RESEARCH

Cloning, Expression, and Purification of HIV-2 gp125

A Target for HIV Vaccination

Samer Sourial,[1,*] Anette Wärnmark,[2] Charlotta Nilsson,[1,3,4] Ewa Björling,[1] Adnane Achour,[5] and Robert A. Harris[6]

Abstract

The envelope of the human immunodeficiency virus (HIV) is the main target for neutralizing antibodies. We report the cloning, purification, and characterization of two recombinant forms of the envelope glycoprotein gp125 from a primary HIV-$2_{SBL-6669}$ isolate. Both constructs were truncated at the N- and C-termini, and in the gp125$\Delta v_1 v_2$ construct the variable V1 and V2 loops were deleted. The recombinant glycoproteins were stably expressed in Chinese hamster ovarian cells, producing soluble gp125 and gp125$\Delta v_1 v_2$ at molecular weights of 74.2 and 56.9 kDa, respectively, and were purified from cell culture supernatants in a single step using *Galanthus nivalis* lectin chromatography. Circular dichroism analysis indicated a similar secondary structure for gp125 and gp125$\Delta v_1 v_2$, and both proteins were recognized by HIV-2 serum antibodies in surface plasmon resonance assays. The high yield and purity of these constructs makes them suitable for structural and functional analyses, as well as vaccine studies.

Index Entries: HIV; envelope; surface unit; lectin; viral glycoprotein.

1. Introduction

The human immunodeficiency virus (HIV) type 2 is a member of the lentivirus subfamily, infection with which leads to the development of AIDS. HIV-$2_{SBL6669}$ belongs to subtype A, which accounts for the majority of HIV-2 infections and is the predominant genotype in Guinea-Bissau and Europe ***(1,2)***. Although HIV-1 is responsible for the steady spread of the ongoing AIDS pandemic, HIV-2 describes a much more limited distribution, where it is less readily transmitted and is generally less pathogenic than HIV-1 ***(3,4)***.

The envelope (Env) proteins of HIV are primarily involved in cell tropism, binding, and entry, using CD4 and different chemokine receptors ***(5,6)***. Although both HIV-1 and HIV-2 infect the same target cells and display similar genomic organizations, HIV-2 is only about 40% similar to HIV-1 in nucleotide sequence and shares an approx 75% similarity with the nucleotide sequence of the simian immunodeficiency virus (SIV) ***(7,8)***.

The surface unit (SU) of HIV-1, glycoprotein 120 (gp120), and the transmembrane protein (TM), glycoprotein 41 (gp41) associate noncovalently into heterodimers, which further assemble into trimers on the surface of the virus ***(9,10)***. Glycoprotein 125 (gp125) and glycoprotein 36 (gp36) are the equivalent SU and TM, respectively, of HIV-2. Both gp120 and gp125 are composed of five variable (V1–V5) and five conserved domains (C1–C5), several N-linked glycosylation sites and conserved disulfide bonds ***(11–13)***.

*Author to whom all correspondence and reprint requests should be addressed.[1]Microbiology and Tumor Biology Center, Karolinska Institutet, S-171 77, Stockholm, Sweden. E-mail: samer.sourial@mtc.ki.se. [2]Department of Biosciences at Novum, Karolinska Institutet, Huddinge, Sweden. [3]Swedish Institute for Infectious Disease Control, Solna, Sweden. [4]Research Center, Stockholm Söder Hospital, Stockholm, Sweden. [5]Center for Infectious Medicine, F59, Department of Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm, Sweden. [6]Center for Molecular Medicine, Karolinska University Hospital, Solna, Sweden.

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Construction of an immunogen that can induce neutralizing antibodies is a primary goal in HIV vaccine development. This requires an understanding of the structure of HIV surface glycoproteins where the conformation and exposure of neutralizing epitopes can be studied. In a previous crystallization study of HIV-1 gp120, the first three loops (V1–V3) were eliminated and the protein was truncated at both the N and C termini ***(14)***. Deletion of the variable loops has also been reported to increase the immunogenicity of HIV-1 gp120, inducing neutralizing antibodies ***(15,16)***. Cross-neutralization of HIV-1 by HIV-2^+ sera has been described ***(17)***, where HIV-2-specific antibodies that cross-react with HIV-1 gp120 have been previously reported using HIV-2^+ sera ***(18)***.

In this study we describe the cloning of the envelope gene from a primary HIV-$2_{SBL6669}$ isolate, and the design of two gp125 constructs. Furthermore, we describe the production and characterization of the corresponding proteins. Both gp125 constructs are truncated at their N and C termini, and the V1 and V2 loops were deleted in gp125Δv_1v_2, thus isolating the V3 loop. The gp125 proteins were expressed in a Chinese hamster ovary cell line and were purified in a single step using *Galanthus nivalis* lectin affinity chromatography. Circular dichroism assays were used to ensure the preservation of secondary structure in both gp125 constructs. The expressed glycoproteins were recognized by HIV-2 positive sera in surface plasmon resonance assays. The high yield and purity of these proteins make them suitable for both structural and functional analyses of HIV-2 immunity as well as vaccine development.

2. Materials and Methods

2.1. Infection of PBMCs With HIV-$2_{SBL6669}$

Peripheral mononuclear cells (2.5×10^6, PBMCs) from blood donors were grown in RPMI medium (Gibco BRL, Paisley, UK), containing 10% fetal calf serum (FCS; Gibco BRL), 0.05% Polybrene (PB; Sigma, St. Louis, MO), 0.08% interleukin (IL)-2 (Amersham, Buckinghamshire, UK), and antibiotics. The 50% tissue culture infective dose ($TCID_{50}$) of primary HIV-$2_{SBL6669}$ was determined as described previously ***(19)***, and 30 $TCID_{50}$ was used to infect the PBMCs in a biosafety laboratory level III. After 24 h the PBMCs were washed and fresh RPMI medium was added. Nine days later the cells were harvested and nuclear DNA was extracted using a genomic DNA purification kit (Promega Corporation, Madison, WI).

2.2. Cloning the HIV-$2_{SBL6669}$ Envelope Gene and Design of gp125 Constructs

A nested polymerase chain reaction (PCR) was used to clone the envelope *(env)* gene from the HIV-2 provirus in the nuclear DNA extracted from the virus-infected PBMCs. The following primers (Gibco BRL, Life Technologies) were used in the first PCR reaction, 5'-GGG CTC GGG ATA TGG TAT GAA CGA AAG GG-3' and 5'-AGT TCT GCC AAT CTG GAA TTA TCC CTT CT-3'. The extracted nuclear DNA (2.5 µL) was mixed with 1.25 U *Pfu*-polymerase (Stratagene, La Jolla, CA) in buffer (according to manufacturer's instructions), 2.5 m*M* dNTPs and 10 µ*M* of each primer in a total volume of 25 µL. The mix was used in the following reaction; 1 min 94°C, 20 cycles of 45 s 94°C, 45 s 50°C, 4 min 72°C, followed by 15 cycles of 45 s 94°C, 45 s 50°C, and 4 min 15 s at 72°C. The same reaction mix and protocol was used in subsequent PCR reactions. The product was amplified in a second PCR reaction using 5'-TTA GAT CTG TCT TCT GCA TCA GAC AAG TGA GTA TG-3' and 5'-TTA GAT CTC ATC CCT TCC AGT CCC CCT TTT TCT TTT A-3' primers.

Cloning of gp125 from the *env* gene excluded the signal peptide and the first 25 N-terminal as well as the 10 C-terminal amino acids. In this PCR, primers 5'-TAT CTA GAG AGA TAC CTG TGA ATG TA-3' and 5'-TTA GGA TCC ACG CGG AAC GAG TTC TAC TAA TTT ATA ATC-3' were used. The cloned gp125 was then used as template for gp125Δv_1v_2 in which the V1 and V2 loops were excluded in three subsequent PCR reactions. In one PCR reaction, primers 5'-GAG ACA TCA ATA AAA CCA GGT GCT GGT AAC ACA TCA GTC ATC ACA-3' and 5'- TTA GGA TCC ACG CGG AAC GAG TTC TAC TAA TTT ATA ATC-3' were

used, and in a second, primers 5'-TAT CTA GAG AGA TAC CTG TGA ATG TA-3' and 5'-TGT GAT GAC TGA TGT GTT ACC AGC ACC TGG TTT TAT TGA TGT CTC-3' were used. Finally, gp125$\Delta v_1 v_2$ was cloned in a third PCR reaction by combining the product of the previous two reactions with the same primers used to produce gp125, as described above. To verify the sequence, the gp125 constructs were sequenced (Cybergene, Stockholm, Sweden), using primers 5'-TGT ACC ATG GGT AAA TGA CAC CTT AAC ACC-3' and 5'-TTC CCG GGC ATC CCT TCC AGT CCC CCT TTT TCT TTT A-3'.

2.3. Cloning of gp125 Constructs Into a Mammalian Expression Vector

Both gp125 constructs were cloned into a pBSKS-vector using restriction sites *Xba*I/*Bam*HI. The vector included the T7 promoter as well as the Ig κ-chain signal peptide, and introduced a hemagglutinin-A (HA) epitope and a thrombin cleavage site into the N-terminal of gp125. The constructs were then subcloned into the expression vector pBJ5/GS ***(20)*** using the restriction sites *Xba*I/*Bam*HI. This vector expresses the glutamine synthetase gene (GS) and includes the SR-α promoter ***(21)***.

2.4. Transfection of Chinese Hamster Ovarian Cells and Selection of Clones

Chinese hamster ovarian (CHO*lec* 3.2.8.1) cells ***(20)*** were grown using minimal essential medium (MEM; Sigma, Irvine, UK) supplemented with glutamine and 10% dialyzed fetal calf serum (FCS) (Sigma, Steinheim, Germany). When the cells were approx. 70% confluent they were transfected with either of the gp125 constructs cloned in pBJ5/GS. Calcium phosphate precipitation was applied using 15 μg of DNA to transfect the CHO cells. The cells were detached 24 h later using trypsin and were grown in glutamine-free MEM containing 25 μ*M* L-methionine sulphoxamine (MSX; Sigma, St. Louis, MO) before being dispensed into 96-well plates at 10×10^4 cells/well. After 3 wk the clones that appeared were screened by immunoprecipitation. Cells from each clone were grown in MEM containing 25 μCi/mL ^{35}S-cysteine and 75 μCi/mL ^{35}S-methionine at 37°C. Supernatants were collected and the expressed proteins were immunoprecipitated as previously described ***(22)*** using anti-HA monoclonal antibody (Roche Molecular Biology, IN) and protein A-Sepharose (Amersham Bioscience, Uppsala, Sweden).

2.5. Production and Purification of gp125 and gp125Δ $v_1 v_2$ Proteins

Clones expressing gp125 or gp125$\Delta v_1 v_2$ were grown in roller flasks (Falcon, NJ), and the supernatants were collected after 7 d. Immobilized lectin from *G. nivalis* (Sigma, St. Louis, MO) was used to trap the different gp125 proteins from the supernatant. The column was washed with phosphate buffered saline (PBS), followed by PBS/1 *M* NaCl to remove nonspecifically bound material. Both gp125 constructs were thereafter eluted with 0.5*M* α-methyl-D-glycoside (Sigma, St. Louis, MO) dissolved in PBS.

2.6. Western Blotting

In all Western blot analyses, 12% polyacrylamide gels were prepared. Purified glycoproteins were treated with a sample buffer (5X) containing 10% glycerol, 0.03% SDS, 10% bromophenol blue, and 200 m*M* Tris at pH 7.4. After SDS-PAGE and subsequent transfer to nitrocellulose membranes (BioTrace NT, Pall Life Sciences, Ann Arbor, MI), blots were blocked using 5% milk in PBS. An anti-HA monoclonal antibody (Boehringer Mannheim) was diluted to 0.1 μg/mL in 5% milk in PBS and 0.05% Tween. After incubation with the monoclonal antibody, the membrane was washed with 0.05% Tween in PBS. The bound murine antibodies were detected using HRP-labeled rabbit anti-mouse antibodies (Dako A/S, Denmark). Signal was detected using ECL (Amersham Bioscience, Uppsala, Sweden) according to the manufacturer's instructions.

2.7. Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy was performed at 25°C using an AVIV model 202SF spectropolarimeter and a quartz cuvet with 0.1-mm path length. Spectra were obtained by measure-

ment of the ellipticity as a function of wavelength for 10 s/nm between 260 and 184 nm. Sample solutions with a protein concentration of 0.5 mg/mL in 20 m*M* Tris at pH 7.4, 9 m*M* NaCl were used. Contributions from cuvet and buffer were removed by subtracting an identically recorded spectrum of buffer without protein.

2.8. Surface Plasmon Resonance Analysis

All experiments were performed at 25°C and at a flow rate of 5 µL/min, using a BIAcore 2000 system and software BIACORE 200 control software 3.1.1 (BIAcore Inc., Uppsala, Sweden). The buffer used contained 50 m*M* Tris at pH 7.4, 150 m*M* NaCl, 1 m*M* EDTA, and 0.05% Tween. Equimolar amounts of the different glycoproteins were covalently attached to individual surfaces on a research grade CM5 chip (Biacore), using the amine coupling kit according to the manufacturer's instructions. A surface coupled with bovine serum albumin (BSA) was used as a negative control. The sera used in the analysis were diluted 1:20 in the same running buffer, and one pulse of 1 min injection of 10 m*M* glycine buffer at pH 2.2 was used to regenerate the surface of the chips.

2.9. Mass Spectrometry Analysis

The gp125 and gp125$\Delta v_1 v_2$ proteins (~1 mg/mL in 20 m*M* Tris at pH 7.4, 0.1 *M* NaCl) were diluted two times with 0.1% (v/v) trifluoroacetic acid and mixed with an equal volume of saturated solution of sinapinic acid in 33% (v/v) acetonitrile and 0.1% (v/v) ethanol. An aliquot of 1 µL of this mixture was applied on a steel target plate that had been prepared with a thin layer consisting of saturated solution of sinapinic acid in ethanol. The MALDI-TOF spectra were acquired using a Ultraflex mass spectrometer (Bruker Daltonics, Bremen, Germany) and calibrated using the protein calibration standard II (Bruker Daltonics). Data processing and evaluation were carried out with the Flexanalysis software from Burker Daltonics.

3. Results

3.1. Cloning of gp125 and gp125Δ v_1v_2

Primary isolate HIV-$2_{SBL6669}$ was used to infect PBMCs and nuclear DNA containing the provirus was used to clone the *env* gene by PCR. The different gp125 constructs were designed by truncating the N and C termini in both constructs, as previously described for HIV-1 gp120 ***(14)***. The V1 and V2 loops were deleted in gp125$\Delta v_1 v_2$, and replaced by a GAG sequence. An HA tag as well as the signal peptide of the Ig κ-chain leader sequence were introduced into the N termini of both gp125 constructs by cloning into the vector pBSKS (**Fig. 1**).

3.2. Transfection and Selection of CHOlec Cells

After sequencing, both gp125 constructs were subcloned into the pBJ5/GS expression vector. Calcium phosphate precipitation was applied to transfect CHO*lec* cells, which were subsequently grown in a selective medium containing 25 µ*M* MSX. After 1 wk colonies of the different clones appeared that were then screened for expression of the gp125 proteins. A fraction of each clone was grown in radioactively labeled medium and the expressed gp125 proteins were immunoprecipitated using an anti-HA monoclonal antibody (**Fig. 2A**).

3.3. Purification and Characterization of gp125 and gp125Δ v_1v_2 Glycoproteins

Cell-free supernatants from CHO*lec* clones were collected and the different gp125 proteins were purified using *G. nivalis* lectin affinity chromatography. More than 2 mg of both gp125 and gp125$\Delta v_1 v_2$ could be purified from 1 L of culture supernatant. **Fig. 2B** depicts the purified gp125 and gp125$\Delta v_1 v_2$ proteins analyzed by SDS-PAGE and Coomassie Blue staining. Mass spectrometry analyses of both constructs indicated that the molecular mass of gp125 is 74.2 kDa, whereas that of gp125$\Delta v_1 v_2$ is 56.9 kDa (data not included). To verify that the multiple species observed with the purified gp125$\Delta v_1 v_2$ were not contaminants trapped by the lectin used, the samples were ana-

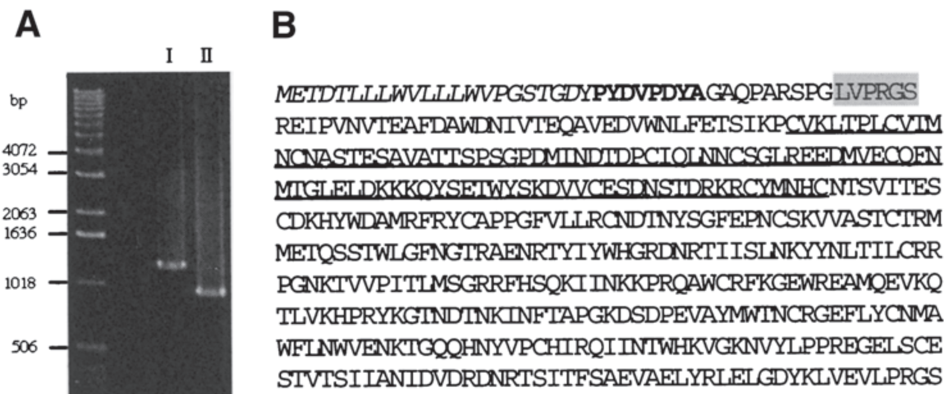

Fig. 1. Cloning of gp125 constructs. **(A)** Gp125 (lane I) and gp125Δv_1v_2 (lane II) are composed of 1269 and 978 bp, respectively. **(B)** The gp125 constructs were cloned into pBSKS using the *Xba*I and *Bam*HI constriction sites. The signal peptide of the Ig k-chain leader sequence (shown in italics), a hemagglutinin tag (shaded), and a thrombin cleavage site (bold) were introduced at the amino termini of both gp125 constructs. The underlined sequence is deleted gp125Δv_1v_2 and replaced with a GAG sequence.

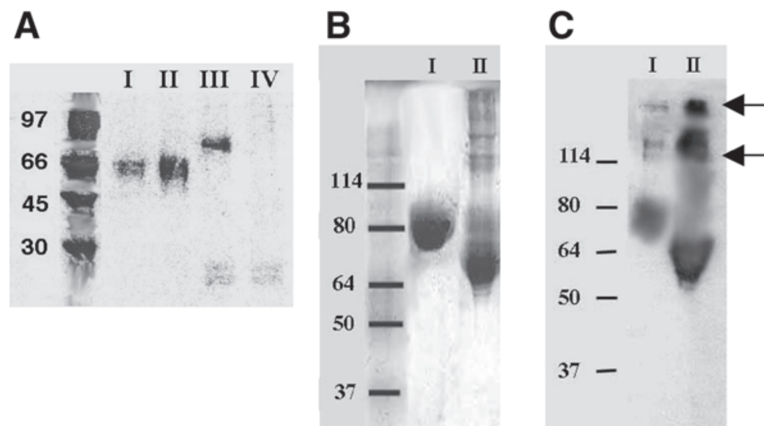

Fig. 2. Production of gp125 and gp125Δv_1v_2 proteins. **(A)** Screening of CHO*lec* clones was performed using immunoprecipitation with an anti-HA monoclonal antibody. The figure demonstrates gp125Δv_1v_2 detected in the supernatant of different CHO*lec* clones (lanes I and II), as well as gp125 detected in one of the clones (lane III). The last lane (IV) is a mock CHO*lec* clone. **(B)** Coomassie blue staining of *Galanthus nivalus*-purified gp125 (lane I) and gp125Δv_1v_2 (lane II) run in a 12% SDS-PAGE gel. **(C)** Western blot analysis of both gp125 proteins using anti-HA antibody. The arrows indicate the polymers formed by gp125Δv_1v_2.

lyzed by Western blotting using the anti-HA monoclonal antibody (**Fig. 2C**). This analysis confirmed that the observed bands corresponded to the oligomerization of gp125Δv_1v_2. It also demonstrated that gp125 polymerized similarly as gp125Δv_1v_2, but at much lower levels. The CD analyses revealed that both proteins acquired secondary structure and no apparent differences were discerned between the two proteins (**Fig. 3**). Surface plasmon resonance analysis of both constructs demonstrated binding capacity of HIV-2-positive sera to both gp125 and gp125Δv_1v_2 (**Fig. 4**), verifying that the expressed glycoproteins display naturally antigenic conformations.

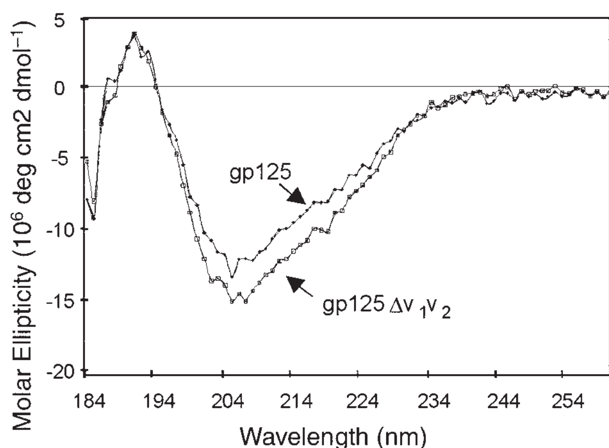

Fig. 3. Circular dichroism analyses of gp125 constructs.

4. Discussion

Owing to its ability to elicit neutralizing antibodies, the surface unit of the viral envelope is a primary target for vaccine development against HIV ***(19,23,24)***. The autologous neutralization capacity apparent in HIV-2 infection ***(25)*** may be attributed to differences in properties observed between HIV-1 gp120 and HIV-2 gp125 component envelope proteins ***(26)***. In an effort to facilitate a comparative investigation of these key viral proteins, we report the production of recombinant gp125 isolated from a primary HIV-2 isolate in two different forms, one including the V1–V3 loops and one truncated form lacking the variable V1 and V2 loops.

A mutant form of Chinese hamster ovarian cells (CHO*lec* 3.2.8.1) was used to express the proteins with minimal glycosylation heterogeneity. Glycoproteins expressed in this system are fully glycosylated, enabling the study of the functional role of sugar moieties in neutralization. Moreover, reduction of heterogeneity and removal of flexible loops are two characteristics that may facilitate crystallization of proteins, making gp125Δv_1v_2 a candidate for structural studies. Although this is the first report of the expression of HIV-$2_{SBL6669}$-derived gp125, the expression of external glycoprotein gp105 of HIV-2_{ROD} using *Pichia pastoris* has previously been reported ***(27)***. The molecular size of this heterologous glycosylated glycoprotein expressed in yeast is approx. 85 kDa, whereas the mass spectrophotometric analysis of gp125 expressed in CHO*lec* cells in our study displayed a homogenous species of 74.2 kDa. This difference is considered to be due to the difference in glycosylation between the yeast- and CHO*lec*-expressed proteins, as well as difference in deletions at the termini of gp125.

CHO*lec* 3.2.8.1 expresses glycoproteins comprising mannose sugars, making it possible for both gp125 constructs to be isolated using mannose-specific lectins ***(28)***. Due to its exclusive α-D-mannosyl specificity, *G. nivalis* allowed selective binding of the glycoproteins expressed by the CHO*lec* cells, both gp125 and gp125Δv_1v_2 being purified in single chromatographic steps. *G. nivalis* has previously been used to purify envelope glycoproteins of HIV-1, HIV-2, and SIV ***(29)***. However, only monomeric glycoproteins were purified from those different virus isolates. The oligomeric forms observed with gp125Δv_1v_2 are most likely due to differences in properties between gp125 and gp125Δv_1v_2, and not to the use of the *G. nivalis* lectin.

The fact that gp125Δv_1v_2 has a higher tendency to spontaneously oligomerize is of importance when designing effective immunogens for vaccine trials. Vaccination with monomeric gp120 has been suggested to be inefficient in eliciting neutralizing antibodies against primary HIV-1 isolates ***(30)***. Attempts have been made to stabilize envelope glycoprotein trimers by modifying the cleavage site between gp120 and gp41, or through the introduction of cysteine cross-links and trimeric motifs ***(31)***. Deletion of the V1 and V2 loops appears to be an alternative for the induction of polymers in HIV-2 gp125. An improvement in elicitation of neutralizing antibodies has also been observed through the deletion of theV1 and V2 loops in gp120, which exposed neutralizing epitopes ***(15)***. Deletion of the V1 and V2 loops did not appear to affect the secondary structure of gp125Δv_1v_2, and a serum from an

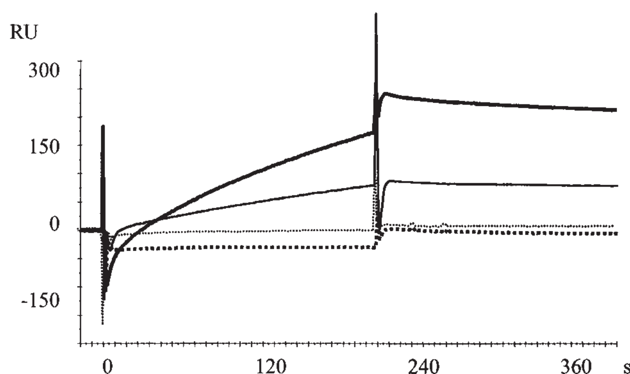

Fig. 4. Surface plasmon resonance analysis of gp125 constructs. The real-time binding sensogram shows injection of diluted (1:20) HIV-2$^+$ (solid line) and healthy (dashed line) sera over surfaces immobilized with equimolar amounts of either gp125 (bold line) or gp125Dv_1v_2 (light line). The data shown have been corrected for bulk and background effects using a surface immobilized with bovine serum albumin.

HIV-2-infected individual recognized the gp125 proteins in surface plasmon resonance, indicating that these proteins are expressed with native antigenic conformation.

Vaccination studies using the two gp125 proteins described in this study will facilitate investigation of neutralization mechanisms, especially the role of the V3 loop in antibody interaction, and the exposure of neutralizing epitopes in HIV-2. Studies of antibodies induced by these gp125 constructs may also provide clues for the design of effective immunogens against HIV-1.

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