

## Cell Proteins Interacting with the Human Immunodeficiency Virus in Immunoblotting can be Detected by R5- or X4- Tropic Human Immunodeficiency Virus Particles

### Abstract

**Introduction:** The present study reported a new immunoblot assay, with revelation by R5- or X4-whole free human immunodeficiency virus (HIV) particles or recombinant gp160. **Materials and Methods:** The assay was optimized to identify cell proteins interacting with HIV. Whole cell lysates were prepared from peripheral blood lymphocytes (PBLs), dendritic cells (DC), monocyte-derived macrophage (MDM), and Henrietta Lacks (Hela, wild-type or transfected with DC-specific intracellular adhesion molecule-3-Grabbing Non-Integrin, HeLa) and Human endometrial cells (HEC-1A) lines; HIV particles used were the R5-tropic HIV-1<sub>JRC5F</sub> and the X4-tropic HIV-1<sub>NDK</sub>. **Results:** Experiments with PBL lysates and both viruses demonstrated different bands, including a unique band at 105–117 kDa in addition to nonspecific bands. The 105–117 kDa band migrated at the same level of that observed in controls using total PBL lysate and anti-CD4 mAb for detection and thus likely corresponds to the cluster difference (CD) 4 complex. Blots using lysates of DCs, MDM, HeLa cell line, and HEC-1A cell line allowed identifying several bands that positions were similar to that seen by recombinant gp160 or whole R5- or X4-HIV particles. **Conclusion:** Blot of whole lysates of various HIV target cells is recognized by free HIV particles and allows identifying a wide range of HIV-interacting cell proteins. Such optimized assay could be useful to recognize new cellular HIV attachment proteins.

**Keywords:** Free human immunodeficiency virus particles, gp160, human immunodeficiency virus, western blot

### Introduction

The human immunodeficiency virus Type 1 (HIV-1) is a retrovirus of high infectivity and high virulence. It is the causative agent of most cases of acquired immunodeficiency syndrome (AIDS).

HIV-1 interacts with a large number of molecules residing on the cell surface, including the cluster difference 4 (CD4) molecule,<sup>[1,2]</sup> the chemokine receptor 5 (CCR5) and (Chemokine (CXC) receptor 4 [CXCR4]) co-receptors,<sup>[3-7]</sup> various heparan-sulfates,<sup>[8,9]</sup> the syndecans,<sup>[10]</sup> and the mannosylated C-type lectin molecules, such as the macrophage mannose receptor<sup>[11]</sup> and the dendritic cell-specific intracellular adhesion molecule-3-Grabbing Non-Integrin (DC-SIGN) molecule.<sup>[12-14]</sup>

The envelope glycoprotein of HIV-1 consists of a complex of gp120 and gp41. Viral gp120 bind to the target cell

receptors that can be either glycolipids or galactocerebrosides and its sulfate derivatives, while gp41 is in the fusion between the viral envelope and host cell membrane.<sup>[15]</sup> Interactions between HIV and its receptor and coreceptors play a critical role in viral tropism, pathogenesis, and disease progression.<sup>[16,17]</sup>

However, molecules specifically blocking HIV receptor and its coreceptors do not completely inhibit the attachment of HIV particles.<sup>[18]</sup> Furthermore, recent observations show that the attachment of HIV on DCs may involve other than adhesion molecules than the DC-SIGN molecule.<sup>[19-21]</sup> These findings strongly suggest that interactions between HIV and the membranes of target cells may likely involve yet nonwell-defined HIV-interacting proteins, in addition to those previously recognized.

The western blot assay is a valuable diagnostic tool and a reliable immunological

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method in which proteins of a HIV-1 lysate are separated according to molecular weight by polyacrylamide gel electrophoresis (PAGE). The viral proteins are then transferred onto nitrocellulose membranes and reacted with the patient's serum.

In the present study, we developed a new western blot assay using nitrocellulose membranes in which various cell lysates were transferred, with further revelation by R5- or X4-whole free HIV-1 particles or recombinant gp160. The present investigation is proposing a novel systematic approach to identify the wide range of cell proteins interacting with HIV.

## Materials and Methods

### Virus stocks, reagents, and antibodies

Primary X4-tropic HIV-1<sub>NDK</sub> was grown in peripheral blood lymphocytes (PBLs) of healthy donors stimulated with phytohemagglutinin (PHA) and Recombinant human interleukin-2 (rhIL-2). R5-tropic HIV-1<sub>JR-CSF</sub> then amplified in monocyte-derived macrophages (MDM) of healthy donors. Tropism of viruses was determined using U87 cells transfected with DNA encoding for human CD4 and CCR5 or CXCR4. HIV titer/particles were quantified in cell culture supernatants by means of the HIV-p24 ELISA assay (HIV-1 p24 ELISA, Ingen, Belgium).

Roswell Park Memorial Institute (RPMI) 1640 (with L-glutamine) and penicillin/streptomycin and Dulbecco's Mod Eagle Medium (DMEM) were purchased from BioWhittaker Europe (Verviers, France) and Gibco-Invitrogen (Scotland, UK), respectively. Lymphocyte separation medium was from Eurobio (Les Ulis, France). Human recombinant macrophage-colony stimulating factor (rhM-CSF), interleukin-2 (rhIL-2), interleukin-4 (rhIL-4), and colony-stimulating factor (rhGM-CSF) were obtained from Peprotech (Rocky Hill, NJ). PHA and bovine serum albumin were from Sigma (St Louis, MO). The following murine antibodies against many cluster of differentiation (CD) were obtained from BD Pharmingen (San Diego, CA): including antiCD14 (My4) FITC, antiCD16FITC, antiCD83FITC, antiCD1aPE, and antiDCSIGNPE (known as anti-Dendritic Cell-Specific Intercellular adhesion molecule 3 -Grabbing Nonintegrin). The recombinant HIV-1 gp160 (MN/LAI2) were obtained from the National Institutes of Health (AIDS Research-US department of health and human services).

### Cell preparations

MDM and monocyte-derived dendritic cells (MDDC), peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy adult donors by Ficoll density gradient centrifugation on MSL. The percentage of monocytes was determined by flow cytometry using forward scatter and side scatter properties. PBMC were resuspended in RPMI 1640 medium supplemented with

glutamine, penicillin (100 IU/ml), and streptomycin (100 µg/ml). Cells were seeded into 24 well-plates (Costar, Cambridge, MA) at the concentration  $1 \times 10^6$  adherent cells/ml and incubated at 37°C for 45 min. Nonadherent cells were removed by four washes. Adherent monocytes were incubated in RPMI medium with 10% fetal calf serum (FCS), glutamine, and antibiotics in the presence of 10 ng/ml rhM-CSF to differentiate to MDM or in the presence of a combination of rhGM-CSF and IL-4 (10 ng/ml) to differentiate to immature MDDC. The medium, including all supplements, was replaced on the 3<sup>rd</sup> day of differentiation. After 6 days of culture, adherent cells corresponding to the enriched fraction of monocyte-derived cells were harvested, washed, and used for subsequent experiments. Flow cytometry analysis (Becton Dickinson, San Jose, CA USA) demonstrated that the CD14<sup>+</sup>CD16<sup>+</sup> MDM and CD14<sup>-</sup>CD16<sup>-</sup>CD1a<sup>+</sup>CD83<sup>-</sup>DC-SIGN<sup>+</sup> immature MDDC were more than 90% pure.

### Autologous T lymphocytes

T cells were subsequently prepared from the monocyte-depleted cell fraction. Thus, PBLs were cultured for 48 h in fresh medium supplemented with PHA (2.5 µg/ml) and rhIL-2 (1 µg/ml). PBLs were then washed and cultured in growth medium containing rhIL-2 (1 µg/ml) for 24 h.

### Epithelial cell lines

The epithelial cell line HEC-1A was maintained in RPMI 1640 containing 10% FCS and antibiotics (100 mg of streptomycin per ml, 100 U of penicillin per ml). HeLa cells expressing DC-SIGN and Hela DC-SIGN-were maintained in DMEM supplemented with 10% FCS.

### Preparation of cell lysates

$2 \times 10^7$  total cells of each primary cells or cell lines were harvested by centrifugation at 1500 rpm at 4°C for 5 min. The supernatant was removed and the cell pellet was gently solubilized in lysis buffer containing 100 mM Hepes pH 7.9, 100 mM KCl, 15 mM MgCl<sub>2</sub>, DTT (1 mM dithiothreitol), and 1% of protease inhibitor cocktail (cellLytic Nuclear extraction Kit, Sigma, Aldrich, Saint Louis, MO-USA). Suspended cells were incubated on ice for 15 min. After the addition of the IGEPAL CA-630 detergent at 0.6% to the swollen cells, the solution was vigorously vortexed for 10 s. The resulting lysate was centrifuged at 14,000 rpm for 30 s, and the supernatant corresponded to the cytoplasmic fraction was stored at -80°C for subsequent use.

### Western blot assay

Protein concentrations of cell lysates were determined using Bradford assay. Ten micrograms of protein from each cell type was then subjected to sodium dodecyl sulfate (SDS)-PAGE (SDS-PAGE), 10% resolving gel at 200 V for 30 min. Separated proteins were subsequently transferred to nitrocellulose membranes (Hybond-ECL, Amersham

Biosciences, UK). The membrane was blocked with 2% skim milk powder dissolved in PBS for 1 h at room temperature with shaking. The membrane was incubated with HIV-1<sub>NDK</sub> or HIV-1<sub>JRCSF</sub> (20 ng of p24 antigen) or recombinant protein gp160 (2 µg/ml). Incubation of the membrane with antihuman CD4 monoclonal antibodies (mAb, 2.10<sup>-3</sup>µg/ml) instead of HIV-1 particles or recombinant protein gp160 constituted the positive control. The immunoblot or the nitrocellulose membrane was washed four times with PBS containing 0.05% Tween 20 and incubated with the secondary biotinylated anti-gp160 (1:500) to reveal bounded virus or gp160, or with the goat biotinylated mouse polyclonal antibodies to detect bounded mAb anti-CD4 for 1 h with shaking. The proteins were detected by incubation for 1 h with 1:2000 of streptavidin-peroxidase (Immunotech Beckman-Coulter, France S. A.). Membranes were then visualized by addition of chemiluminescent substrate (ECL-western blotting detection reagents, Amersham Pharmacia Biotech, UK-Limited) and the subsequent exposure to X-ray Kodak film for 1–20 min.

## Results

Systematic screening of HIV-interacting molecules is critical for understanding the early steps involved in HIV cell interactions and for the development of new viral inhibitors of HIV adsorption on target cells, such as microbicide molecules.

In the present investigation, a new western blot assay of whole lysates of various HIV target cells elucidated or found by free HIV particles in order to identify a wide range of HIV-interacting cell proteins has been developed. Western blot analyses were carried out with 10 µg of proteins from whole cell lysates prepared from PBL, DCs, MDM, and human epithelial cell lines HEC-1A, DC-SIGN<sup>+</sup> HeLa, and DC-SIGN<sup>-</sup> HeLa cells, resolved on SDS-PAGE and transferred on nitrocellulose membrane, and exposed using recombinant gp160, HIV-1<sub>NDK</sub>, or HIV-1<sub>JRCSF</sub>.

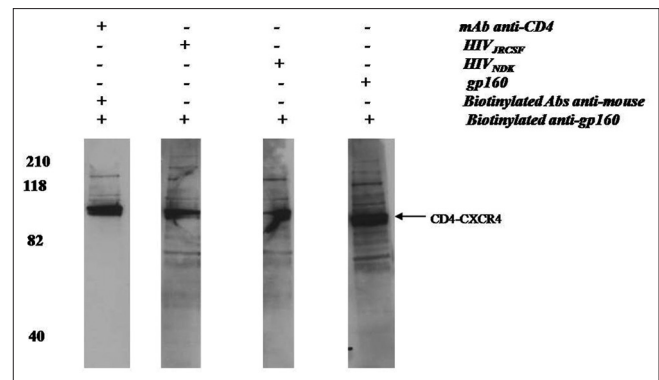
*HIVgp 160 is an envelope gp precursor of high immunogenicity, antibodies to gp 160 are the earliest to appear after infection followed shortly by antibodies to gp41, p55, p66 and p51. A positive result has been interpreted by Centers for Disease Control to be presence of any two bands p24, gp 41, gp 120/160* <http://www.clinlabnavigator.com/human-immunodeficiency-virus-western-blot.html>.

Another study has shown that a solid positive result of HIV infection could be based only on the presence of the two bands gp160 and p31.<sup>[22]</sup>

All western blots in this report were repeated at least six times with reproducible results. Representative images are shown.

### Western blots with peripheral blood lymphocyte lysates

The western blot patterns obtained with PBL lysates on nitrocellulose strips, HIV-1<sub>NDK</sub>, HIV-1<sub>JRCSF</sub> and recombinant gp160 for the detection or visualization is shown in



**Figure 1: Western blot analysis of human immunodeficiency virus-interacting cluster difference 4 complex** 10 µg of total membrane protein extracts of activated peripheral blood lymphocytes were purified. Purified proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Western blotting was carried out using mouse primary monoclonal antihuman cluster difference 4 antibody, recombinant protein gp160, human immunodeficiency virus-1<sub>NDK</sub>, or R5-human immunodeficiency virus-1 (identified by JRCSF). The bounded mAb anticluster difference 4 was labeled with biotinylated goat polyclonal antibodies antimouse immunoglobulin G and the bounded gp160 with the secondary biotinylated anti-gp160. After exposure to chemiluminescent reagents, membranes were exposed to X-ray film for 5 min

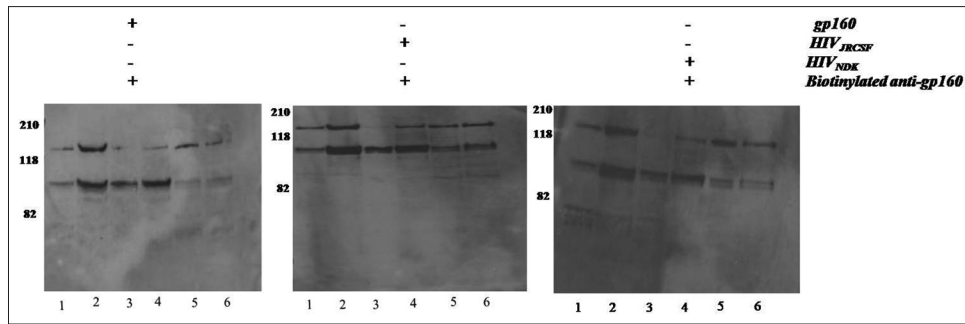
Figure 1. Different patterns of migration were observed, including a unique and main band at 105–117 kDa and various nonspecific bands. The presence of these faint nonspecific bands could be attributed to the presence of unknown glycosylated or posttransitionally modified proteins. It is worth mentioning that a major limitation of immunoblotting is that many antibodies exhibit off-target effects by interacting with other proteins. These bands could also be due to less than perfect compatibility between homogenization buffer, blocking agents, and antibodies; however, the main bands of significance are clearly visible and proving the concept. The 105–117 kDa bands migrated at the same level as that observed in western blot control using total PBL lysate and anti-CD4 mAb for revelation, and thus likely corresponding to the CD4 complex.

### Western blots using lysates of dendritic cells, MDM, Hela, and HEC-1A cell lines

On the other hand, lysates of DCs, MDM, Henrietta Lacks (Hela), and HEC-1A cell lines on nitrocellulose strips and HIV-1<sub>NDK</sub>, HIV-1<sub>JRCSF</sub> and recombinant gp160 for the detection showed the pattern presented in Figure 2. Each pattern comprised several bands with similar positions whatever the revelation was by recombinant gp160 or whole R5- or X4-HIV particles. The intensity of bands on nitrocellulose strips might vary from cell lysate to another according to the mode of detection.

## Discussion

Western blot patterns obtained with PBL lysates and HIV-1<sub>NDK</sub>, HIV-1<sub>JRCSF</sub> and recombinant gp160 for revelation contained a unique and main significant band at 105–117 kDa, migrating at the same level as that



**Figure 2:** Human immunodeficiency virus-interacting proteins from primary cells and cell lines were detected by immunoblotting purified proteins were obtained from primary cells (dendritic cell, macrophages, or cluster difference 4 T-cells) or from cell lines (HEC-1A cells and HeLa cells expressing or not dendritic cell-Specific Intracellular adhesion molecule-3-Grabbing Non-Integrin molecule). Western blotting was carried out by recombinant protein gp160, human immunodeficiency virus-1<sub>NDK</sub> or R5-human immunodeficiency virus-1. Biotinylated goat polyclonal antibodies, antimouse immunoglobulin G, and the bounded gp160 were detected with the secondary biotinylated anti-gp160. After exposure to chemiluminescent reagents, membranes were exposed to X-ray film for 5 min

observed in western blot control using total PBL lysate and anti-CD4 mAb for revelation. This band likely corresponds to the CD4-CXCR4 complex.<sup>[23]</sup> Unstimulated T-cell membranes exhibit multiple CXCR4 forms of 47, 50, 64, and 90–100 kDa. In activated PBL, the expression of the 47-kDa CXCR4 isoform is greatly decreased, whereas the expression of the 50–62 kDa isoform is significantly stronger.<sup>[23]</sup> T-cell activation also induces the increase in the coprecipitation of CXCR4 with CD4 (55 kDa), forming a complex at 105–117 kDa. In addition, several other bands of different intensities could be observed on western blot using various HIV target cells and recombinant gp160 or whole R5- or X4-tropic HIV particles for revelation, confirming the identity of several cellular proteins able to bind to HIV. The similarity of patterns obtained with recombinant gp160 and whole HIV particles binding toward cellular proteins likely suggests that free HIV mostly interacted with a range of cell surface proteins through the gp160. Taken together, these findings highlight the significance to use the cell-free HIV particles to detect cellular HIV-1 attachment proteins instead of using a recombinant protein gp160.

Many approaches may be used to evaluate the interactions between HIV and membrane cellular proteins. Recently, a single C-type lectin receptor (CLR), DC-SIGN, has been reported to be the predominant receptor on MDDCs rather than CD4. To determine whether CLR or CD4 was predominant receptors on MDDCs, a novel biotinylated gp120 assay was used. Purified HIV gp120 from the BaL isolate was biotinylated with EZ-Link succinimidyl-6-biotinamido hexanoate (NHS-LC-Biotin). Cells were preincubated with biotinylated-gp120 at various concentrations for 40 min at 4°C in binding media. The detection of biotinylated-gp120 was through streptavidin-FITC.<sup>[20]</sup> However, biotinylated gp120 binding assay was routinely used because it saved one additional antibody staining step, reduced the variability of antibody binding, and allowed for more flexibility when working with blood DCs, which are labeled with multiple antibodies for the detection of multiple DC subsets.

Other groups studied binding assays using gp120-Fc. In order to facilitate binding assay, the authors used a HIV-1 gp120 construct fused to the Fc region of human immunoglobulin G1. Secondary reagents against the human Fc region were used to detect binding of this gp120-Fc fusion in a Fluorescence-activated cell sorting (FACS)-based assay. This strategy avoids modifying gp120 itself (e.g., via biotinylation) or the use of anti-Env reagents that might themselves interfere with DC-SIGN/Env binding.<sup>[24]</sup>

A cell surface gp120 binding assay was used to evaluate gp120-coreceptor interactions for a variety of R5- and X4-tropic isolates of HIV-1 and HIV-2. Secreted gp120 proteins were generated in 293T human cells. Target cells were incubated with gp120 proteins for 1 h at 37°C. Cell pellets were lysed with ice-cold lysis buffer and then cell lysates were analyzed by SDS-PAGE.<sup>[25]</sup>

Western blot of cell lysates using whole free HIV particles for the formation of detectable complexes may have some advantages. Thus, whole cell-free HIV-1 particles allow to study cellular HIV-1 attachment proteins in more physiological condition. Our novel technique may be valid on a wide range of HIV target cells using various virus isolates regardless of their tropism. Finally, this western blot approach is relatively easy to perform and definitely less expensive when compared with western blotting using recombinant gp160. Our new technical approach using whole HIV particles offers three important advantages over the common western blot: (i) it can be used with a broad range of HIV strains including both R5-HIV-1<sub>JRCSF</sub> and X4-HIV-1<sub>NDK</sub> particles, (ii) cell-free HIV-1 particles, that are easily obtained in large quantities, could be used instead of the extremely expensive gp160, and (iii) our technique allows us to study cellular HIV-1 binding proteins under more physiological conditions. Finally, our study highlights the importance or significance of using cell-free HIV particles to detect new cellular HIV-1 attachment proteins instead of using a recombinant protein gp160.

## Conclusion

Western blot assay using HIV target cell lysate and the cell-free HIV particles or recombinant protein gp160 may be useful to screen new cellular HIV-1 binding proteins.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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