Identification of V3 Loop-binding Proteins as Potential Receptors Implicated in the Binding of HIV Particles to CD4⁺ Cells*

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The binding of human immunodeficiency virus (HIV) type 1 particles to CD4⁺ cells could be blocked either by antibodies against the V3 loop domain of the viral external envelope glycoprotein gp120, or by the V3 loop mimicking pseudopeptide 5[Kφ(CH2N)PR]-TASP, which forms a stable complex with a cell-surface-expressed 95-kDa protein. Here, by using an affinity matrix containing 5[Kφ(CH2N)PR]-TASP and cytoplasmic extracts from human CEM cells, we purified three V3 loop-binding proteins of 95, 40, and 30 kDa, which after microsequencing were revealed to be as nucleolin, putative HLA class II-associated protein (PHAP) II, and PHAP I, respectively. The 95-kDa cell-surface protein was also isolated and found to be nucleolin. We show that recombinant preparations of gp120 bind the purified preparations containing the V3 loop-binding proteins with a high affinity, comparable to the binding of gp120 to soluble CD4. Such binding is inhibited either by 5[Kφ(CH2N)PR]-TASP or antibodies against the V3 loop. Moreover, these purified preparations inhibit HIV entry into CD4⁺ cells as efficiently as soluble CD4. Taken together, our results suggest that nucleolin, PHAP II, and PHAP I appear to be functional as potential receptors in the HIV binding process by virtue of their capacity to interact with the V3 loop of gp120.

HIV is an enveloped virus that infects target cells by the fusion of viral and cellular membranes. This fusion requires first the binding of HIV external and transmembrane envelope glycoprotein complex to the CD4 receptor, and is dependent on the presence of cofactors on the cell surface (for reviews, see Refs. 1 and 2). The external envelope glycoprotein contains the binding site for the CD4 receptor and an hypervariable region of about 36 amino acids referred to as the V3 loop. The transmembrane glycoprotein contains a potential fusion peptide at its amino terminus that is implicated in the membrane fusion process. The external and transmembrane glycoproteins (gp120-gp41 for HIV-1) are associated in a noncovalent manner to generate a functional complex in which the V3 loop plays a critical role (3).

Throughout the years, several potential cofactors of CD4 have been proposed in order to explain why CD4 molecule is essential but not sufficient for HIV entry and infection. Accordingly, several cell-surface proteins (reviewed in Ref. 4) and heparan sulfates (5, 6) have been proposed to be implicated in the viral entry process. More recently, however, convincing evidence was provided by several laboratories to show that several protein-coupled chemokine receptors, such as fusin/CXCR4 and CCR5, serve as essential cofactors for the entry of T cell (lymphotropic) and macrophage-tropic HIV-1 isolates, respectively (7–13). Consequently, chemokines specific for CXCR4 and CCR5 receptors inhibit efficiently T cell and macrophage tropic HIV-1 isolates, respectively. Interestingly, chemokines inhibit HIV entry without affecting the binding of HIV particles to cells (14, 15). Chemokines therefore block HIV infection by interfering at a post-binding event, namely fusion between viral and cellular membranes. Several groups have proposed that HIV-1 binding to CD4⁻ cells creates a high affinity interaction site for the cofactor CCR5/CXCR4, and that in this mechanism, the V3 loop plays an important role (7, 9, 15–19). However, no evidence has been provided for a direct binding event between the chemokine receptors and the V3 loop. Consequently, as these latter experiments were carried out by investigating the binding of soluble gp120 to the cell surface, it remains difficult to eliminate the possibility for the existence of complementary interactions of gp120 with other cell-surface proteins.

Previously, it has been considered that the expression of human CD4 in human or heterologous cells is sufficient for the binding of HIV particles to cells (20). However, we and others have recently demonstrated that this latter conclusion is not correct and was due to the use of soluble gp120 instead of HIV particles (21, 22). Indeed, HIV particles, which do not infect CD4-negative human or heterologous cells, have the capacity to bind such cells. Interestingly, monoclonal antibodies directed against the V3 loop inhibit significantly HIV binding to CD4 human or murine cells (22). On the other hand, in the CD4⁺
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HIV permissive human cell lines, HIV binding is blocked by monoclonal antibodies directed against either the gp120 binding site in CD4 or against the V3 loop (21, 22). It should be emphasized that the inhibition of HIV binding in the presence of anti-V3 loop antibodies is not the direct consequence of gp120 shedding from HIV particles or of a decreased affinity of CD4 or gp120 for binding to its surface counterpart (22). Consequently, anti-V3 loop antibodies bind HIV virions and prevent the binding of HIV to CD4+ cells, despite the fact that the CD4-binding domain of gp120 remains accessible for a potential interaction with CD4 (21, 22). These observations have indicated that the neutralizing effect of anti-V3 loop antibodies is not a post-binding event as it has been considered before, and have suggested the existence of other cell-surface molecules besides CD4 for stable binding of HIV particles to cells. Moreover, the inhibition of virus binding to CD4+ cells in the presence of anti-V3 loop antibodies have pointed out the importance of the V3 loop in the HIV binding process. Accordingly, HIV binding appear to require both CD4 and cell-surface components interacting directly with the V3 loop (21, 22).

Recently, we reported that the pseudopeptide 5[K(\text{CH}_2\text{N})-\text{PR}]-TASP, which mimics the V3 loop, is a potent inhibitor of HIV entry by a specific interaction with protein components on the cell surface, other than the CD4 molecule (4, 23). Furthermore, we demonstrated that 5[K(\text{CH}_2\text{N})\text{PR}]-TASP binds and becomes complexed with a cell-surface-expressed 95-kDa protein (4). This pseudopeptide inhibitor blocks the binding of HIV particles to CD4+ cells, as neutralizing antibodies against the CD4 receptor or antibodies against the V3 loop (4, 22). 5[K(\text{CH}_2\text{N})\text{PR}]-TASP inhibits infection of cells by HIV-1 or HIV-2 but not by SIV-mac (23) and has no effect on HIV-1 pseudotyped with MMLV envelope proteins (results herein), thus demonstrating its specific action on the HIV-envelope-mediated entry process. Here, by using an affinity matrix containing either 5[K(\text{CH}_2\text{N})\text{PR}]-TASP or a synthetic V3 loop peptide, we report the isolation of nucleolin, PHAP II and PHAP I as three V3 loop-binding proteins (V3-BPs). The 95-kDa cell-surface protein, by virtue of its capacity to bind 5[K(\text{CH}_2\text{N})\text{PR}]-TASP and form a stable complex with it (4), was also isolated and identified to be nucleolin. The identity of nucleolin, PHAP II, and PHAP I was confirmed by their respective reactivity with polyclonal antibodies against synthetic peptides corresponding to the NH2 terminus of each one of them. These three proteins were demonstrated to bind a synthetic peptide corresponding to the sequence of HIV-1 V3 loop; consequently, they were referred to as V3-BPs. Recombinant preparations of gp120 were then used to demonstrate the specific binding of gp120 to purified preparations containing the three V3-BPs. No binding was observed with the transfemoral membrane glycoprotein gp41 of HIV-1, or with histone H3, which is rich in basic positively-charged amino acids. Interestingly, monoclonal antibodies directed against the V3 loop domain block the binding of gp120 to V3-BPs. The binding of gp120 to V3-BPs is also inhibited by 5[K(\text{CH}_2\text{N})\text{PR}]-TASP. These results, and the observation that the V3-BPs block HIV entry as efficiently as soluble CD4, are consistent with the implication of nucleolin, PHAP II, and PHAP I as potential V3 loop receptors functioning in the process of HIV particle binding to CD4+ cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant gp120 and gp41 (produced by the baculovirus and *Escherichia coli* expression system, respectively) of HIV-1 Lai (IIIB), and recombinant soluble CD4 (produced in CHO cells or by the baculovirus expression system) were purchased from NeoSystem Laboratories, Strasbourg, France. Other recombinant preparations of gp120 corresponding to that of HIV-1 isolates, LAV (or Lai), MN (both from *MicroGeneSys*, Inc., Meriden, CT), SF2, and the unglycosylated gp120-SF2 (Env 2–3; from Dr. K. Steimer; Chiron Corp. were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health. The gp120 MN and Lai (LAV) were produced in insect cells using baculovirus, gp120 SF2 was produced in CHO cells, whereas the unglycosylated gp120 SF2 was produced in the yeast. The concentration of p24 was measured by p24 Core Profile ELISA (DuPont).

**Cells**—CEM cells (clone 13) derived from human lymphoid cell line CEM (provided by L. Montagnier, Institut Pasteur, France) were cultured in suspension medium RPMI 1640 (Bio-Whittaker, Verviers, Belgium). Peripheral blood mononuclear cells (PBMCs) from healthy donors were prepared by Ficoll Hypaque density gradient centrifugation and suspended in RPMI 1640 medium containing 10% fetal calf serum (23). Human HeLa-CD4-LTR-β-gal cells (from Dr. F. Clavel, Institut Pasteur, Paris) were cultured in Dulbecco’s medium (24). All cells were cultured with 10% (v/v) heat-inactivated (56 °C, 30 min) fetal calf serum.

**Preparation of Cell Extracts**—Cells were first washed extensively in PBS before lysis in buffer E (100 μl/3 × 107 cells) and the nuclei were pelleted by centrifugation (350 g for 5 min). Buffer E contains 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM MgCl2, 0.2 mM PMSF, 5 mM β-mercaptoethanol, aprotinin (1000 units/ml) and 0.5 mM Triton X-100. The nuclei-free supernatant was further centrifuged at 12,000 × g for 10 min, and the supernatant was stored at −80 °C. Routinely, cell extracts were harvested 48 h after cell passage.

**The Production of HIV-1 Pseudotyped MMLV Virus**—HeLa cells were cotransfected by electroporation with plasmids pNL4–3 defective in env gene (25) and pE-4070A expressing amphotrope envelope glycoproteins of MMLV (26). Electroporation (27) was performed at 200 V, 960 microfarads, using a 4-mm wide cuvettes in a Bio-Rad Gene Pulser. The pseudotyped virus was recovered from the culture supernatant after 48 h of culturing. Plasmids were kindly provided by Dr. S. Le Gall (Institut Pasteur, Paris).

**Antibodies**—The mAb CC98 directed against human nucleolin (28) was generously provided by Dr. N.-H. Yeh, Graduate School of Microbiology and Immunology, National Yang-Ming Medical College, Shih-Pai, Taiwan, Republic of China. The mAb specific to human CD4 and reacting with the gp120 binding domain was kindly provided by Dr. E. Boomsma (clone CP-T4–2; Eurogenetics, Tessenderlo, Belgium). The mAb specific to human CD45 (clone HB10AB21B6) was generously provided by Dr. R. Siragandian (NIDR/NIH, Bethesda; Ref. 29). mAbs N11–20 (also referred to as 110-H), 110-C, 110-D against gp120 Lai, and mAb 41-A against gp41 Lai, were provided by Dr. J. C. Mazie, Hybridlab, Institut Pasteur. Other mAbs specific to gp120 included mAbs 110–4 and 110–1 provided by Genetics Systems, Seattle, WA (30, 31), mAb ADF930 (from Drs. J. Cordell and C. Dean) provided by MRC AIDS Directed Program Reagent Repository (32), mAbs AD3 (from Drs. K. Ugen and D. Weiner), V3–21 (from Dr. J. Laman), and b12 (from Drs. D. Burton and C. Barbas), obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (33–35).

**Synthesis of TASP Constructs and the Biotin-labeled V3 Loop Peptide**—The purification and characterization of the different unlabeled and biotin-labeled TASP constructs were as described previously (4, 23). The V3 loop sequences corresponded to that from the HIV-1 Lai NC-TRPNNNTKRRISIQGRGPRAFVTVGGKNRMQAHCNIS and HIV-1 Ba-L NC-TRPNNNTKRRISIQGRGPRAFVTVGGKNRMQAHCNIS iso- late. The V3 loop peptides were synthesized using classical Fmoc chemistry. The biotin was added at the NH2 terminus of the peptide. The loop structure was generated by air oxidation at pH 8, in a 10% MeSO water solution under vigorous stirring for 4 days at room temperature. The final products were more than 90% pure. Fluorescence-activated cell sorting (FACS) analysis was carried out as described previously (4).

**Purification of the V3-BPs**—Avidin-agarose (3 ml; ImmunoPure immobilized avidin, Pierce) in PBS was incubated (18 h, 4 °C) in the presence of the biotin-labeled 5[K(\text{CH}_2\text{N})\text{PR}]-TASP (20 μM) before washing extensively in PBS-EDTA (PBS containing 1 mM EDTA). Cytosplasmic extracts (material corresponding to 2 × 107 CEM cells; Ref. 4) were added to the affinity matrix and after 2 h of incubation at 4 °C, the samples were washed extensively with PBS-EDTA. The purified proteins were eluted by 1 ml NaCl solution containing 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM PMSF, 5 mM β-mercaptoethanol, and 20% (v/v) glycerol. The eluted sample was dialyzed overnight against PBS containing 0.1 mM EDTA and 1 mM PMSF and aliquots stored at −80 °C. For microsequencing, the purified proteins (100 μg) were analyzed by SDS-PAGE, and the different protein bands were visualized after a slight staining with Amido Black. The respective bands were excised from the gel and digested with endo-lysin C, which cleaves peptides adjacent to lysine residues. The peptides were purified...
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**RESULTS AND DISCUSSION**

**Inhibition of HIV Infection by 5(Kr(CH2N)PR]-TASP Is Specific to the HIV Envelope Glycoproteins**—We have previously demonstrated that 5(Kr(CH2N)PR]-TASP binds specifically to the surface of CEM cells and blocks the binding of HIV particles to cells (4, 23). Consequently, HIV-1/Lai infection is inhibited by 5(Kr(CH2N)PR]-TASP as drastically as by the addition of AZT, the well known reverse-transcriptase inhibitor of HIV (Fig. 1). The monoclonal antibody CB-T4 against the gp120 binding domain in CD4 also inhibits HIV-1 infection, whereas the control monoclonal antibody against the cell-surface-expressed antigen CD45 has no apparent effect (Fig. 1A). In order to demonstrate the specificity of 5(Kr(CH2N)PR]-TASP in respect to the HIV-envelope glycoprotein mediated infection of CD4+ cells, we investigated infection with an HIV-1 pseudotyped virus harboring amphotropic MMLV envelope proteins. Infection of CEM cells by this pseudotyped virus is inhibited by AZT but not by the anti-CD4 mAb CB-T4 (Fig. 1B), as the viral binding and entry in this case is mediated by the MMLV envelope proteins. Infection of CEM cells by this pseudotyped virus (Fig. 1B) is specific to the HIV Envelope Glycoproteins—24. Cells were plated at 10⁴ cells/well in 96-well plates, and at 24 h later cells monolayers were infected with HIV-1 Lai (a dose containing 20 ng of p24). At 48 h, cell monolayers were washed with PBS before lysis of cells in 100 µl/well of buffer-L containing 0.1% Nonidet P-40 (v/v), 60 mM NaH2PO4, 40 mM NaHPO4, 10 mM KCl, 10 mM MgSO4, 1 mM EDTA, and 50 mM β-mercaptoethanol. After 10 min of incubation at room temperature, 100 µl of the reaction mix containing 10 µM phosphate buffer at pH 7.4, 10 mM MgCl2, 10 mM β-mercaptoethanol, and 600 mM chlorophenol red-β-D-galactopyranoside was added in each well. The 96-well plate was then incubated at 37 °C, and the β-galactosidase activity was measured at 10-min intervals in a microplate reader using a 570 nm filter.

**FIG. 1.** The action of 5(Kr(CH2N)PR]-TASP is specific to the HIV envelope glycoproteins. CEM cells (2 × 10⁶) were first incubated (37 °C, 15 min) with 5 µg/ml mAbs (the symbols α for anti-CD4 (CBT4); (22) and anti-CD45 (29), or AZT (5 µM), or 5(Kr(CH2N)PR]-TASP (at 1 and 5 µM) before infection either with the HIV-1 Lai isolate (0.2 synchronous dose; Ref. 23) in A or the HIV-1 pseudotyped MMLV virus (at a dose corresponding to 95 ng of p24/ml; see “Experimental Procedures”) in B. Virus production was then monitored by measuring the concentration of p24 in culture supernatants at 5 and 2 days after infection for the experiment described in A and B, respectively. The mean ± S.D. of duplicate samples is given. The sample Control represents infections without any prior treatment of cells. Note: the infection by the HIV-1 pseudotyped MMLV is a one-cycle infection.

by HPLC (DEAE-C18) using a gradient of acetonitrile/trifluoroacetic acid 0.1%. The microsequencing was carried out by the Micro-Sequencing Laboratory at Institut Pasteur.

**Purification of the Cell-surface-expressed p95 for Microsequencing—** Twenty four h after passaging, CEM cells (10⁷ cells) were washed extensively with PBS before incubation (4 °C, 30 min) in 10 ml of FACS buffer with 15 µM biotin-labeled 5(Kr(CH2N)PR]-TASP. Cells were then washed in FACS buffer (2 × 100 ml) and nucleus-free cell extracts were prepared using buffer E (3 ml). Such extracts were first diluted in PBS (12 ml) prior the addition of 1 ml of avidin-agarose to capture the biotin-labeled TASP complexed to p95. These suspensions were incubated at 4 °C for 2 h, and then washed batchwise with PBS (6 × 60 ml). Finally, the avidin-agarose pellet was resuspended in 2 ml of 2-fold concentrated electrolysis sample buffer (125 mM Tris-HCl, pH 6.8, 2 M urea, 1% SDS, 0.1% β-mercaptoethanol, and 20% glycerol, (v/v)) and heated at 95 °C for 5 min. Three 250-µl aliquots of the purified preparation were analyzed by SDS-PAGE using polyvinylidene difluoride sheets (Bio-Rad) before microsequencing the NH₂ terminus.

**Production of Polyclonal Antibodies against Nucleolin, PHAP II, PHAP I, and CXC9—** Peptides corresponding to the NH₂ terminus of nucleolin (residues 1–26), PHAP II (residues 1–23), PHAP I (residues 1–20), and CXC9 (residues 1–27) were synthesized according to conventional Fmoc chemistry on a multichannel peptide synthesizer. The peptides were conjugated to ovalbumin through the cysteine residue added at the COOH terminus of each peptide. Rabbits were immunized five times at 2-week intervals by intramuscular injections with the coupled material (150 µg of peptide/injection/animal). The first injection was performed in the presence of complete Freund’s adjuvant (Difco, West Molesey, UK), and the following injections were done with incomplete Freund’s adjuvant. The antisera were titrated by enzyme-linked immunoabsorbent assay (ELISA) for the production of antibodies by monitoring reactivity with the respective peptides.

**ELISAs to Show the Binding of gp120 to V3-BPs—** The microtiter plates were coated with the purified preparation of the V3-BPs at different concentrations (12.5–200 ng/ml) before incubation with either gp120 (1 ng/ml), gp41 (2 ng/ml), or histone H3 (5 ng/ml). After extensive washing, the binding of different reagents was monitored using specific antibodies: mAb 110-D (1 µg/ml) specific for residues 381–394 of gp120 of HIV-1, mAb 41-E (1 µg/ml) specific for gp41, and mAb specific for histone H3 (2 µg/ml) (36). As control antibodies, we used mAb ORT4A specific for CD4 and a rabbit antiserum against CXC9 (at 1:200 dilution).

**Biosensor Measurements—** For real-time binding experiments, a BIAcore™ biosensor system (Pharmacia Biosensor, AB, Uppsala, Sweden) was used. Experimental procedures were as described previously (36, 37). The purified preparation of the V3-BPs (100 ng/ml) was immobilized on the sensor chips to characterize the binding with the peptides 5(Kr(CH2N)PR]-TASP and 5(KP)-TASP, or the gp120 preparations (each at 800 nM).

**Assay of HIV Entry in HeLa CD4+ Cells—** The HIV-1 entry was monitored indirectly in HeLa-CD4-LTR-β-gal cells containing the bacterial lacZ gene under the control of HIV-1 LTR. HIV-1 entry and replication results in the activation of the HIV-1 LTR leading to the expression of β-galactosidase (24). Cells were plated at 10⁴ cells/well in 96-well plates, and at 24 h later cell monolayers were infected with HIV-1 Lai (a dose containing 20 ng of p24). At 48 h, cell monolayers were washed with PBS before lysis of cells in 100 µl/well of buffer-L containing 0.1% Nonidet P-40 (v/v), 60 mM NaH2PO4, 40 mM NaHPO4, 10 mM KCl, 10 mM MgSO4, 1 mM EDTA, and 50 mM β-mercaptoethanol. After 10 min of incubation at room temperature, 100 µl of the reaction mix containing 10 µM phosphate buffer at pH 7.4, 10 mM MgCl2, 10 mM β-mercaptoethanol, and 600 mM chlorophenol red-β-D-galactopyranoside was added in each well. The 96-well plate was then incubated at 37 °C, and the β-galactosidase activity was measured at 10-min intervals in a microplate reader using a 570 nm filter.

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**5(Kr(CH2N)PR]-TASP Affects the Interaction of a Synthetic V3 Loop Peptide with the Surface of CEM Cells—** Recent observations have suggested that the V3 loop in gp120 plays an important role in the HIV binding process, by interacting with cell-surface proteins other than CD4 and the chemokine receptors (22). In view of this, we synthesized a biotin-labeled V3 loop peptide corresponding to the sequence of the HIV-1 Lai isolate. At 25 µM V3 loop peptide, a significant labeling of cells was obtained by FACS analysis (Fig. 2). Such labeling by the...
The capacity of the V3 loop peptide to bind a cell-surface ligand is inhibited partially by the pseudopeptide 5[Kφ(CH2N)PR]-TASP inhibitor. CEM cells were incubated with 25 μM of the biotin-labeled V3 loop peptide Lai in the absence (peak V3 Loop/B*) or presence of 25 μM unlabeled 5[Kφ(CH2N)PR]-TASP (peak 5[KφPR]-TASP) before processing for FACS analysis under the experimental conditions as described previously (4). Peak C gives the fluorescence of cells incubated with the unlabeled 5[Kφ(CH2N)PR]-TASP construct (25 μM). The ordinate gives the relative cell number, whereas the abscissa gives the relative fluorescence intensity. Under similar experimental conditions, the control biotin-labeled 5[QPOP]-TASP construct does not give a positive signal (4).

Biotin-labeled V3 loop peptide was significantly reduced in the presence of 25 μM unlabeled 5[Kφ(CH2N)PR]-TASP, suggesting that the V3 loop and 5[Kφ(CH2N)PR]-TASP interact with similar cell-surface ligands. However, it should be noted that the affinity of the V3 loop peptide to bind CEM cells is probably severalfold lower compared with 5[Kφ(CH2N)PR]-TASP. Indeed, significant labeling of CEM cells could be obtained at 0.5 μM biotin-labeled 5[Kφ(CH2N)PR]-TASP (see Ref. 4 in Fig. 4). In contrast cell labeling with the biotin-labeled V3 loop peptide requires 25–50-fold higher concentrations.

Purification of Several Proteins from Crude Cell Extracts Using the Biotin-labeled 5[Kφ(CH2N)PR]-TASP—Several experiments were carried out to optimize the experimental conditions for the purification of 5[Kφ(CH2N)PR]-TASP-binding proteins from crude cell extracts using the biotin-labeled TASP construct coupled to avidin-agarose. No proteins were recovered by the avidin-agarose in the absence of the biotin-labeled 5[Kφ(CH2N)PR]-TASP or when cell extracts were incubated with the biotin-labeled control 5[QPOP]-TASP construct (data not shown). Thus, the biotin-labeled 5[Kφ(CH2N)PR]-TASP coupled to avidin-agarose represents an efficient affinity matrix for the purification of 5[Kφ(CH2N)PR]-TASP-binding proteins. Fig. 3 shows the profile of the purified proteins revealed by staining the SDS-PAGE gel with Coomassie Blue and by ligand blotting using either the biotin-labeled 5[Kφ(CH2N)PR]-TASP or the biotin-labeled V3 loop peptide. By using this experimental procedure, four major proteins of 95, 60, 40, and 30 kDa (p95, p60, p40, and p30, respectively) were purified. By ligand blotting, each one of these proteins was shown to bind 5[Kφ(CH2N)PR]-TASP and the V3 loop peptide, thus pointing out that they are V3-BPs.

Identification of the V3-BPs: p95/Nucleolin, p40/PHAP II, and p30/PHAP I—The four proteins purified from cell extracts (Fig. 3) were analyzed by microsequencing after digestion with endo-lysine C, which cleaves peptides adjacent to lysine residues. The peptides were purified by HPLC, and some of the peaks were processed for microsequencing (see “Experimental Procedures”). The amino acid sequences obtained from the different peptides indicated that p95 is nucleolin (38), p40 is PHAP II, and p30 is PHAP I isoform 2a (39, 40), whereas p60 corresponds to a partially degraded product of nucleolin (Table I). Recently, we reported the isolation of a 95-kDa cell-surface protein, which binds 5[Kφ(CH2N)PR]-TASP and forms a stable complex with it (4). To confirm that this latter protein is nucleolin, the 95-kDa protein was isolated from the surface of CEM cells and was processed for NH2-terminal microsequencing (Table I). The 15-amino acid sequence obtained was found to be 100% identical with that of the NH2-terminal sequence of human nucleolin (38). Nucleolin is the major non-histone protein of the nucleolus, which has been suggested to shuttle between nucleus and cytoplasm (38, 41). Although primarily localized in the cell nucleoli, nucleolin has been reported to be also expressed on the cell surface and serve as a binding protein to different ligands (see Refs. 42–44 and references therein). On the other hand, although PHAP I and PHAP II have been isolated as putative HLA class II associated proteins, as yet there is no direct evidence to elucidate their precise function (39). PHAP I is most likely the human homologue of the rat “leucine-rich acidic nuclear protein” (45), whereas PHAP II is identical to a phosphoprotein named SET and localized predominantly in the nucleus (46, 47). Purified preparations corresponding to PHAP I and PHAP II have been reported to inhibit the activity of the protein phosphatase 2A (48, 49).

The common feature among nucleolin, PHAP II, and PHAP I is their polyamionic nature due to the presence of extended stretches of amino acids composed of aspartic and glutamic residues (38, 39). The comparison of the amino acid sequences of these three proteins revealed conserved acidic domains overlapping 14–38 residues (data not shown). These acidic domains in nucleolin, PHAP II, and PHAP I are probably responsible for the interaction with the V3 loop peptide and the pseudopeptide...
TABLE I
Homology of the amino acid sequence of the different peptides from the V3-BPs to nucleolin, PHAP II, and PHAP I

| Protein | HPLC fraction | Amino acid sequence | Homology (aa) |
|---------|---------------|---------------------|---------------|
| p95*    | Peak 24 (K)QGTEIDGRSISLYYT | Nucleolin (447–463) |               |
| p40*    | Peak 27 (K)KLESE | PHAP I-2a (67–73) |               |
| p30*    | Peak 33 (K)SLDLFXENVLNDY | PHAP I-2a (116–131) |               |
| p95**   | Peak 24 (K)QGTEIDGRSISLYYT | Nucleolin (447–463) |               |
| p40**   | Peak 27 (K)KLESE | PHAP I-2a (67–73) |               |
| p30**   | Peak 33 (K)SLDLFXENVLNDY | PHAP I-2a (116–131) |               |

*The four proteins purified from crude cell extracts using the affinity matrix containing 5\((\text{CH}_2\text{N})\text{PR})\]-TASP (see Fig. 3), were recovered individually from the SDS-PAGE gel, digested with endo-lysin C, and the peptides were purified by an HPLC column (see “Experimental Procedures”). Several peptides of each protein were microsequenced. The homology of the obtained amino acid (aa) sequences to that deduced from the nucleotide sequence of cDNAs corresponding to known proteins is given. As the endolysine C cleaves peptide bonds after lysine residues, the (K) at the beginning of the sequences of the different peptides, and at the end of some peptides, points out that indeed in the homologous protein sequence, these peptides are adjacent to a lysine residue. The results show that p95 and p60 are homologous to human nucleolin (38), whereas p40 and p30 are homologous to PHAP II and PHAP I. The NH\(_2\)-terminal amino acid sequence of the cell-surface p95 (referred to as p95*) was also carried out as described under “Experimental Procedures.”

5\((\text{CH}_2\text{N})\text{PR})\]-TASP. In this respect, it is worthwhile to mention here that polyanions such as heparin, dextran sulfate, synthetic double-stranded RNAs, and synthetic aspartate/glutamate-rich peptides, are potent inhibitors of HIV entry and infection (50). Interestingly, the mechanism of the inhibitory effect of polyanions has been proposed to be related to their capacity to bind the V3 loop domain in gp120 (51).

Nucleolin, PHAP II, and PHAP I Bind the Pseudopeptide 5\((\text{CH}_2\text{N})\text{PR})\]-TASP and the Synthetic V3 Loop Peptide—in order to confirm the identity of the purified proteins, rabbit antibodies were generated against synthetic peptides corresponding to the NH\(_2\)-terminal sequences of nucleolin, PHAP II, and PHAP I. Such antibodies were shown to be highly specific, since by immunoblotting each rabbit antisera reacted only with the protein corresponding to the peptide that was used for immunization. Accordingly, in crude CEM cell extracts, the antisera against the nucleolin, PHAP II, and PHAP I peptides revealed the 95-, 40-, and 30-kDa bands, respectively (Fig. 4, A, B, and C, lanes 1). The preimmune sera from the different rabbits did not show any signal (data not shown). In the purified preparations using the affinity matrix containing 5\((\text{CH}_2\text{N})\text{PR})\]-TASP or the V3 loop peptide, the different antisera confirmed that nucleolin, PHAP II, and PHAP I indented bands of V3 loop (Fig. 4, A, B, and C, lanes 2 and 3). The mAb CC98 specific for human nucleolin, reacted with p95 and p60, thus further demonstrating that p60 is a partial degradation product of nucleolin (Fig. 4D).

On the whole, these experiments illustrate that 5\((\text{CH}_2\text{N})\text{PR})\]-TASP and the V3 loop peptide bind a similar pattern of proteins, namely p95, p40, and p30. Furthermore, they provide further confirmation concerning the identity of the V3-BPs as being nucleolin, PHAP II, and PHAP I, respectively. Moreover, they point out that CXC4, the cofactor of CD4 required for the entry of lymphotropic HIV-1 isolates, does not bind the V3 loop peptide (Fig. 4E).

On the Cell-surface Expression of V3-BPs—We have shown that 5\((\text{CH}_2\text{N})\text{PR})\]-TASP forms a stable complex with a cell-surface-expressed nucleolin could be detected by using the biotin-labeled 5\((\text{CH}_2\text{N})\text{PR})\]-TASP, either by FACS analysis of cells or by the recovery of the complex formed on the surface of intact cells (4). This latter property of the pseudopeptide 5\((\text{CH}_2\text{N})\text{PR})\]-TASP was used as a tool to investigate the cell-surface expression of PHAP II and PHAP I. For this purpose, phytohemagglutinin-activated PBMcs from an healthy individual were incubated with different concentrations of the biotin-labeled 5\((\text{CH}_2\text{N})\text{PR})\]-TASP at 4°C in the...
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FIG. 5. Recovery of the cell-surface-expressed V3-BPs by the capacity of 5[K(\text{CH}_2\text{N})\text{PR}]-TASP to bind and form a stable complex with them. PBMCs from a healthy donor were activated by phytohemagglutinin as described previously (22). Four days after activation, cells were washed extensively with PBS before incubation (30 min, 4 °C) with different concentration of the biotin-labeled 5[K\text{(CH}_2\text{N})\text{PR}]-TASP (lanes 0, 2.5, 5, 10, and 20 nM) in PBS containing 1% bovine plasma albumin and 0.01% sodium azide. For a control, cells were incubated with the biotin-labeled 5[\text{QPQ}]-TASP construct (20 nM, lane C/B*). Cells were then washed extensively in the incubation buffer prior to the preparation of nucleus-free cell extracts, which were then purified using avidin-agarose to capture the biotin-labeled 5[K\text{(CH}_2\text{N})\text{PR}]-TASP to bind and form a stable complex with them. The capacity of stable complex formation between 5[K\text{(CH}_2\text{N})\text{PR}]-TASP and gp120 for the V3-BPs and to CD4 was determined using a biacore instrument (BIAcore; see “Experimental Procedures”). The preparation of the V3-BPs containing nucleolin, PHAP II, and PHAP I was as described in Fig. 3. The gp120 preparations corresponded to that of lymphotropic HIV-1 isolates Lai, MN, and SF2. The gp120 HIV-1 SF2 2/3 represents an unglycosylated form of gp120 SF2. The CD4 represented a soluble form of recombinant CD4.

The ultrastructural localization of nucleolin, PHAP II, and PHAP I by electron microscopy revealed the expression of these proteins in both nuclear and cytoplasmic compartments in all of these cells, 5[K\text{(CH}_2\text{N})\text{PR}]-TASP complexed to PHAP II, and PHAP I by electron microscopy revealed the expression of these proteins in both nuclear and cytoplasmic compartments in addition to their presence at the plasma membrane.**

High Affinity Binding of gp120 to the V3-BPs—In an ELISA type experiment, HIV-1 gp120 but not gp41, corresponding to that of the HIV-1 Lai isolate, was shown to bind in a dose-dependent manner the purified V3-BPs (data not shown; described under “Experimental Procedures”). It could be argued that the binding of gp120 to the V3-BPs is simply the consequence of a nonspecific interaction between basic amino acid residues in the gp120 and the acidic domains in nucleolin, PHAP II, and PHAP I. However, this is most unlikely, since histone H3, which is rich in basic amino acids, does not bind the V3-BPs. This latter fact and the observation that binding does not occur with gp41 confirm that the binding of gp120 to the V3-BPs is specific. It is also of interest to note that the preparation of V3-BPs in such ELISA type experiments using specific antibodies, was shown not to be contaminated with the CD4 receptor or the chemokine receptor CXCR4 (consistent with the results of immunoblotting analysis shown in Fig. 4).

The gp120 from three lymphotropic HIV-1 isolates manifested a high affinity binding to the V3-BPs, with equilibrium affinity constant $K_a$ values of $2.1 \times 10^8$ M$^{-1}$, $4.3 \times 10^8$ M$^{-1}$, and $2.3 \times 10^8$ M$^{-1}$ for gp120 of Lai, MN, and SF2, respectively (Table II). Interestingly, although the unglycosylated form of gp120-SF2 manifested about 10-fold reduction compared with the glycosylated counterpart, its affinity was still high with a $K_a$ value of $1.6 \times 10^8$ M$^{-1}$ (Table II). This result suggests that the polysaccharide side chains of the native gp120 molecule are probably not absolutely necessary for its binding to the V3-BPs. Under similar experimental conditions, the $K_a$ values of gp120-Lai, gp120-MN, and gp120-SF2 for soluble CD4 were 10.8, 7.7, and $2.1 \times 10^9$ M$^{-1}$, respectively (Table II), in accord with previously published values (52). Therefore, the affinity of gp120 to bind CD4 and the V3-BPs was of the same order.

The $K_a$ value of 5[K(\text{CH}_2\text{N})\text{PR}]-TASP and 5[K\text{PR}]-TASP to bind the V3-BPs was $9.6 \times 10^9$ M$^{-1}$ and $1.5 \times 10^9$ M$^{-1}$, respectively (Table II). The higher $K_a$ value observed for 5[K(\text{CH}_2\text{N})\text{PR}]-TASP is correlated with its higher activity on HIV entry, con-
pared with the non-reduced counterpart 5[KPR]-TASP (4, 23). Two synthetic V3 loop peptides, corresponding to the amino acid sequence of the T-cell tropic HIV-1 Lai and of the macrophage-tropic HIV-1 Ba-L isolate (see “Experimental Procedures”), were investigated for their capacity to interact with the purified V3-BPs. Both of these V3 loop peptides were found to bind V3-BPs; however, in contrast to gp120 and to 5[Kφ(CH2N)PR]-TASP, they manifested somewhat lower affinity of binding. The $K_a$ value of V3 loop-Lai and V3 loop-Ba-L to bind the V3-BPs was $5.1 \times 10^{-6} \text{ M}^{-1}$ and $1.5 \times 10^{-6} \text{ M}^{-1}$, respectively. By extrapolation, therefore, the affinity of the V3 loop to bind the V3-BPs might be at least 3-fold higher for the T-cell tropic compared with the macrophage-tropic HIV-1 isolates.

The presence of a high number of basic residues in the V3 loop-Lai compared with the V3 loop-Ba-L could account for this difference (53). Interestingly, the 50% inhibitory concentration of 5[Kφ(CH2N)PR]-TASP to inhibit entry of HIV-1 Lai and Ba-L in HeLa CD4+ /CXCR4+/CCR5+ cells is 0.4 and 0.8 $\mu$M, respectively (data not shown).

**Figure 6. Characterization of gp120 binding to the V3-BPs.** These experiments were carried out using biosensor technology as described under “Experimental Procedures.” The purified preparation of the V3-BPs was as described in Fig. 3. The sample containing the V3-BPs (100 ng/ml) was immobilized on the sensor chip to characterize the binding with either 5[Kφ(CH2N)PR]-TASP or gp120 (HIV-1 Lai). A, gp120 prevents binding of 5[Kφ(CH2N)PR]-TASP to the V3-BPs. The binding of 5[Kφ(CH2N)PR]-TASP (800 nM) to the V3-BPs was carried out in the presence of increasing concentrations of gp120 (the *absissa*). The ordinate gives the percent inhibition of 5[Kφ(CH2N)PR]-TASP binding to the V3-BPs; the 0% inhibition value represents the degree of binding in the absence of gp120. The IC$_{50}$ value for the inhibition of 5[Kφ(CH2N)PR]-TASP binding to the V3-BPs is around 3 nM gp120. B, 5[Kφ(CH2N)PR]-TASP prevents the binding of gp120 to the V3-BPs. The binding of gp120 (800 nM) to the V3-BPs was carried out in the presence of increasing concentrations of 5[Kφ(CH2N)PR]-TASP (the *absissa*). The ordinate gives the percent inhibition of gp120 binding to the V3-BPs; the 0% inhibition value represents the degree of binding in the absence of the antibody.

**Purified Preparations of the V3-BPs Inhibit HIV-1 Infection—**The results presented above demonstrated the high affinity interaction of soluble gp120 with the V3-BPs, thus suggesting the implication of these V3-BPs as potential receptors of the V3 loop. If this was the case, then soluble V3-BPs, by
interacting with the gp120 expressed on the surface of HIV particles, could block virus infection as has been reported for the soluble CD4 (54, 55). Indeed, purified preparations of the V3-BPs were consistently shown to inhibit HIV infection in a dose-dependent manner. For example, HIV-1 Lai infection of CEM cells was inhibited by 91 and 67% at 10 and 5 \( \mu \)g/ml, respectively, of the purified preparation of the V3-BPs (data not shown).

In order to investigate whether this preparation of the V3-BPs block the HIV entry process, we studied its effect on the HIV infection in the HeLa-CD4-LTR-\( \beta \)-gal cell model. Upon HIV entry and replication, the viral transactivator Tat induces the lacZ gene to express \( \beta \)-galactosidase, which then can be monitored by an enzymatic assay. Consequently, \( \beta \)-galactosidase activity is directly correlated to the HIV entry and its replication, since the expression of such activity does not occur in the presence of anti-CD4 mAb CB-T4, which blocks HIV entry, or AZT, which blocks viral replication (Fig. 7). In this experimental model, the V3-BPs had the capacity to block HIV entry in a dose-dependent manner. Interestingly, the degree of inhibition by the V3-BPs was comparable to that observed with soluble CD4 (sCD4) preparation produced in CHO cells (Fig. 7). The inhibitory effect of the V3-BPs on HIV entry was abolished when the purified preparation was preheated at 56 or 95 °C, thus confirming the protein nature of the inhibitory component. It should be noted that sCD4 produced in the baculovirus expression system had no effect on HIV entry, and even higher concentrations resulted in an enhancing effect. The difference between the two sCD4 preparations might be the degree of glycosylation in the two expression systems used.

The capacity of the purified V3-BPs to inhibit HIV infection suggests their potential interaction with HIV particles, and provides a mechanism by which the purified proteins could block what might be happening under normal conditions, i.e. the interaction of HIV particles with the cell-surface-expressed V3-BPs.

The V3-BPs (Nucleolin, PHAP II, and PHAP I) as cofactors in the mechanism of HIV binding—The pseudopeptide 5K8(CH2N)PR-TASP, designed to mimic the conserved RP dipeptide motif and basic lysine and arginine residues in the V3 loop of HIV isolates, is a potent and specific inhibitor of HIV infection (23). Here, we demonstrate that an identical pattern of proteins composed of nucleolin, PHAP II, and PHAP I can be purified from cells using either the pseudopeptide 5K8(CH2N)PR-TASP or a synthetic V3 loop peptide (Fig. 4, lanes 3 and 4), suggesting that 5K8(CH2N)PR-TASP can indeed mimic the V3 loop. This observation, together with the fact that 5K8(CH2N)PR-TASP is a potent inhibitor of HIV entry by binding to cell-surface components of protein in nature (4), suggests that the V3-BPs described here are targets of this pseudopeptide inhibitor. The interaction of 5K8(CH2N)PR-TASP with the V3-BPs is of high affinity (Table II). Otherwise, the purification of these V3-BPs by just a single step would not have been possible. The control peptide 5K0Q0Q-TASP construct does not bind the V3-BPs, whereas the tetravalent 4KPR-TASP construct, which has very little activity against HIV, binds poorly the V3-BPs but along with many other proteins. These observations therefore emphasize the unique specific nature of the pentavalent 5K8(CH2N)PR-TASP construct. In addition, the anti-body to bind the V3-BPs and the anti-HIV activity of the different TASP constructs (23) are tightly correlated. These V3-BPs therefore appear to be implicated as co-factors in the mechanism of HIV binding to permissive CD4+ cells. Such a cofactor role is enforced by several observations: 1) specific binding of 5K8(CH2N)PR-TASP to cell-surface proteins (4); 2) inhibition of HIV particle binding to CD4+ cells in the presence of 5K8(CH2N)PR-TASP (4, 23); 3) binding of 5K8(CH2N)PR-TASP and the V3 loop peptide to...
each one of the V3-BPs, nucleolin, PHAP II and PHAP I (Fig. 3, panels 3 and 4); 4) formation of a stable complex between 5[κ(CH2N)PR]-TASP and cell-surface-expressed nucleolin, PHAP II, and PHAP I (Fig. 5); 5) demonstration that gp120 binds the V3-BPs via its V3 loop (Tables II and III); 6) competition between gp120 and 5[κ(CH2N)PR]-TASP to bind the V3-BPs (Fig. 6); 7) inhibition of HIV infection and entry using purified preparations of the V3-BPs (Fig. 7). By virtue to bind the V3 loop domain, the V3-BPs could interact with the gp120 on the surface of HIV particles and thus become implicated in the HIV binding process. Consequently, agents that can interfere in the interaction of the V3 loop domain of gp120 with the cell-surface-expressed V3-BPs block HIV binding and thus entry (results herein; Refs. 4 and 22). For examples, HIV binding to CD4+ cells could be blocked either by 5[κ(CH2N)PR]-TASP, which binds the cell-surface-expressed V3-BPs, or by anti-V3 loop mAbs, which bind the V3 loop in gp120 exposed on HIV particles. We found out that all human and murine cells of lymphoid or non-lymphoid origin which were investigated express nucleolin, PHAP II, and PHAP I (data not shown). In view of this, and the fact that the expression of human CD4 and CXCR4 or CCR5 is sufficient for efficient entry of different HIV-1 isolates,2 thus pointing out the wider significance of V3-BPs in the mechanism of viral binding. Accordingly, the V3-BPs represent novel targets for the development of potential anti-HIV reagents.

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