing sequences

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thereof, have shown that gp120 contains epitopes that can be targets of neutralizing antibodies against the virus.[8–10] However, the role of gp41 in this process remains uncertain even though there have been reports showing that antibodies made against synthetic peptides from the gp41 region neutralize viral infection.[11,12] The role of gp41 has been hard to establish since it is difficult to purify this protein either by recombinant expression systems or from virus cultures due to its extremely hydrophobic nature.

The nucleotide sequences of a number of different isolates of HIV-1 are now known. A comparison of these sequences in the *env* gene of the virus established the presence of a number of common hypervariable regions.[13–15] These regions may code for epitopes in these proteins which may give rise to type-specific neutralizing antibodies against the virus. In fact, with the use of synthetic peptides it has been shown that the region between amino acids 310 and 335, known as RP-135, contains the dominant neutralizing epitope of the virus and lies in the middle of one of the hypervariable regions of the *env* gene.[10] Although the nucleotide sequence of a number of HIV-1 isolates is known, the amino acid sequence of the highly divergent gp120 is experimentally determined for only two of the HIV-1 isolates, namely HTLV-III_B and HTLV-III_{RF}.[16] It is, however, essential to characterize the purified gp120 proteins of a number of HIV-1 isolates to understand the structure-function relationship among these viral isolates. Such a characterization is even more important since antibodies raised against the gp120 of the above two isolates have shown that they possess mainly type-specific neutralizing activities against the virus.[7,17]

This report describes the purification and properties of soluble gp160 and gp120 from the strain HTLV-III_{451} of HIV-1. Nucleotide sequence analysis has previously shown that relative to the prototype HTLV-III_B, HTLV-III_{451} is one of the most divergent of the North American HIV-1 isolates.[13] Furthermore, HTLV-III_{451} was chosen for the study because we have previously shown that a cell line, $6D5_{451}$, chronically infected with this isolate has an unusual property of secreting the highly hydrophobic precursor protein gp160, in addition to gp120, in the extracellular medium.[18] The availability of monoclonal and hyperimmune antibodies to these proteins has allowed us to isolate and purify these proteins. They remained soluble in the absence of detergents. This is in contrast to the gp160 expressed in insect cells which required the use of a powerful denaturing agent such as sodium dodecyl sulfate (SDS) for solubilization.[19] Studies reported here further show that both gp120 and gp160 have strong affinity for CD4 and have group-specific mitogenic effect on the mononuclear cells of HIV-1-infected gibbons.

MATERIALS AND METHODS

Cells

Development of $6D5_{451}$ cells chronically infected with HTLV-III_{451} has been previously described.[20] $6D5_{451}$, MOLT-3 cells producing HTLV-III (MOLT-3/III_B), and CEM_{50} cells were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS). For growth in serum-free medium, $6D5_{451}$ cells grown in fetal bovine serum were centrifuged and resuspended in serum-free HB104 medium (Du Pont) and grown further in the same medium.

Antibodies

Polyclonal goat antibodies to the purified gp120s of HTLV-III_B and HTLV-III_{RF} and rabbit antibody to HTLV-III_B gp41 were kindly provided by Dr. L. Arthur of Frederick Cancer Research Facility. Defined monoclonal antibodies to HIV-1 gp120 and gp41 were obtained from Du Pont. Development of monoclonal antibody to HTLV-III_{451} gp41 used in this study has been described elsewhere.[21]

Purification of HTLV-III_{451} gp120 and gp160

Forty liters of $6D5_{451}$ culture (80 roller bottles) grown in serum-free HB104 medium were clarified by low-speed centrifugation. Triton X-100, NaCl, sodium phosphate pH 7.5, and phenylmethylsulfonylfluoride (PMSF) were added to the supernatant to final concentrations of 0.5%, 500 mM, 20 mM, and 0.1 mM,

respectively. The solubilized medium was concentrated 50-fold by filtration through a Pellicon Cassette (Millipore). The concentrated medium was equilibrated overnight at 4°C with Sepharose-bound goat antibody (IgG) to the proteins in the HB104 medium. The medium was clarified by filtration and passed through a 30 ml column of lentil–lectin Sepharose overnight at 4°C. The column was washed with phosphate-buffered saline (PBS) and the glycoproteins were eluted at room temperature with 400 mM α-methyl mannoside. The eluted protein fraction was adjusted to pH 8.5, and KCl, Triton X-100, and PMSF were added to final concentrations of 1 M, 0.1% and 0.2 mM, respectively. The sample was equilibrated overnight at 4°C with 40 ml of anti-gp41 Sepharose. The latter was prepared by treating 50 ml of CNBr-activated Sepharose 4B with 250 mg of anti-HTLV-III_{451} gp41.[21] The Sepharose equilibrated with the glycoproteins was packed in a column, washed extensively with PBS, and the proteins eluted with 100 mM Na_2CO_3 containing 0.1 mM PMSF. The eluted protein fraction was neutralized with 1 M HCl and concentrated using a Centricon-30 (Amicon). The SDS-polyacrylamide gel electrophoretic profile of this fraction revealed nearly pure gp160.

The unadsorbed fraction from the anti-gp41 Sepharose column was equilibrated overnight at 4°C with IgG-Sepharose prepared by using a human serum containing high-titer antibodies to HIV-1 gp120. The equilibrated IgG-Sepharose was packed in a column, washed with PBS, and eluted with 100 mM Na_2CO_3 containing 0.1 mM PMSF. The eluted protein fraction after neutralization with 1 N HCl was once again equilibrated with anti-gp41 Sepharose to remove the last traces of gp160. The unadsorbed fraction was concentrated using Centricon-30. SDS polyacrylamide gel electrophoresis demonstrated that this fraction contained nearly pure gp120. The average yield of gp120 and gp160 from 40 liters of the medium was 2 mg and 8 mg, respectively.

Western blot analysis

The proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose. The nitrocellulose was treated with specific antibodies, washed, and subsequently treated with ^{125}I-labeled appropriate second antibodies as described earlier.[22] The reacted proteins were visualized by autoradiography.

Endoglycosidase digestion of gp120 and gp160

Five micrograms of purified gp120 or gp160 were digested for 3 h with 5 mU endoglycosidase H or 100 mU endoglycosidase F (Boehringer Mannheim) in 50 mM sodium acetate buffer, pH 5.5, containing 0.02% SDS and 0.1 mM PMSF. The proteins were similarly digested with 100 mU of *N*-glyconase (Genzyme) in 50 mM sodium phosphate buffer pH 8.0 containing 0.1 mM PMSF and 0.02% SDS. The digested proteins were analyzed in 9% SDS-polyacrylamide gels.

Amino acid sequence analysis

The HTLV-III_{451} gp120 and gp160 isolated as above were further purified by high-performance liquid chromatography (HPLC). Trifluoroacetic acid (TFA) was added to the proteins to a final concentration of 0.1%, and the mixture loaded on a μBondapak C-18 column (Waters). The column was then eluted with a 0–60% linear gradient of acetonitrile in 0.1% TFA. The major protein peak containing gp120 or gp160 was subjected to automated Edman degradation in an Applied Biosystems gas-phase sequanator. The amino acids were identified as phenylthiohydantoin derivatives using the Applied Biosystems PTH analyzer.

Binding of labeled gp120 to CEM_{50} cells

The purified gp120 was labeled with ^{125}I to a specific activity of 2000 cpm/ng in the presence of Iodogen. Four million CEM_{50} cells were washed once with the binding medium (RPMI-1640 medium containing 10 mM BES, 2 mg/ml of bovine serum albumin, and 0.1 mM PMSF) and incubated at room temperature with 50,000 cpm of gp120 in 500 μl of the binding medium for 2 h. The cells were washed twice with PBS and the radioactivity in the cell pellet was measured in an LKB gamma counter. In control assays unlabeled gp120 was included to determine the nonspecific binding which was nearly 1% of the labeled protein in the reaction.

Syncytia inhibition assay

In 200 μl medium 1×10^4 MOLT-3/III_B cells were cocultured for 24–36 h with 1×10^5 CEM cells in a 96-well microtiter plate and the cultures were photographed to visualize syncytia. Alternately, the CEM cells were preincubated with 1 μg of either gp120 or gp160 for 1 h prior to cocultivation with MOLT3/III_B cells.

T-cell proliferation assay

Peripheral blood mononuclear cells were isolated from HTLV-III_B-infected and uninfected gibbon apes by Ficoll-Hypaque gradient centrifugation. Cells were washed twice in cold PBS and resuspended at the concentration of 1×10^6 ml^{-1} in RPMI-1640 culture medium supplemented with 10% autologous serum for each animal. Triplicate cultures were established for each antigen by seeding 100 μl of the cell suspension and adding 100 μl of the relevant antigen at the appropriate concentration. After five days, the cultures were pulsed with 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA), cultured for an additional 18 h, then harvested, and the cell-associated radioactivity determined in a liquid scintillation counter. Data were calculated as the arithmetic mean from the triplicate cultures. The stimulation index (SI) represents the ratio between stimulated and unstimulated control cultures. The antigens used in the assay were: HTLV-III_{451} gp120 or gp160 used at 1.0 μg ml^{-1} and tetanus toxoid fluid (Wyeth Labs, Marietta, PA), used at 1:10 final dilution. No animal included in the study had been previously immunized against tetanus.

RESULTS

Characterization of the properties of gp120 and gp160

Both gp120 and gp160 were purified starting with the soluble conditioned media from cultures of $6D5_{451}$ as described in Materials and Methods. It is extremely important to note that throughout the purification the glycoproteins remained soluble without the need for harsh detergents and denaturants. The use of the affinity procedures described yielded apparently homogeneous gp120 and gp160 (Fig. 1). The purified proteins were stable for several months when stored at −70°C. Purified gp160, however, underwent some degradation to 70K and 55K proteins with time. While gp120 reacted specifically with monoclonal and polyclonal antibodies to gp120 of HIV-1 strains HTLV-III_B and HTLV-III_{RF}, gp160 reacted with antibodies to both gp120 and gp41.[16,21]

Amino acid sequence analysis by Edman degradation indicated that a 33-residue signal peptide was cleaved off to generate gp160 and subsequently gp120 having a common amino terminal sequence of NLWVTV—for the proteins (Fig. 2). The two proteins had apparent molecular sizes of 110 and 140 kD, while the predicted protein backbones of these proteins were only 54 and 96 kD, respectively. The nature of the glycosylation was analyzed by digestion with specific glycosidases. Digestion of gp120 with either endo F or endo H resulted only in partial deglycosylation, yielding a protein of 89 kD (Fig. 3A, lanes 2 and 3). Similarly, gp160 also underwent limited digestion with these enzymes giving rise to a protein of 106 kD (Fig. 3B, lanes 2 and 3). However, digestion with *N*-glyconase (glycopeptidase F) resulted in the complete removal of the sugars from gp120 and gp160 and the reduction in molecular weights to 62 (Fig. 3A, lane 4) and 89 (Fig. 3B, lane 4), respectively. This showed that the purified gp120 and gp160 had hybrid sugars which are only partially susceptible to endo F and H digestion.

Relative interactions of gp120 and gp160 with CD4

HTLV-III_{451} gp120 labeled with ^{125}I bound strongly to CEM_{50} cells and this binding was completely inhibited by excess unlabeled gp120 or the OKT4a monoclonal antibody to CD4. In order to determine the relative affinities of gp120 and gp160 to CD4, serial twofold dilutions of either HTLV-III_{451} gp120 or gp160 were mixed with ^{125}I-labeled gp120, and the extent of binding to CEM_{50} cells was measured under standard assay conditions as described under *Materials and Methods*. As shown in Figure 4A, both unlabeled proteins

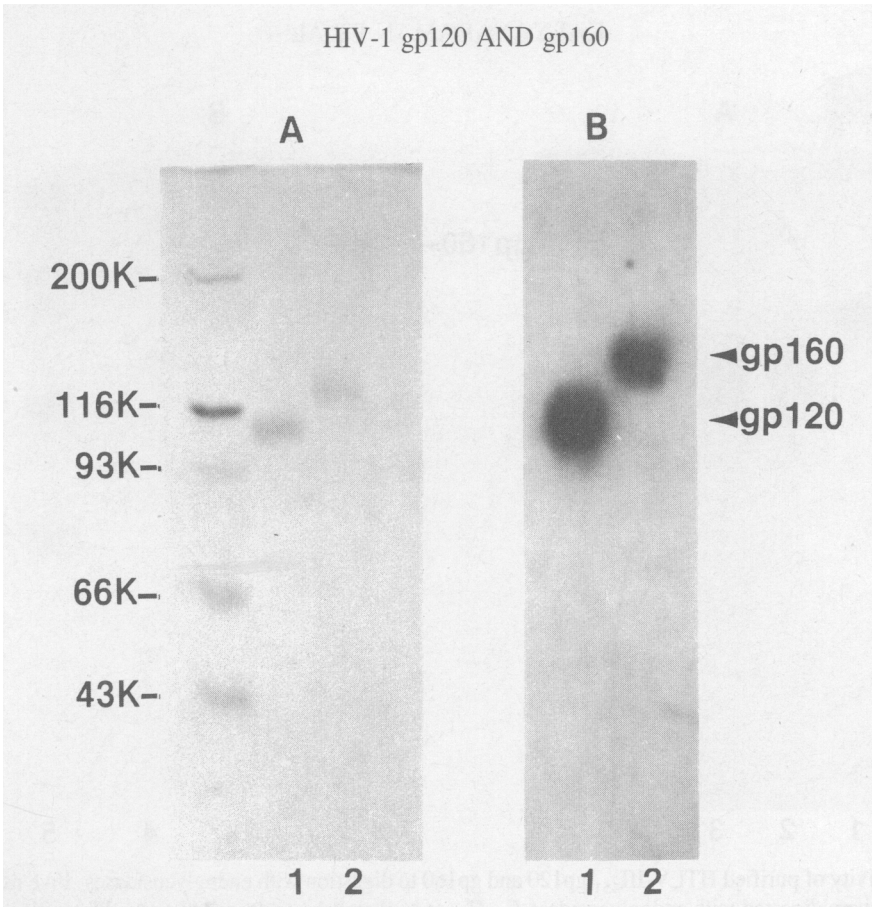

FIG. 1. SDS polyacrylamide gel electrophoresis of HTLV-III$_{451}$ gp120 and gp160. Five μg of gp120 and gp160 were run on 7.5% polyacrylamide gels and (A) stained with Coomassie blue or (B) analyzed by Western blot using an HIV-1-positive human serum. 1. gp120; 2. gp160.

competed out totally the binding of labeled gp120 to the cells. The slope of the competition curves was nearly identical, showing that the two proteins were reacting with the same group of sites. However, a nearly fivefold higher concentration of gp160 was required for the same degree of competition as gp120, which probably reflects a lower affinity of gp160 to CD4. This is in agreement with our previous observation using metabolically labeled gp120 and gp160. Although both proteins bind to CD4, at limiting CD4 concentrations preferential binding of gp120 was observed.[18] Figure 4B shows the Scatchard analysis of the binding data.

		1 30	40
Deduced Sequence	gp160	MAM..... S A A A N L W V T V Y Y G V P	
Experimentally Determined Sequence	gp160	N L X V T V Y Y G V	
	gp120	N L X V T V	

FIG. 2. Amino acid sequence of HTLV-III$_{451}$ gp120 and gp160. The proteins purified by HPLC were subjected to automated Edman degradation and sequence analysis as described in Materials and Methods. The gp160 sequence predicted from the known nucleotide sequence of the *env* gene[13] is given in the top line. The residue numbers starting with the initiator methionine is indicated on the top. The bold arrow indicates the site of cleavage of the signal peptide.

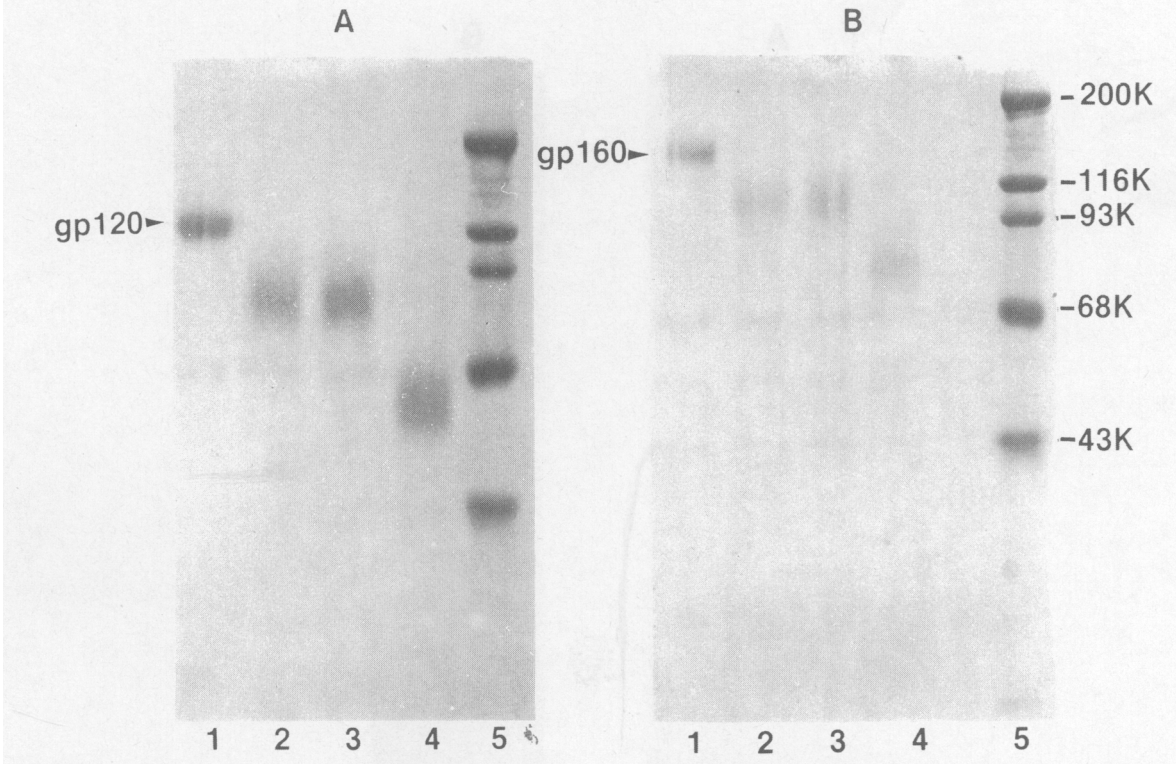

FIG. 3. Sensitivity of purified HTLV-III$_{451}$ gp120 and gp160 to digestion with endoglycosidases. Five micrograms of gp120 or gp160 were digested with endoglycosidase *F*-, *H*-, or *N*-glyconase as described under Materials and Methods, separated in 10% polyacrylamide gel and stained with Coomassie blue. (A) gp120; (B) gp160. Proteins digested with Endo F- (lane 2), H- (lane 3), or *N*-glyconase (lane 4). Lane 1 represents undigested proteins and lane 5 molecular weight standards.

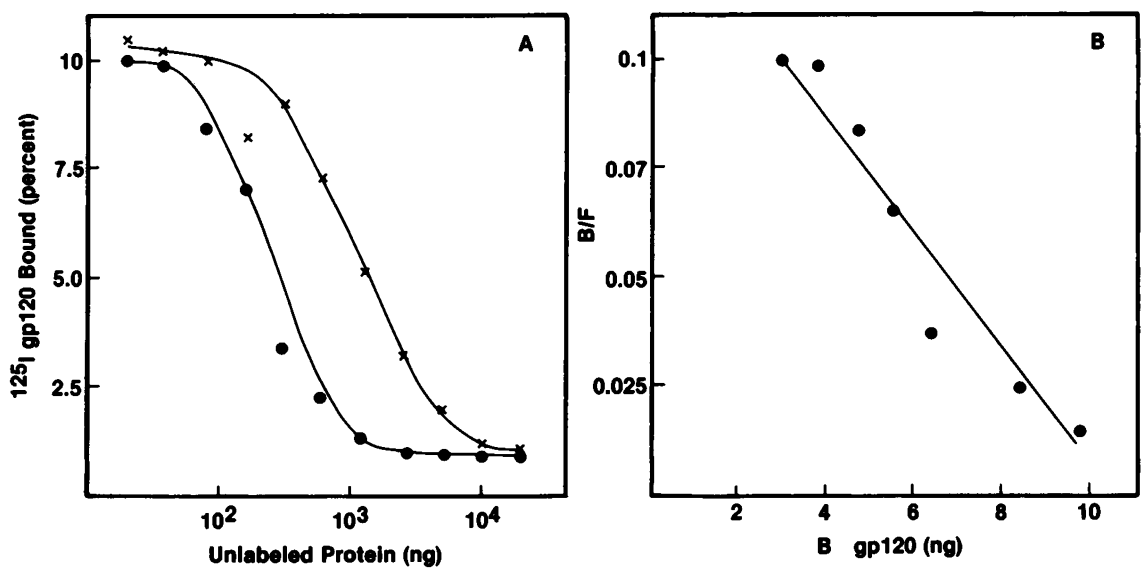

FIG. 4. Competitive inhibition of the binding of labeled HTLV-III$_{451}$ gp120 to CEM$_{50}$ cells by purified gp120 or gp160. Different concentrations of unlabeled gp120 or gp160 were mixed with 50,000 cpm of ^{125}I-labeled gp120 and the extent of binding of the labeled gp120 CEM$_{50}$ cells was determined under standard binding conditions. (A) inhibition of binding by serial dilutions of gp120 (●—●) or gp160 (×—×). (B) Scatchard plot analysis (●—●) of the binding of gp120 to CEM$_{50}$ cells.

The dissociation constant (K_d) for the gp120 CD4 complex was calculated from the slope of the curve to be 1.2×10^{-9} M, and the number of binding sites per CEM_{50} cell was determined to be 13,200. These values are in the same range as the reported values of 2 to 5×10^{-9} M and 15,000 for the HTLV-III_B gp120.[3,23] The interaction of purified gp120 and gp160 with CD4 was further examined by the ability of purified gp120 and gp160 to inhibit the formation of syncytia when HIV-1-infected cells were mixed with $CD4^+$ cells. For this purpose 1×10^5 CEM_{50} cells were pretreated with 1 μg of either purified gp120 or gp160 and then mixed with 1×10^4 HTLV-III_B-infected MOLT-3 cells. As shown in Figure 5, extensive large multinucleated cells were seen 24–36 h after mixing CEM and MOLT-3/III_B cells. However, pretreatment of CEM cells with either gp120 or gp160 completely prevented syncytia induction.

Mitogenic effect of gp120 and gp160

We have previously shown that the mononuclear cells from gibbons infected with HTLV-III_B can be stimulated with inactivated virus to show extensive proliferation. There was, however, no effect on the cells from uninfected gibbons. It was unclear which specific viral antigen mediated this response. As shown in Table 1, both gp120 and gp160 had a strong proliferative effect on the cells from infected but not uninfected gibbons. There was no quantitative difference in the response to either gp120 or gp160, which indicated that the effect is elicited by the gp120 portion of gp160.

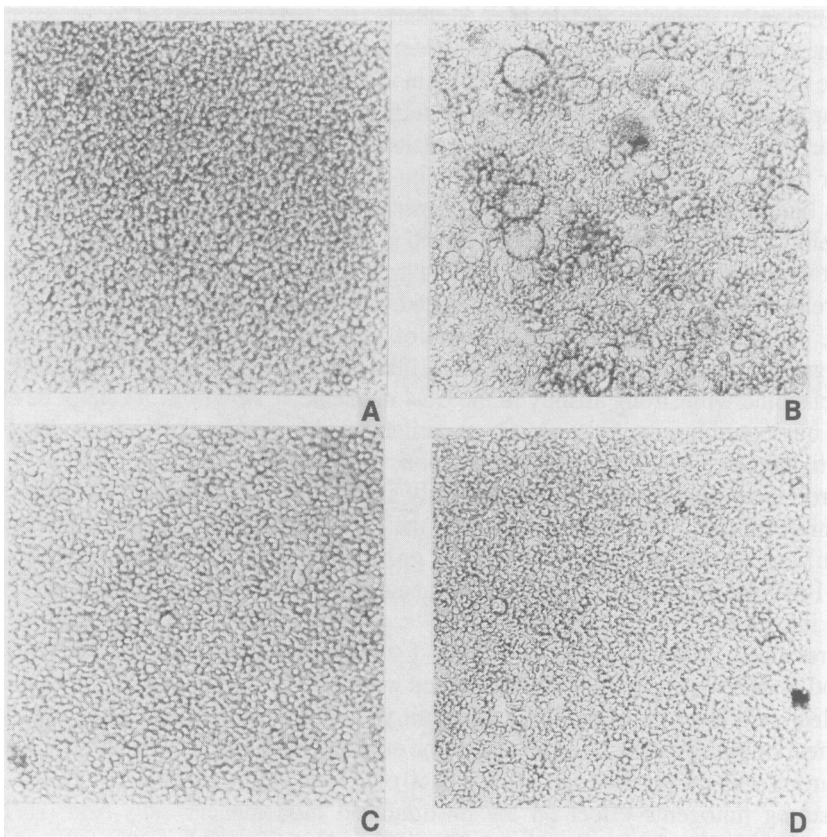

FIG. 5. Inhibition of HIV-1 induced syncytia formation by purified HTLV-III_{451} gp120 and gp160. 1×10^5 CEM_{50} cells were pretreated with 2 μg of either gp120 or gp160 for 1 h and then mixed 1×10^4 MOLT-3/III_B cells and the cells were photographed 36 h later. (A) CEM_{50} cells. (B) CEM_{50} cells plus MOLT-3/III_B cells. (C) CEM_{50} cells pretreated with HTLV-III_{451} gp120 or (D) gp160 before mixing with MOLT-3/III_B cells.

TABLE 1. T-CELL PROLIFERATIVE RESPONSE TOWARD HTLV-III$_{451}$ gp120 AND gp160 IN GIBBON APES CHRONICALLY INFECTED WITH HTLV-III$_B$

Gibbon ape	*HTLV—III$_B$*[a]	*gp160*[b]	*gp120*[b]	*TT*[c]
Uninfected control	1.9	1.0	1.4	<1
HIV-1-infected #1	28.8	17.4	20.6	<1
HIV-1-infected #2	34.3	28.3	27.1	1.3

The values represent the stimulation index, calculated as the ratio between [^{3}H]thymidine incorporation in stimulated and unstimulated cultures as described in Materials and Methods.

[a] Whole sucrose-banded virions, inactivated for 60′ at 56°C and used at 25 μg/ml.

[b] HTLV-III$_{451}$ gp120 and gp160, present at a concentration of 5 μg/ml.

[c] Tetanus toxoid, used as irrelevant control antigen.

DISCUSSION

The present study demonstrates that native soluble HIV-1 envelope glycoproteins can be purified from the culture media of virus-producing cells. Amino acid sequencing of purified gp120 and gp160 demonstrated the loss of the signal peptide. Comparison of the amino terminal sequence of the HTLV-III$_{451}$ glycoproteins with that of two other HIV-1 strains (HTLV-III$_B$ and HTLV-III$_{RF}$) reveal no conservation of the cleavage site sequence.

In HIV-1-infected cell lines the intracellular gp160 is present only in the mannose-rich form and is fully sensitive to digestion by endoglycosidase-H.[24] Similarly, the gp160 expressed by recombinant expression systems in Chinese hamster ovary cells was also shown to contain only endo-H-sensitive carbohydrate.[25] In contrast to these observations, the secreted gp160 from vaccinia virus recombinants has undergone extensive addition of complex carbohydrates.[26] In the present study we have shown that the gp160 purified from 6D5$_{451}$ cells is only partially sensitive to endo-H. The possibility that the addition of complex sugars may be a requirement for the secretion of the viral glycoproteins in the medium is being investigated. There is some support for this line of thinking from pulse-chase experiments which showed that a considerable lag (~2 h) between intracellular synthesis and secretion of gp160 in these cells (data not shown).

In this report we clearly show that both gp120 and gp160 bind to CD4 and block the syncytia induced by MOLT-3/III$_B$ cells. Both proteins effectively competed with the binding of labeled gp120 by CD4 molecules on CEM cells, even though higher concentrations of gp160 were needed to achieve the same level of competition as gp120 (Fig. 4). This confirmed our earlier finding that metabolically labeled gp120 and gp160 from 6D5$_{451}$ cells bind with strong affinity to the CD4 molecule.[18] However, it cannot be excluded that the lower levels of competition seen in the present experiments with gp160 may be due to the lower number of functionally active molecules in our gp160 preparation.

Using in vitro mutagenesis, it has been previously shown that HIV-1 having uncleaved gp160 on their surface are noninfectious.[27] This cannot be due to the inability of gp160 to bind CD4 antigen on the cell surface because we have demonstrated the ability of gp160 to bind to CD4. Proteolytic cleavage of gp160 to gp120 and gp41 may be necessary for virus-membrane fusion, which allows the necessary subsequent steps resulting in the infection.

It has been previously shown that inactivated HIV-1 stimulated the growth of unstimulated T cells from the peripheral blood of animals experimentally inoculated with HIV-1.[28] A similar effect has also been observed with mononuclear cells derived from HIV-I seropositive humans. It has been speculated that most of this proliferative effect of the virus can be attributed to the envelope proteins gp120 and gp41. We examined this phenomenon further with the purified gp120 and gp160 of HTLV-III$_{451}$. As shown in Table 1, both gp120 and gp160 had a strong mitogenic effect on the unstimulated mononuclear cells from HTLV-III$_B$-infected gibbons, but not from cells of normal gibbons. The effect was comparable to that elicited with inactivated virus. Since HTLV-III$_B$ and HTLV-III$_{451}$ are two of the most divergent HIV-1 isolates from North America,[13] it is reasonable to conclude that the mitogenic effect of gp120 is mediated through group-specific determinants. This report describes a unique system in which the cleaved and uncleaved envelope glycoproteins of an HIV-1 strain were obtained in the native form and their functional integrity was verified

by demonstrating their ability to bind to the viral receptor, block syncytia formation during cell to cell infection, and elicit a proliferative response in in vivo sensitized target cells.

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