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A post-CD4-binding step involving interaction of the V3 region of viral gp120 with host cell surface glycosphingolipids is common to entry and infection by diverse HIV-1 strains

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Abstract

The V3-loop region in the envelope protein gp120 of HIV is critical for viral infection, but its interaction with the target cells is not clear. Using synthetic peptides, representing linear V3 sequences as reagents, we obtained evidence to show inhibition of infection by both T-cell- and macrophage-tropic strains of human immunodeficiency virus type 1 (HIV-1) (X4 and R5, respectively), without interfering with gp120–CD4 interaction, by the V3 peptides through binding to host cell membrane glycosphingolipids (GSL). Synthetic peptides mimicking the central 15–21 amino acid sequence of the V3-loop region in both X4 and R5 strains of HIV-1 competed with and blocked the entry of both types of HIV isolates. These HIV-inhibitory V3 peptides exhibited specific binding to target cells that was not competed by antibodies to either the primary receptor CD4 or the co-receptors CXCR-4 and CCR5. However, R15K, the V3 peptide from HIV-1 IIIB gp120 exhibited specific binding to three distinct cell surface GSL: GM3, Gb3, and GalCer. Further, R15K inhibited GSL binding of gp120 from both HIV-1 IIIB (X4, Gb3-binding strain) and HIV-1 89.6 (X4R5, GM3-binding strain). Together, these results suggest a critical V3-mediated post-CD4-binding event involving cell surface GSL binding represented by the HIV-inhibitory V3 peptides, that is common for the entry of diverse HIV-1 strains and may be targeted for the development of novel HIV therapeutics aimed at blocking viral entry.
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1. Introduction

Infection of human cells by the human immunodeficiency virus type 1 (HIV-1) involves sequential interaction of the viral envelope surface protein gp120 with cellular receptors that leads

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to the fusion of the viral and cellular membranes mediated by the viral transmembrane protein gp41. The CD4 molecule serves as the primary receptor for HIV binding to human cells, even though HIV infection of $CD4^-$ cells has also been reported (Fauci, 1996; Olinger et al., 2000). However, successful infection of human cells also requires binding of gp120 to a co-receptor belonging to the CXC- or CC-chemokine receptor family (reviewed in Berger et al., 1999). In general, the T-cell-tropic HIV-1 isolates use CXCR-4 as the co-receptor (X4 strains), those with macrophage-tropism use mainly CCR5 (R5 strains), while still others (X4R5 strains) use either or both types of receptors (Berger et al., 1999; Berson and Doms, 1998; Dimitrov, 1997; Moore et al., 1997). It has also been reported that the interaction between gp120 and CD4 is of high-affinity, while the interaction with the coreceptor is of much lower affinity, so additional interactions with the coreceptor and CD4 may aid in stabilizing the complex (Lee et al., 2000).

In addition to the CD4 receptor and co-receptor, recent reports have shown that glycosphingolipids (GSL), lipid rafts, may also be involved in HIV-1 entry mechanisms (Campbell et al., 2001). In the plasma membrane, GSL are found in highly specialized microdomains (lipid rafts) characterized by a distinct lipid composition that includes high concentrations of cholesterol and sphingolipids (Masserini and Ravasi, 2001). Interaction of viral gp120 with certain cell surface glycoshingolipids like globotriaosylceramide (Gb3) and monosialoganglioside (GM3), was shown to be essential for viral entry and fusion (Puri et al., 1998; Hammache et al., 1999). CXCR4 receptor association with GM3 was also observed when gp120 bound to the cell surface of Sup T1 cells induced CXCR4 recruitment to the glycosphingolipid-enriched microdomain (Sorice et al., 2001). Also, CD4-independent infection of human colonic epithelial cells (HT-29) was shown to involve the use of galactosylceramide (GalCer) as an alternate receptor for HIV gp120 (Yahi et al., 1992). Moreover, Alfsen et al. (2001) demonstrated that gp120 and gp41 of HIV-1 bind GalCer and part of the lipid raft structures at the apical membrane of epithelial cells. It was also shown that gp120 and gp41 use these lipid structures as a transport mechanism to travel from the apical side of the cell to the basolateral side. Further, Hug et al. (2000) reported that blocking of membrane GSL biosynthesis in human cells results in inhibition of infection by both X4 and R5 strains of HIV-1 despite normal expression and association between CD4 and the respective co-receptors. Based on these results it has been proposed that target cell GSL play a key role in the assembly at the host cell membrane of HIV-1 fusion complex that consists of viral envelope gp120–gp41, and host cell CD4 and a chemokine receptor (Hammache et al., 1999; Manes et al., 2000). Also, it has been shown that treatment of cells with beta-cyclodextrins results in cholesterol depletion and raft dispersion, and that removal of cellular cholesterol rendered primary cells and cell lines highly resistant to HIV-1-mediated syncytium formation and to infection by both CXCR4- and CCR5-specific viruses (Liao et al., 2001). Taken together, these data strongly suggest that intact and functional lipid rafts are required for infection of cells by HIV-1.

While the region in the HIV gp120 that binds to CD4 has been well characterized (Berger et al., 1999; Berson and Doms, 1998; Dimitrov, 1997; Moore et al., 1997; McDougal et al., 1986; Olshevsky et al., 1990), it is not clear as to what are the critical sites in the viral envelope protein that interact with the co-receptors and/or GSL. Indirect evidence from several reports suggested that the V3-loop region of gp120 is essential for these interactions because antibodies to the principal neutralizing domain in the V3-loop region inhibit HIV infection of $CD4^+$ human cells without interfering with the binding of gp120 to CD4 (Linsley et al., 1988; Rusche et al., 1988; Javaherian et al., 1989). Additionally, antibodies directed against conformational epitopes in gp120, in particular those involving the V3-loop region, were shown to inhibit HIV-infection indicating that conformational changes subsequent to CD4–gp120 interaction, are involved in HIV infection (Linsley et al., 1988; Moore and Nara, 1991; McKeating et al., 1992; Sattentau and Moore, 1991; Lapham et al., 1996; Wu et al., 1996; Trkola et al., 1996). In this regard, it is reported that cyclized V3 peptides (V3-BH10, V3-ELI, V3-ADA

and V3-89.6), but not the linear sequences (CTR36) corresponding to the entire V3-loop region from X4 and R5 strains bind to CXCR4 and CCR5 (Sakaida et al., 1998; Rabehi et al., 1998). Further, binding of cyclized V3-loop peptide from X4 or X4R5 strains to CXCR4 co-receptor resulted in inhibition of infection by X4 strains (Sakaida et al., 1998). These reports suggest that the interaction of the V3-loop region in gp120 with the co-receptors is a conformationally dependent event. Interestingly, CD4-independent infection of human colonic epithelial cells (HT-29), that use GalCer, the cell surface GSL, as alternate receptor for HIV gp120, is inhibited by suramin through blocking of the binding of V3 region to GalCer (Yahi et al., 1994b). Further, binding of recombinant gp120 to GalCer was blocked by V3-specific monoclonal antibodies suggesting direct interaction between the V3-loop region in gp120 and GalCer (Cook et al., 1994). Finally, the V3 domain was characterized as a major determinant controlling HIV-1 tropism for $CD4^-$/$GalCer^+$ cells (Harouse et al., 1995; Fantini et al., 1996; Trujillo et al., 2000). Together, these reports provide compelling evidence for the involvement of the V3 region during HIV infection, particularly viral entry and membrane fusion, through interactions with several cell surface molecules (co-receptor proteins and GSL).

Studies from our group (Nehete et al., 1993) along with others reported previously showed that synthetic peptides corresponding to the central region in V3-loop blocked infection and syncytium formation by different primary and laboratory isolates of HIV-1. Previously published studies employed V3 peptides of different length (18 amino acids, Zanotto et al., 1995) or as multi-branched peptides (Yahi et al., 1994a, 1995; Delezay et al., 1996) and template assembled synthetic peptide constructs (TASP, Benjouad et al., 1995; Callebaut et al., 1993). In these studies, the inhibition of viral infection was measured in terms of virus production by lower p24 levels in treated cultures, or inhibition of virus-induced syncytium formation. Using linear V3 peptides as reagents, we extended our studies to demonstrate that the V3-loop region in gp120, in particular the central 15–21 amino acid sequence, is involved in the entry of both X4 and R5 strains of HIV-1 through host cell membrane GSL binding, as detected through PCR techniques.

2. Experimental procedures

2.1. Materials

The $CD4^+$ T-lymphoblastoid cell lines were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS). Primary human peripheral blood mononuclear cells (PBMCs) were prepared by density gradient centrifugation of whole blood over Ficoll-Hypaque and cultured in RPMI-1640 medium containing 10% FBS and phytohemagglutinin (PHA) at 1 μg/ml for 48–72 h before using for infection. Various plasmids representing the molecular clones of HIV-1 such as pNL4-3, pYK-JRCSF, pYU-2, and $pMenv^{(-)}$, and HIV envelope gene constructs like HXB2 were obtained through the AIDS Research Reference Reagent program, division of AIDS, NIAID, NIH (pNL4-3 from Dr Malcom Martin, pYK-JRCSF from Irvin Chen and Yoshio Koyanagi, pYU-2 from Beatrice Hahn and George Shaw, $pMenv^{(-)}$ from Dr Reza Sedaie, and, HXB2-env from Dr Kathleen Page and Dr Littman). The plasmids 91US712 and 92BR020, encoding envelope sequences from primary syncytium inducing (SI) and non-syncytium inducing (NSI) HIV strain, respectively, were obtained from Dr Feng Gao, University of Alabama at Birmingham, Birmingham, Alabama. The plasmid MD.6 encoding the VSV glycoprotein was obtained from Dr Inder Verma, Salk Institute, San Diego, CA. Glycolipids were obtained from Sigma.

In the present investigation we used linear peptides between 15 and 21 amino acids in length derived from the V3-loop regions in the T-cell- and macrophage-tropic HIV-1 strains (X4 and R5, respectively): R15N, (RIHIGPGRAFYTTKN) from HIV-1 MN, R15K (RIQRGPGRAFV-TIGK) from HIV-1 IIIB, and R21D (RKSIHIGP-GRALYTTGEIIGD) from HIV-1 BAL. The peptides were made in the institutional synthetic antigen core facility as reported earlier (Nehete et al., 1993) using the Merrifield solid-phase method

(Merrifield, 1963) either on a modified Vega 250 automatic peptide synthesizer (Vega Biochemicals, Tucson, AZ) or by the 'bag' method as described by Houghten (1985). The purity of the peptides used was determined to be >95% by high-pressure liquid chromatography and was validated by mass spectrometry. In some experiments, we also used R15K peptide (>95% pure) obtained from three other sources: Multiple Peptide Systems (San Diego, CA), Advanced Chem. Tech. (Louisville, KY), and Therapeutic Peptides Inc. (Harahan, LA). Peptide L275 consisting of a scrambled sequence of R15K (IFPGKRTIVAGQRGR) and another peptide D167 with a scrambled sequence close to R15K (IFPGKRTIVAGIRGM) were used as control reagents. As additional controls, we used several gp120 peptides that are from regions other than V3, and also peptides from HIV-1 gag and pol. Also, we used one peptide from the c-mos protooncogene (STRTPEDSNSLGT) and another from the C-terminal region of Bcr (PAPDSKRQSILFSTEV) as controls in some experiments. In monolayer experiments, a linear 6-mer V3 peptide derived from the clade-B consensus motif GPGRAF was used as control. In addition, synthetic peptides corresponding to the extracellular N-terminal part of CXCR4 (MEGISIYTSDNYTEEMGSGDYD) and CCR5 (MDYQVSSPIYDINYYTSEPCQK) were obtained from the Centre d'Immunologie de Marseille-Luminy. To increase water solubility of the CCR5 peptide, two arginine residues were added at the C-terminal end. Stock solutions of peptides were prepared in phosphate buffered saline (PBS) (pH 7.0) and filter sterilized. Further, we tested the effects of the V3 peptides on lipid associations with unrelated proteins.

2.2. Production of virus stock

Infectious virus stock was prepared from each of the different molecular clones of HIV-1 strains used in the study by transfecting Cos-1 cells with 15 μg of the plasmid DNA using either DEAE-dextran or LipofectAmine 2000. For preparing the pseudotyped viruses, plasmid DNA (8 μg) encoding the env-defective HIV-1 (pMenv-) and the VSV, A-MuLV, or the HIV-1 envelope sequences from the T-cell-tropic, SI or NSI strains (HXB2, 91US712, and 92BR020, respectively) were co-transfected into Cos-1 cells using either DEAE-dextran or LipofectAmine 2000. The culture supernatants containing infectious virus were collected 48 h after transfection, quantified by determining the reverse transcriptase activity or p24 antigen, and either used immediately or stored at −80 °C.

2.3. Assay for HIV infection (entry)

We used a PCR-based method for analyzing the early stages of virus entry in a 4 h infection assay employing HIV-1 molecular clones representing X4 and R5 strains (Deng et al., 1996). We also performed an assay involving single round of infection by HIV-1 pseudotyped with envelope sequences from either VSV or primary SI, NSI isolates, or a laboratory HIV strain (Deng et al., 1996). A positive infection in the presence or absence of various peptide inhibitors was assayed by PCR using either gag or LTR primers for HIV-1. The virus stock in each case was treated with DNAse (200 u/ml, Boehringer Mannheim Corporation, Indianapolis, IN) for 30 min at 37 °C, to remove any contaminating viral DNA. The target cells for infection were human PBMCs activated with PHA or human cell lines (PM-1 and Jurkat) in log phase growth. Aliquots of 1×10^6 cells were incubated in RPMI-1640 medium containing 10% FBS, along with either the control or V3 peptides at various concentrations for 30 min at 37 °C. At the end of the incubation, cells were infected with various viruses (equivalent to 40–800 pg p24 for the cloned viruses, and 0.1–2 ng p24 for the pseudotyped viruses). The cultures were incubated for 4 h at 37 °C before harvesting by lysing cells for DNA extraction and PCR analysis. The DNA was extracted by overnight incubation of cells at 65 °C in 50 μl of lysis buffer (10 mM Tris–HCl, pH 8.0, 100 mM KCl, 2.5 mM $MgCl_2$, 0.5% Nonidet P-40, 0.5% Tween 20, and 0.3 mg/ml proteinase K). The PCR was carried out using 4 μl of cell extract in 50 μl PCR buffer (50 mM KCl, 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$), primers for HIV-1 gag SK38 and SK39 or HIV-1 LTR M667 and AA55, (each at 50 pmol,

from Life Technologies, Grand Island, NY), 10 mM of each of the dNTPs and 0.25 units of *Taq* DNA polymerase (Perkin–Elmer, Norwalk, CT). The samples were subjected to 30 cycles of PCR in a thermocycler (Perkin–Elmer, Norwalk, CT) with the denaturing step at 94 °C for 45 s, annealing step at 55 °C for 45 s, and an extension step at 72 °C for 2 min. As a control for the amount of DNA used, we also performed amplification of the β-actin gene in the PCR cocktail containing the β-actin primers B10 and B11 (50 pmol each, from Life Technologies, Grand Island, NY) and subjected to 30 cycles of amplification. The PCR products were visualized by agarose gel electrophoresis (1.8% gel) and ethedium bromide staining. The intensities of the PCR products were quantitated using a Personal Densitometer SI (Molecular Dynamics Sunnyvale, CA) and ImageQuant version 5.0 software. The percent inhibition of HIV infection by the peptides in terms of decreases in the PCR product intensities were calculated, by comparing to the values in the absence of peptide additions.

2.4. Cellular binding studies

For testing whether the V3 synthetic peptides specifically bind to cells, we employed ^{125}I-labeled V3 peptide R15N from the HIV-1 MN strain because of the presence of a tyrosine residue in the peptide that enabled radio-iodination. The HPLC-purified R15N peptide prepared in the Institutional core synthetic-antigen laboratory was custom ^{125}I-labeled by NEN-Dupont (Boston, MA) and obtained as an aqueous solution with a specific activity of 2200 Ci/mmol. Human T lymphoblastoid cell lines (e.g. Jurkat, H9, CEM, and PM1), and PHA-activated PBMCs were used as target cells for testing the binding of ^{125}I-labeled-R15N. Cells (1×10^6) were washed and resuspended in 200 μl of binding buffer (Hanks balanced salt solution supplemented with 0.1% BSA and 1 mM Tris–HCl, pH 7.0) before adding ^{125}I-labeled-R15N (1.75 nM). After incubation at 37 °C for 45 min on a nutator (Clay Adams, Parsippany, NJ), 1 ml of cold PBS was added to the cells, centrifuged and the supernatant discarded before estimating the bound radioactivity in the cell pellet using the gamma counter (Wallac Inc., Gaithersburg, MD). Typically, total bound radioactivity was in the range of 3000–10,000 cpm. For competition assays, aliquots of target cells (1×10^6) were preincubated for 10 min at 37 °C with serial dilutions of unlabeled R15N peptide, other V3 peptides, or unrelated control peptides. Subsequently, the cells were washed with sterile PBS, and resuspended in 200 μl of binding buffer before adding the ^{125}I-labeled R15N. We also tested the effect of the V3 peptide R15K (40 ng) on the binding of anti-CXCR4 and -CCR5 monoclonal antibodies to cell surface CXCR4 and CCR5, respectively, by the flow cytometric assay as described before (Endres et al., 1996). We used four different anti-CXCR4 antibodies (12G5, 44717.111, 44708.111, and 44716.111) and three different anti-CCR5 antibodies (2D7, 45531.111 and 45523.111) obtained from the AIDS Research and Reference Reagent Program Repository of the NIH. Each of the antibodies was used at different concentrations ranging from 62 ng/ml to 10 μg/ml. We used recombinant stromal cell-derived factor 1 (rSDF-1), the natural ligand for CXCR4 (1000 ng/ml) as a positive control reagent in these experiments. The incubation was for 45 min on ice and the second antibody was an FITC-conjugated goat-*anti*-mouse IgG. The cells were fixed and analyzed on Coulter Epics Elite flow cytometer using the Coulter Elite software (Beckman Coulter, Inc., Miami, FL).

2.5. Interaction of HIV-1 gp120 and R15K with membrane GSL

To analyze GSL–gp120 and GSL–R15K interactions, a reconstituted monolayer of purified GSL was prepared at the air–water interface as a model for GSL membrane microdomain (Hammache et al., 1998b). Additionally, the monolayer of GSL extracted from plasma membrane microdomains as described earlier (Fantini et al., 2000) was also employed in certain experiments. Briefly, plasma membrane microdomains were isolated from 2×10^9 PBMC from seronegative donors by centrifugation of Triton X-100 insoluble material on a sucrose density gradient (Brown and Rose, 1992). The microdomains were recovered as mo-

lecular complexes from the buoyant fractions (12.8–14.0% sucrose) in agreement with previously characterized detergent insoluble membranes (DIMs) (Brown and Rose, 1992). Following purification on sucrose density gradient, the DIMs were dissolved in chloroform–methanol (1:1, v/v), dried under a chemical hood, and eventually resuspended in hexane–chloroform–ethanol (11:5:4, v/v/v). The lipids were spread at the air–water interface of a fully automated computerized microtensiometer trough (μTrough SX, Kibron Inc.) and the variations of surface pressure ($\Delta\pi$, expressed in mN/m) induced by gp120 (or the indicated synthetic peptide) added in the aqueous subphase were measured as a function of time. In all experiments, the GSL monolayers were prepared at an initial pressure of 10 mN/m. The surface envelope glycoproteins gp120 from HIV-1 (IIIB) or HIV-1 (89.6) were purified by lectin affinity chromatography (Hammache et al., 1998a) and incubated in the aqueous phase underneath the monolayer at a final concentration of 8 nM. The R15K peptide was added at a concentration of 100 nM. For competition experiments, 8 nM gp120 and 100 nM R15K were added simultaneously in the aqueous phase. The maximal surface pressure increase ($\Delta\pi$ max) was measured after reaching equilibrium. To evaluate the inhibitory effect of the peptide on gp120–glycolipid interactions, a percentage of inhibition was calculated according to the following formula: $1-[(\Delta\pi \text{ max}(\text{R15K}+\text{gp120})-\Delta\pi \text{ max}(\text{R15K alone})]/[\Delta\pi \text{ max}(\text{gp120 alone})] \times 100$.

3. Results

3.1. The V3 region in viral gp120 is involved in the entry of HIV into cells

Others and we have reported that peptides corresponding to the central portion of the V3-loop region in the HIV envelope protein inhibit HIV infection and syncytium formation (Nehete et al., 1993; Callebaut et al., 1993; Yahi et al., 1994a, 1995; Benjouad et al., 1995; Delezay et al., 1996). Using linear V3 peptides as reagents and the method based on the polymerase chain reaction (PCR) to detect HIV-1 early-DNA in various target cells, we investigated the potential role of V3 region for the entry of HIV-1 into target cells. Kinetic analyses of early DNA reverse transcripts after infection of cells with NL4-3 (a typical X4 virus, Fig. 1A) or YU-2 (a typical R5 virus, Fig. 1B) revealed that 4 h period of infection was adequate for detecting a 115-bp PCR product representing the HIV-1 gag sequence. Further, when we used heat-inactivated virus preparation no gag-specific PCR product was observed confirming that the PCR analysis was detecting only products from active virus infection (data not shown). Pretreatment of human PBMC with R15K at doses ranging from 10 to 100 μg/ml for 30 min at 37 °C resulted in strong inhibition of viral DNA synthesis (Fig. 1C). Densitometric analyses of the PCR product intensities using the ImageQuant software revealed inhibition of HIV infection by 80, 86, 95, and 13% at 100, 40, 10, and 1 μg/ml concentrations of R15K, respectively. Inhibition of HIV infection, in terms of reduction in PCR signal, was also observed when the target cells were treated with R15K (40 μg/ml) for 0.5–2 h before (83–96%), or at the time of infection (84%), but not after the addition of the virus (Fig. 1D). These results suggest potential involvement of this region of the V3-loop in gp120 during the early stage(s) of HIV entry into cells.

Employing R15K as a representative peptide of the X4 strains, and an analogous peptide R21D from HIV-1 BAL, a typical R5 strain, we further investigated the role of the V3 region of gp120 in the infection by X4 and R5 strains of HIV-1. Infection of human PBMC by a molecular clone of an X4 HIV-1 strain, derived from the plasmid pNL4-3, was inhibited by both R15K and R21D peptides as indicated by reduction in the intensity of the 115-bp PCR band representing the gag sequence (Fig. 2A). Control peptides L275 and D167 were not inhibitory to the infection by the NL4-3 virus. The 5- and 10-fold dilutions of the infected cell lysate used for PCR analysis clearly showed the reduction in the PCR band in V3 peptide-treated cells compared to those treated with the control peptide. The 110-bp β-actin PCR band amplified from the same DNA samples served as the control. We also observed that

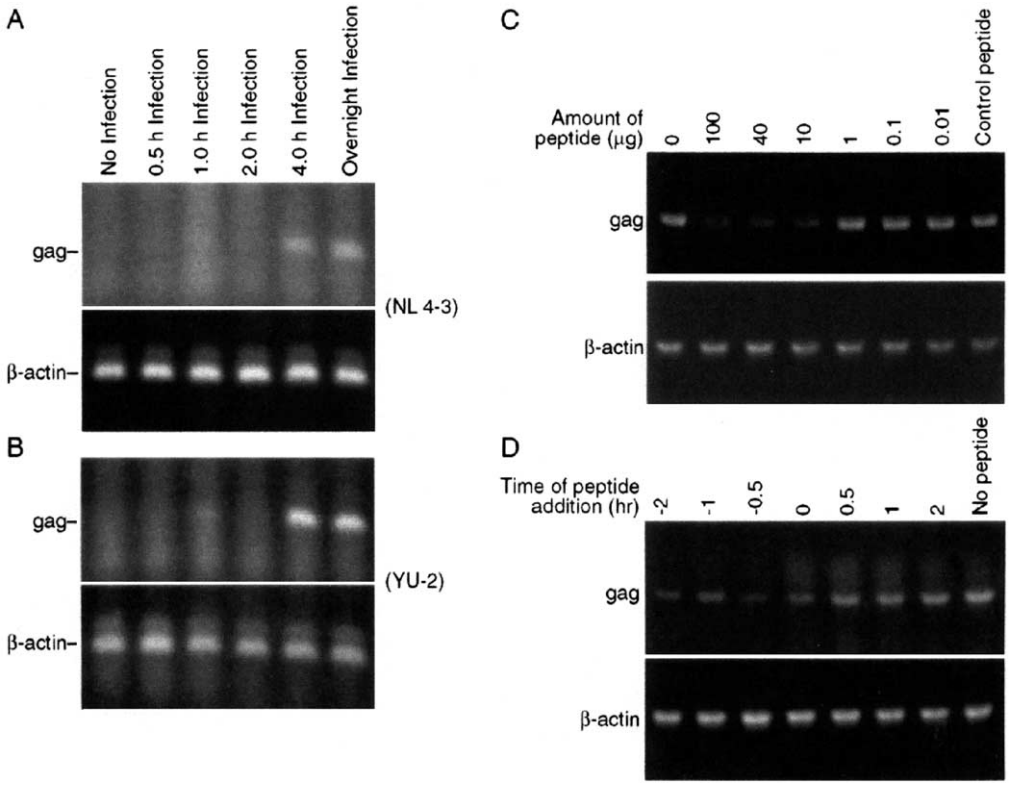

Fig. 1. Kinetics of HIV-1 infection of cells and inhibition of HIV-1 entry into cells by R15K. Panel A: PBMC from HIV-seronegative healthy individuals (1×10^6 cells) were infected with HIV-1 NL4-3 (equivalent to 40–800 pg p24), and at different time intervals after infection at 37 °C the cells were washed, lysed and used for PCR analyses for detection of the HIV-1 gag and β-actin sequences as described in Section 2. Panel B: PBMC (1×10^6 cells) were infected with HIV-1 YU-2 (equivalent to 40–800 pg p24), and at different time intervals after infection the cells were washed, lysed and used for PCR analyses for detection of the HIV-1 gag and β-actin sequences. Panel C: Aliquots of PBMC (1×10^6 cells) were pretreated with the indicated amounts of the peptide for 30 min at 37 °C, and infected with HIV-1 NL4-3 for 4 h at 37 °C before harvesting. DNA isolation and PCR analysis for detection of the HIV-1 gag and β-actin sequences was performed. The control peptide was with a scrambled amino acid sequence to R15K, and was used at 100 μg/ml concentration. Panel D: PBMC (1×10^6 cells) were pretreated with R15K at 40 μg/ml for the indicated amount of time before or after infection with HIV-1 NL4-3. After 4 h of incubation at 37 °C cells were harvested, DNA isolated, and used for PCR analysis for detection of the HIV-1 gag sequences. The no peptide lane indicated PCR signals in cells infected in the absence of R15K pretreatment.

R15K and R21D peptides exhibited 71 and 76.3% inhibition, respectively of infection by a molecular clone of an R5 strain of HIV-1, derived from the plasmid pYU-2 (Fig. 2B). However, control peptides L275 and D167 were not inhibitory to the infection by these viruses. Similarly, infection by another R5 strain, HIV-1 JR-CSF, was also efficiently inhibited by the V3 peptides, R15K and R21D, but not the control scrambled peptides (data not shown).

The primary patient HIV-1 isolates are also classified on the basis of their capacity to induce syncytium formation as, SI and NSI phenotypes (Tersmette et al., 1988; de Jong et al., 1992). We tested the inhibitory effect of V3 peptides in an assay involving an envelope-defective virus pseudotyped by the envelope sequence from one of three different types of viruses; a lab-adopted SI virus (HXB2), a primary SI isolate (91US71), and a primary NSI isolate (92BR020). In this assay where only a single round of infection occurs, both R15K and R21D peptides inhibited the infection of human PBMC by each of these three pseudotyped viruses (Fig. 2C–E). Densitometric analyses

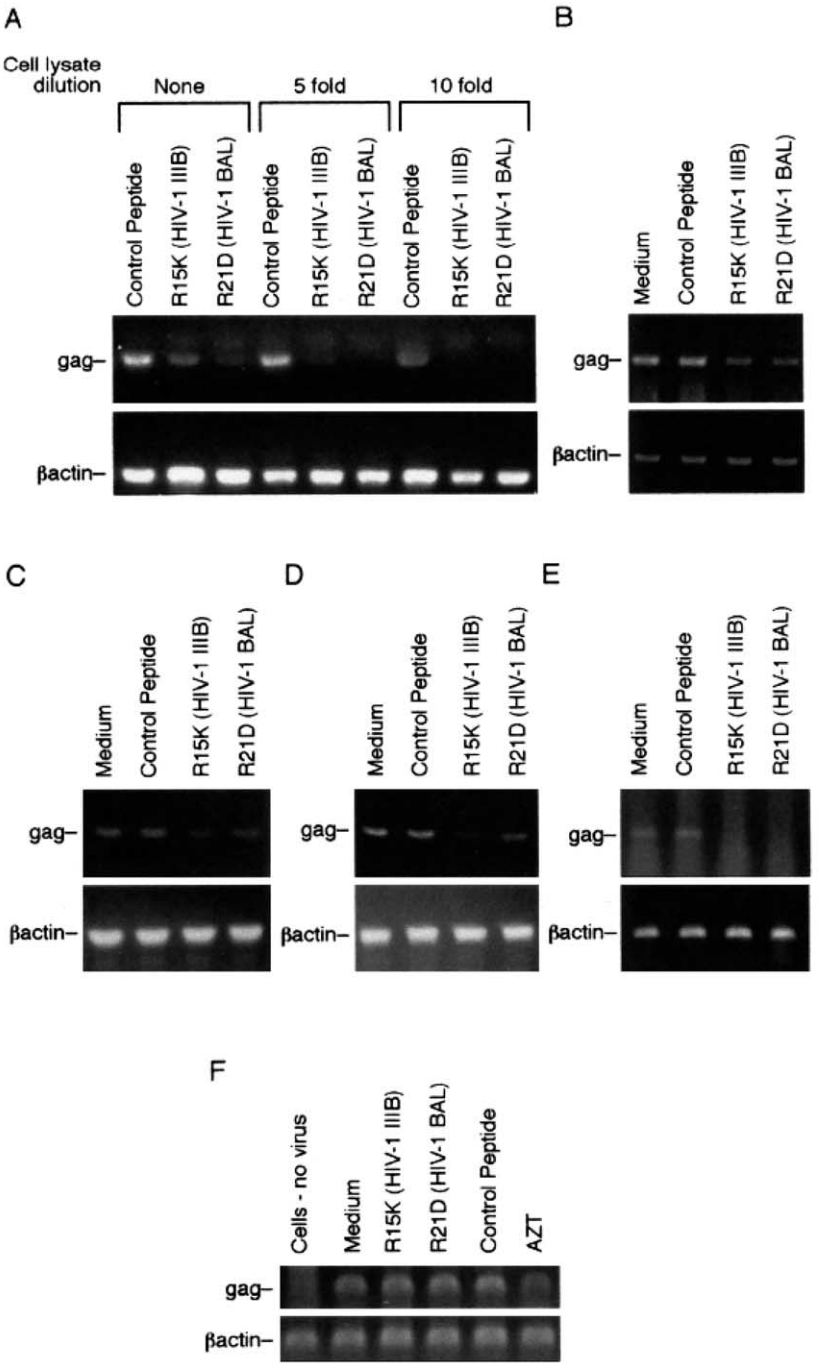
A
Cell lysate dilution
None
5 fold
10 fold
Control Peptide
R15K (HIV-1 IIIB)
R21D (HIV-1 BAL)
gag–
βactin–
B
Medium
Control Peptide
R15K (HIV-1 IIIB)
R21D (HIV-1 BAL)
C
D
E
F
Cells - no virus
AZT

Fig. 2

of the PCR signals in R15K-treated cultures revealed inhibition of infection by 95, 93, and 99% for the three viruses pseudotyped with envelopes from HXB2, 91US71, and 92BR020, respectively. Similar analysis of cells treated with R21D revealed 81, 65, and 99% inhibition of infection by the three viruses, respectively. Importantly, infection of various human cells by envelope-defective HIV pseudotyped by the envelope protein from VSV was not inhibited by the V3 peptides, while inhibition was observed with AZT (Fig. 2F). Similar results were obtained in several repeat experiments using an envelope-defective HIV pseudotyped by the envelope protein from A-MuLV (data not shown). In these experiments, peptide L275 with scrambled amino acid sequence to R15K (shown as control peptide in all the panels of Fig. 2) did not show inhibition of infection by any of the viruses employed. These data further support the important role of distinct V3 sequences in gp120, mimicked by the peptides employed (R15K and R21D), during the entry of different HIV-1 strains. Further, these results also suggest a V3-mediated event during the early stages of infection that is common to the entry of phenotypically different strains of HIV-1.

To determine whether the V3 region also plays an active role in post-entry stages in HIV infection, we tested the V3 peptides for their effect on HIV replication in terms of modulating the expression of a reporter gene, chloramphenicol acetyl transferase (CAT), under the control of HIV-1 LTR promoter (pLTR-CAT). Jurkat cells were co-transfected with plasmids pLTR-CAT and pC-tat (the HIV-1 tat gene under the control of cytomegalovirus promoter), and incubated with varying concentrations of R15K, R21D or a control peptide. No reduction in CAT activity was observed in cells treated with any of the peptides (data not shown). These results together with data presented in Figs. 1 and 2 indicate that the V3 peptide-mediated inhibition of HIV infection is manifested in terms of blocking the early step(s) of the virus entry into host cells but not at the level of HIV transcription, a post-entry stage. Additionally, we reported earlier that the V3 peptides used in the study did not exhibit direct virucidal effects because, experiments carried out to preincubate virus inoculum with the peptides for various time points followed by removing the peptide before adding to the cells showed no loss of infectivity (Nehete et al., 1993).

3.2. Cell-surface interaction of the V3 region in HIV envelope protein

We predicted that the observed inhibition of HIV entry into cells by the V3 peptides might involve competition by the peptide with analogous V3 region in the virion for specific binding to the target cells. To test this hypothesis, we first determined the binding of the V3 peptide to host cells by employing ^{125}I-labeled R15N peptide from the X4 strain HIV-1 MN. We observed that binding of the labeled R15N to Jurkat cells was specific, as indicated by proportional decrease in cell-bound radioactivity in the presence of increasing amounts of the unlabeled R15N, but not a control peptide with scrambled amino acid sequence of the V3 peptide (Fig. 3). However, the requirement of 1000-fold excess unlabeled R15N (1750 nM) for 50% inhibition suggests a low-affinity binding. Similar results were obtained when a number of other human T cell lines (H9,

Fig. 2. Inhibition of an early step in entry of dierse strains of HIV-1 into cells by the V3 peptides. Aliquots of PBMC 1106 cells were actiated by treatment with PHA for 4872 h and treated with either R15K, R21D, a control peptide with scrambled amino acid sequence each at 40 gml, or medium alone before infection by arious HIV-1 strains. See Section 2 for details. Panel A: Cells pretreated with the peptides were infected with HIV-1 NL4-3 X4 strain for 4 h at 37C and the cell lysates, either undiluted or diluted 5- and 10-fold, were subjected to PCR analysis for the detection of HIV gag and -actin sequences. Panel B: Cells were infected with HIV-1 BAL R5 strain. Panel C: Cells were infected with HIV-1 pseudotyped with the enelope sequence from HXB2, a laboratory-adopted SI HIV-1 isolate. Panel D: Cells were infected with HIV-1 pseudotyped with the enelope sequence from a primary SI isolate of HIV-1 91US71. Panel E: Cells were infected with HIV-1 pseudotyped with the enelope sequence from a primary NSI isolate of HIV-1 92BR020. The DNA intensities were quantitated using ImageQuant ersion 5.0 software and the alues for peptide-mediated inhibition of infection by the arious iruses employed were calculated data included in Section 3. Panel F: Cells were infected with HIV-1 pseudotyped with the esicular stomatitis irus VSV enelope.

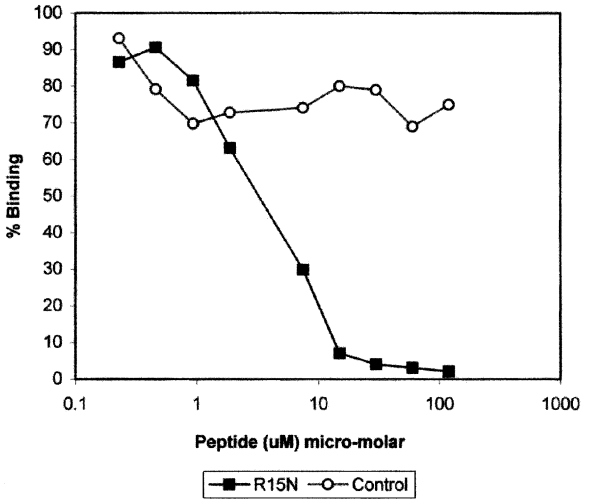

Fig. 3. Binding of ^{125}I-labeled V3 peptide to cells. Jurkat cells (1×10^6 cells) were pretreated with varying concentrations of unlabeled V3 peptide (R15N) or a peptide with scrambled amino acid sequence to R15K (control) in binding buffer. The cells were subsequently washed and incubated with a fixed amount of ^{125}I-labeled V3 peptide, R15N (1.75 nM) for 45 min at 37 °C with constant mixing. After the incubation, the cells were centrifuged and the supernatants were discarded before estimating the cell-bound radioactivity. Data shown as percentage of ^{125}I-labeled V3 peptide binding in the presence of varying amounts of unlabeled R15N and a control peptide with scrambled amino acid sequence of the V3 peptide. See Section 2 for details.

CEM, and PM1) and PHA-activated PBMC were used for binding studies (data not shown). Further, R21D, the peptide corresponding with the V3 region of BAL, an R5 HIV-1 strain, exhibited competition with labeled R15N for cellular binding indicating cross-reactivity between these peptides from phenotypically different HIV-1 strains (Table 1). Additionally, we observed that cellular binding of the R15N peptide from HIV-1 MN, a clade B isolate, was efficiently competed by V3 peptides corresponding to not only members of clade B HIV-1 strains, but also two others belonging to clade D (Table 1). However, control unrelated peptides, and those corresponding to other areas of gp120, gp41, gag or pol proteins of HIV-1 did not compete with R15N for cellular binding (data not shown). Since the V3-loop region in gp120 consists of a number of positively charged residues, it is formally possible that during the entry phase of infection the viral envelope protein engages in ionic-interaction with certain cell-surface molecules that is different from binding to the primary and secondary receptors. However, as shown in Table 1, peptides that competed effectively with labeled R15N for cellular binding as well as those that did not, exhibited a similar variation in the overall positive charge (between 1 and 5) suggesting that the V3 peptide interaction with cells we observed is not charge-based.

We also tested whether the HIV-inhibitory V3 peptides interact with the co-receptor molecules on host cells and observed that the interaction between CXCR4 and a monoclonal antibody specific to CXCR4 (44708.111) was not inhibited by the V3 peptide R15K (Fig. 4B). However, stromal cell-derived factor 1 (SDF-1), the ligand for CXCR4, exhibited strong inhibition (Fig. 4C). Fig. 4A represents the normal binding of an antibody to CXCR4 to cells expressing CXCR4. Similar to the published results (Sakaida et al., 1998), we also observed that a cyclic peptide corresponding with the complete V3-loop of an X4 strain inhibited anti-CXCR4 antibody form binding to cell surface CXCR4 (data not shown). We also tested three other anti-CXCR4 monoclonal antibodies and three different anti-CCR5 monoclonal antibodies where R15K did not show inhibitory effect (data not shown). These results suggest distinct, but differential, interactions for central portion and the complete V3-loop region in viral gp120 with host cells during HIV-1 entry. In reciprocal experiments we also tested several monoclonal and polyclonal antibodies specific to the CD4, CXCR4, and CXCR5, and observed no competition with labeled V3 peptide for cellular binding (data not shown). Further, in a typical solid-phase binding assay the V3 peptides did not interfere with gp120 for binding to CD4, and binding of the V3 peptides to $CD4^+$ cells did not change the cell-surface expression of CD4 (data not shown). Together, these results suggest that the smaller size linear V3 peptides capable of inhibiting entry of diverse HIV-1 strains, unlike cyclic peptides representing the complete V3-loop, did not bind to either the primary receptor or the co-receptor molecules. But, this does not exclude the possibi-

Table 1
Inhibition of binding of ^{125}I-labeled V3 peptide from HIV-1 MN (R15N) to Jurkat cells by different control peptides and V3 peptides from various HIV-1 strains

HIV-1 strain	Sequence	Charge[a]	Clade	% Inhibition at different concentrations (nmol)					
				24	12	6	3	1.5	0.75
RF	SITKGPGRVIYATGQ	2+	B	76	52	19	29	13	32
NY5	GIAIGPGRTLYAREK	3+	B	82	84	66	44	10	10
CDC4	RVTLGPGRVWYTTGE	2+	B	90	87	80	64	52	27
SC	SIHIGPGRAFYATGD	2+	B	92	91	74	70	62	39
SF-2	SIYIGPGRAFHTTGR	3+	B	80	67	68	62	36	27
IIIB	RIQRGPGRAFVTIGK	4+	B	76	71	55	49	28	20
BAL	RKSIHIGPGRALYTTGEIIGD	4+	B	68	67	37	39	0	0
MAL	GIHFGPGQALYTTGI	1+	D	74	68	65	56	49	31
Z6	STPIGLGQALYTTRG	1+	D	60	63	43	33	23	22
Control peptides[b]									
D167	IFPGKRTIVAGIRGM	3+	B	25	31	21	20	1	0
D112	YNKRKRIHI	5+	B	nd	0	nd	nd	0	nd

nd, not determined.
[a] Number of amino acid residues with positive charge.
[b] Peptide D167 was with scrambled amino acid sequence to the V3 peptide, and the control peptide D112 is N-terminus extension to the R15N peptide.

lity that the V3 peptides may bind to receptors/co-receptors in a way that was not detectable by the methods employed.

3.3. Specific interaction of the V3 region with cell membrane GSL

A number of reports in the literature described a role for GSL in HIV-1 entry into both $CD4^-$ and $CD4^+$ cells, and GalCer was identified as an alternative receptor allowing HIV-1 infection of human epithelial intestinal cells (Puri et al., 1998; Hammache et al., 1999; Yahi et al., 1992; Hug et al., 2000; Manes et al., 2000; Fantini et al., 2000; Brown and Rose, 1992; Hammache et al., 1998a; Tersmette et al., 1988). More recently, it has been shown that two other GSL, namely GM3 and Gb3 interact with both CD4 and gp120, leading to the formation of a multimolecular complex in specialized areas (GSL microdomains or rafts) of the lymphocyte plasma membrane (Puri et al., 1998). Since the linear V3 peptides in our studies did not exhibit binding to either the primary receptor or the co-receptors, we tested their specific binding to cell surface GSL extracted from PBMC microdomains as a possible mechanism for their inhibition of HIV-1 entry. These lipids were spread at the air–water interface of a microtensiometer trough and the variations of surface pressure ($\Delta\pi$ max expressed in mN/m) induced by gp120 added in the aqueous subphase were measured as a function of time. The insertion of gp120 in the lipid monolayer resulted in a compression of the monomolecular film that could be measured with the sensor probe of the microtensiometer. As shown in Fig. 5, gp120 purified from HIV-1 IIIB (X4 isolate) or HIV-1 89.6 (X4R5 isolate) reacted with GSL recovered from PBMC plasma membrane microdomains. The maximal surface pressure increase ($\Delta\pi$ max) induced by IIIB and 89.6 gp120 was, respectively 8.2 and 7.5 mN/m. The R15K peptide also interacted with these GSL, with a $\Delta\pi$ max of 7.8 mN/m (Fig. 5). Noteworthy, the initial rate of interaction was greater for the peptide than for the viral glycoproteins. The shorter linear 6-mer V3-loop peptide (GPGRAF) did not interact significantly with the monolayer ($\Delta\pi$ max = 0.8 mN/m). The standard deviation for these experiments never exceeded 15% of the mean value.

To identify the GSL molecules recognized by R15K, a monomolecular film of pure GSL species

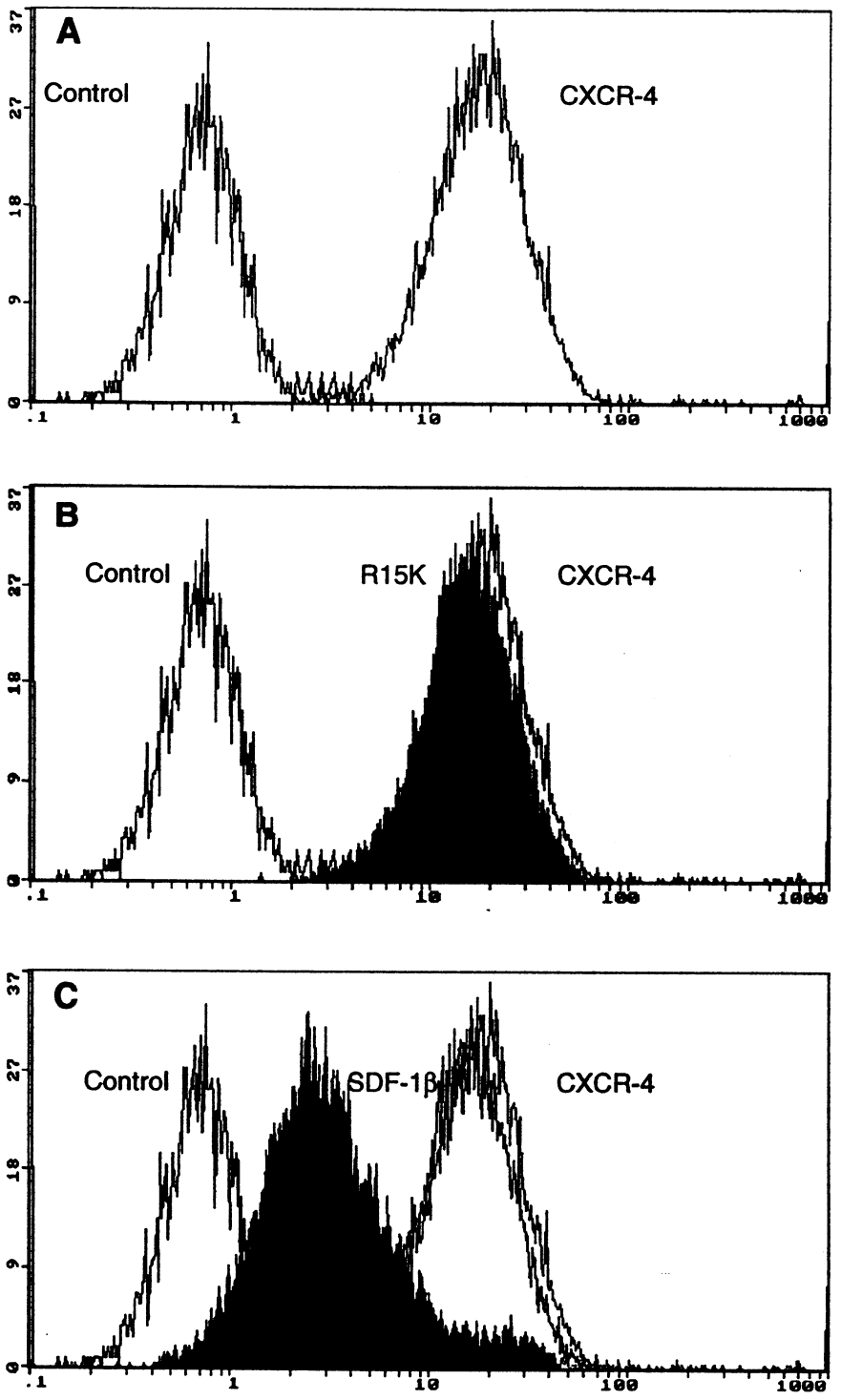
A
Control
CXCR-4
B
Control
R15K
CXCR-4
C
Control
SDF-1β
CXCR-4
0
9
18
27
37
.1
1
10
100
1000

Fig. 4

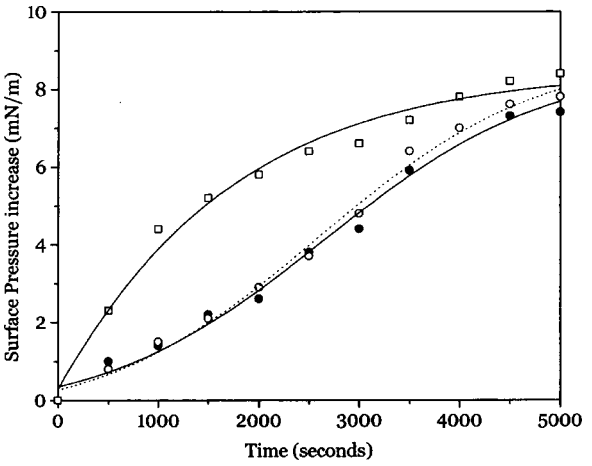

Fig. 5. Gp120 from HIV-1 (IIIB) and HIV-1 (89.6) isolates and the R15K peptide interact with GSL extracted from PBMC microdomains. A monolayer of GSL extracted from PBMC microdomains (DIMs) was prepared at the air–water interface (initial surface pressure range 8–10 (mN/m). The surface envelope glycoprotein gp120 from HIV-1 (IIIB) (full circles) or HIV-1 (89.6) (open circles, dot line) was added at a final concentration of 8 nM. The R15K peptide (open squares) was added at a concentration of 100 nM. The kinetics of the surface pressure increase induced by the viral glycoproteins or the R15K peptide was shown. HIV-1 IIIB is a T-cell line adaped X4 isolate, and HIV-1 89.6 is a primary cloned X4R5 isolate. The results shown are representative of six distinct experiments and the standard deviation values did not exceed 15% of the mean value.

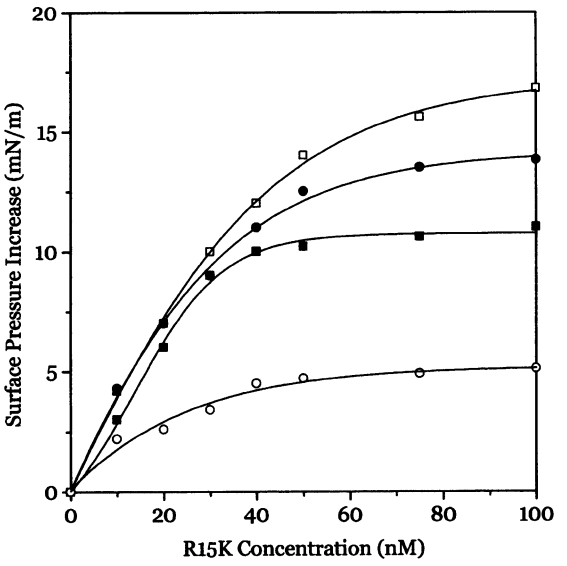

Fig. 6. The R15K peptide interacts with GalCer, GM3, and Gb3. Monomolecular films of GalCer (full circles), GlcCer (open circles), Gb3 (full squares), and GM3 (open squares) were prepared at an initial surface pressure of 10 mN/m. The R15K peptide was then added in the aqueous subphase at the indicated concentration and the maximal surface pressure increase ($\Delta\pi$ max) was determined after reaching equilibrium. The results shown are representative of six distinct experiments and the standard deviation values did not exceed 15% of the mean value.

was prepared at the air–water interface at an initial surface pressure of 10 mN/m. The peptide was then added in the aqueous subphase and the increase in surface pressure was measured until reaching a stable plateau value. Glucosylceramide (GlcCer), which is not recognized by gp120, was used as a GSL negative control. As shown in Fig. 6, the R15K peptide interacted with GM3 ($\Delta\pi$ max = 16.8 mN/m) and GalCer ($\Delta\pi$ max = 13.8 mN/m) with a similar efficiency, and, to a lesser extent, with Gb3 ($\Delta\pi$ max = 11.0 mN/m). However, the peptide showed only limited interaction with GlcCer ($\Delta\pi$ max = 5.1 mN/m). Finally, neither the shorter 6-mer V3-loop peptide, nor peptides corresponding with the N-terminal sequences in either CXCR4 or CCR5 exhibited significant binding to any of the GSL tested ($\Delta\pi$ max < 5 mN/m). The standard deviation for these experiments never exceeded 15% of the mean value. These data show that the R15K peptide interacts specifically with GalCer, GM3, and Gb3. Since GM3 and Gb3, but not GalCer, are abundantly expressed in human PBMC (Fantini et al., 2000), it can be reasonably concluded from these experiments that GSL-enriched microdomains on

Fig. 4. The V3 peptide R15K does not inhibit binding of anti-CXCR4 antibody to cells. Jurkat cells 1106 cells were pretreated with medium, or arying concentrations of the V3 peptide R15K, or a control peptide with scrambled amino acid sequence to R15K, or SDF-1 the natural ligand for CXCR4 in binding buffer. Subsequently, the cells were washed and incubated with a fixed amount of an anti-CXCR4 monoclonal antibody 44708.111. After incubation for 45 min at 4C, the cells were washed and treated with the second antibody FITC-conjugated goat-anti-mouse IgG before fixing and analyzing on FACScan flow cytometer using the FACS-Caliber software Becton and Dickinson, San Jose, CA. Flow cytometry histograms showing binding of mAb 44708.111 to Jurkat cells in medium Panel A, with R15K Panel B, or with SDF-1 Panel C. The X-axis is mean fluorescence intensity MFI and Y-axis is cell count.

the surface of human PBMC bind HIV-1 through gp120-V3 interactions with Gb3 and/or GM3.

We also investigated the relevance of the GSL binding of R15K peptide to viral infection by testing for competition between R15K and gp120 for binding to various GSL. As shown in Table 2, R15K effectively inhibited GSL binding of gp120 from both HIV-1 IIIB (X4, Gb3-binding strain) and HIV-1 89.6 (X4R5, GM3-binding strain). In contrast, the shorter 6-mer V3-loop peptide did not inhibit the insertion of gp120 into any of the GSL monolayers tested. To demonstrate that the V3 peptide R15K does not block GSL-association of other unrelated proteins, we performed two additional experiments. In the first one, we studied the effect of R15K on the interaction of CD4 with Gb3 and GM3, and observed no competition by R15K at concentrations where it showed inhibition of gp120 association with GSL (Table 2). Secondly, we observed that R15K neither exhibited specific binding to another GSL, GM1, nor it competed with the cholera toxin for binding to GM1 (Table 2). These results provide further support to the contention that HIV-1 entry into cells involves interaction between the central 15 amino acid region of the V3-loop in gp120 and GSL-enriched plasma membrane microdomains.

Table 2
The R15K peptide inhibits the interaction of HIV-1 gp120 with GSL monolayers

Monolayer	Incubation	Inhibition (%)
GSL extracted from PBMC microdomains (DIMs)	R15K+gp120 (IIIB)	88.0
GSL extracted from PBMC microdomains (DIMs)	6-mer V3+ gp120 (IIIB)	2.0
GSL extracted from PBMC microdomains (DIMs)	R15K+gp120 (89.6)	98.0
GSL extracted from PBMC microdomains (DIMs)	6-mer V3+ gp120 (89.6)	1.0
Gb3	R15K+gp120 (IIIB)	78.4
Gb3	6-mer V3+ gp120 (IIIB)	3.0
GM1	R15K+cholera toxin	0.5
GM3	R15K+gp120 (89.6)	71.2
GM3	6-mer V3+ gp120 (89.6)	2.0
GM3	R15K+CD4	1.3

R15K or the shorter 6-mer GPGRAF V3 peptides (100 nM) were added in competition with the indicated gp120 (8 nM) underneath the monolayer. The percentage of inhibition was calculated as described in Section 2.

4. Discussion

The discovery of certain chemokine receptors as co-receptors for HIV infection has greatly increased understanding of the HIV infection process and also provided new targets for therapeutic intervention (Fauci, 1996; Olinger et al., 2000; Berger et al., 1999; Berson and Doms, 1998; Dimitrov, 1997; Moore et al., 1997; Puri et al., 1998; Hammache et al., 1999; Yahi et al., 1992; Hug et al., 2000). However, the HIV infection process is complex and many new interactions between the host cell and the virion are being discovered and investigated. For example, evidence from several recent reports suggests that the V3-loop region, either alone or in combination with other regions like V1 and V2 in the gp120, is involved in HIV infection, in particular for HIV binding to the co-receptor and determining the cellular tropism (Freed et al., 1990, 1991; Ivanoff et al., 1991; Kwong et al., 1998; Wyatt et al., 1998; Rizzuto et al., 1998). In the present investigation we obtained data in further support of the critical role of the V3 region in HIV infection, and also identified sequences within the V3 region involved in binding to distinct host cell membrane GSL that are potentially important for the cellular entry of diverse HIV-1 strains. We observed that the R15K peptide corresponding to the central 15 amino acid region in the V3-loop exhibited specific binding to host cell membrane GSL like GalCer, GM3, and Gb3, and effectively competed with gp120 from X4 and X4R5 strain of HIV-1 for GSL binding. Further, our results showed that R15K, and R21D (analogous to R15K, but corresponding with HIV-1 BAL, an R5 strain) exhibited inhibition of entry of both X4 and R5 strains into human cells. These data are in accordance with several reports in the literature indicating the involvement and importance of cell membrane GSL for infection by HIV

isolates exhibiting different cellular tropism (Puri et al., 1998; Hammache et al., 1999; Manes et al., 2000; Fantini et al., 2000). Importantly, CD4-induced interaction between HIV-1 gp120 and GSL like Gb3 and GM3 could be inhibited by V3-specific monoclonal antibodies (Hug et al., 2000).

Using chimeric molecular clones of HIV-1 representing the X4 and R5 strains, Cocchi et al. (1996) demonstrated that the V3-loop region determines the cellular tropism, and is also essential for chemokine-mediated blockade of HIV infection. Further, cyclic peptides representing the entire V3-loop regions in X4 and R5 strains of HIV-1 were shown to bind to CXCR4 and CCR5, respectively without cross-reactivity (Sakaida et al., 1998; Rabehi et al., 1998). However, in these studies linear peptides corresponding to the complete V3-loop region in either of the virus strains did not bind to respective co-receptor molecules. In contrast, we observed that shorter and linear V3 peptides of 15 and 21 amino acids in length from the X4 and R5 strains of HIV-1, respectively showed cross-reactivity in terms of blocking the entry of either type of virus into human cells by binding to cell surface GSL. While these results seem conflicting for the role of V3 region during HIV infection in terms of its binding to different cell surface molecules, it is possible that both types of interactions are important for viral entry. First, it is clear from a number of studies that binding of viral gp120 to host cell CD4 results in the exposure of conformational epitopes in gp120, in particular those involving the V3-loop region, that are important for subsequent interaction with the co-receptor molecules (Linsley et al., 1988; Moore and Nara, 1991; McKeating et al., 1992; Sattentau and Moore, 1991) and virion-cell fusion (Chan and Kim, 1998; Wyatt and Sodroski, 1998). Thus, the cyclic V3-loop peptides potentially possessing the required secondary structural and/or conformational features might be best suited for in vitro demonstration of these events. However, it is also known that gp120 from different HIV isolates bind to host cell surface GSL, like Gb3 and GM3 and anti-V3 antibodies inhibit this interaction (Puri et al., 1998; Hammache et al., 1999; Yahi et al., 1992). Further, cells cultured in the presence of inhibitors of GSL synthesis are not permissive to infection by both X4 and R5 isolates despite normal levels of expression of both the CD4 and the relevant co-receptor (Hug et al., 2000). Our results are in accordance with these reports because linear V3 peptides of 15–21 amino acids in length inhibited entry of diverse HIV-1 strains into cells and also efficiently competed with the envelope proteins from both X4 and X4R5 strains of HIV-1 for binding to host cell surface GSL. Thus, our results together with the literature reports further support the hypothesis that host cell surface GSL function as membrane rafts that move the virion, bound to CD4 through gp120, on the target cell surface towards the appropriate co-receptor for binding and subsequent gp41-mediated fusion (Hammache et al., 1999; Delezay et al., 1996; Hammache et al., 1998b). In this scenario, it is possible to envision multiple interactions for distinct sequences and structural features of the V3-loop region in HIV-1 gp120 with different cell surface molecules like the membrane GSL and the chemokine receptors.

While the co-receptor interaction of the V3-loop region is reported to be dependent on the viral sequence/strain, our results showed that the cell-surface binding mimicked by the linear small V3 peptides lack this stringency and exhibit cross-reactivity. This may be because, the 15–21 amino acid sequence corresponding to the central portion of the V3-loop region exhibits reasonably conserved primary and/or secondary structural features that would allow interaction with membrane GSL. In this regard, it is also known that even though the V3 region is a highly variable sequence, several conserved sub-domains exist (LaRosa et al., 1990). We observed that, binding of the radiolabeled V3 peptide R15N corresponding to the 15 amino acid sequence in the X4 strain of HIV-1 (MN) was competed by analogous sequences from other X4 strains, as well as peptide R21D from BAL, an R5 strain (Table 1). Additionally, cellular binding of R15N, belonging to clade B was competed by analogous V3 peptides corresponding to two isolates of clade D (Table 1). These results suggest that the V3 interaction with host cell membrane we observed is a potentially common event mediated by envelope proteins of HIV-

1 strains differing in phenotype (cellular tropism), and geographical distribution (clades).

It has been shown that dextran sulfate inhibits HIV infection by interfering with the ionic interaction between the positively charged residues in the V3-loop region and cell surface molecules like heparin sulfate (Callahan et al., 1991). In this regard, we observed that dextran sulfate competed efficiently with radiolabeled V3 peptide for cellular binding and blocked HIV entry into cells (data not shown). Therefore, the HIV-inhibitory function of the V3 peptides used in the present investigation may be similar to that of dextran sulfate. However, other peptides with similar or higher net positive charge from either different portions of the V3-loop or other regions in gp160, or other HIV proteins, did not exhibit HIV-inhibitory property nor competed with radiolabeled V3 peptide for cellular binding (Table 1, and data not shown). Further, it is known that polyanionic saccarides strongly inhibit infection by X4 strains but not R5 strains of HIV (Lynch et al., 1994). In our studies, the V3 peptides R15K and R21D from the X4 and R5 strains of HIV-1, respectively, inhibited entry of both types of viruses. Therefore, we believe that a simple charge phenomenon is not responsible for the cellular interaction of the V3 sequences that is mimicked and competed by the HIV-inhibitory V3 peptides.

In summary, our studies employing linear synthetic peptides corresponding to the central portion of the V3-loop demonstrated that V3 binding to the host cell membrane GSL is an important event common for cellular entry of both X4 and R5 stains of HIV-1. Our data shows that interactions between gp120 and CD4 or gp120 and co-receptors were not perturbed by V3 binding to host cell GSL, under the conditions employed. While binding to co-receptor is known to be an HIV strain-dependent event in that the X4 and R5 strains use distinct co-receptors for entry, the GSL-binding represented by the V3 peptide sequences employed in the present study is common to both X4 and R5 strains of HIV-1. Further, peptides representing the V3 sequence(s) involved in GSL binding exhibited cross reactivity in terms of blocking infection of diverse HIV-1 strains. Based on these data, we predict that the cellular interaction of the V3 region in the virion envelope protein gp120 we observed in our studies may be at a step subsequent to CD4-binding, but prior to co-receptor interaction. The V3 peptides employed in our studies should be useful for further elucidation of the mechanism of HIV entry into cells and potentially for exploring novel HIV therapeutic strategies.

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