The Anti-HIV Pseudopeptide HB-19 Forms a Complex with the Cell-surface-expressed Nucleolin Independent of Heparan Sulfate Proteoglycans*

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The HB-19 pseudopeptide 5[K(CH2N)PR]-TASP, ψ(CH2N) for reduced peptide bond, is a specific inhibitor of human immunodeficiency virus (HIV) infection in different CD4+ cell lines and in primary T-lymphocytes and macrophages. Here, by using an experimental CD4+ cell model to monitor HIV entry and infection, we demonstrate that HB-19 binds the cell surface and inhibits attachment of HIV particles to permissive cells. At concentrations that inhibit HIV attachment, HB-19 binds cells irreversibly, becomes complexed with the cell-surface-expressed nucleolin, and eventually results in its degradation. Accordingly, by confocal immunofluorescence microscopy, we demonstrate the drastic reduction of the cell-surface-expressed nucleolin following treatment of cells with HB-19. HIV particles can prevent the binding of HB-19 to cells and inhibit complex formation with nucleolin. Such a competition between viral particles and HB-19 is consistent with the implication of nucleolin in the process of HIV attachment to target cells. We show that another inhibitor of HIV infection, the fibroblast growth factor-2 (FGF-2) that uses cell-surface-expressed heparan sulfate proteoglycans as low affinity receptors, binds cells and blocks attachment of HIV to permissive cells. FGF-2 does not prevent the binding of HB-19 to cells and to nucleolin, and similarly HB-19 has no apparent effect on the binding of FGF-2 to the cell surface. The lack of competition between these two anti-HIV agents rules out the potential involvement of heparan sulfate proteoglycans in the mechanism of anti-HIV effect of HB-19, thus pointing out that nucleolin is its main target.

The external envelope glycoprotein gp120 of HIV plays a key role in the capacity of virus particles to infect CD4+ target cells as a result of the fusion of the viral and cellular membranes. The gp120 has a complex secondary structure in which five conserved regions (C1 to C5) and five hypervariable regions (V1 to V5) have been deﬁned. It exists as an oligomer associated noncovalently with the transmembrane glycoprotein gp41 which at its amino terminus contains a hydrophobic domain essential for the fusion process (1–3). Studies on the mechanism of HIV tropism have revealed that chemokine receptors such as CCR5 and CXCR4 serve as essential cofactors for the entry of macrophage (M)- and T-lymphocyte-tropic HIV-1 isolates, respectively (reviewed in Refs. 3–5). Consequently, infection of cells by both types of HIV-1 isolates could be inhibited by chemokines interacting specifically with their respective receptors (5–7). Besides CCR5, additional β-chemokine receptors including CCR2b, CCR3, and CCR8 are used by some HIV-1 variants (5). It should be noted that chemokines inhibit HIV entry without affecting the attachment of HIV particles to cells (8, 9). Therefore, chemokines appear to block a step following attachment of HIV particles to cells, namely fusion between viral and cellular membranes. By incubation of cells with soluble preparations of gp120, several groups have demonstrated the formation of a complex between gp120, CD4 and CCR5, or CXCR4 and have proposed that HIV-1 attachment to CD4+ cells creates a high affinity interaction site for the coreceptor and that during this event the V3 loop plays an important role by a mechanism that remains to be elucidated (3, 9).

The degree of involvement of CD4 in the initial attachment of HIV particles could be variable according to the cell type (10). In CD4+ T-cells, HIV attachment is mediated by both CD4-dependent and -independent interactions (11). Neutralizing mAbs specific for the V3 loop inhibit both CD4-dependent and -independent interactions, whereas neutralizing mAbs against the gp120 binding domain in CD4 or against the CD4 binding domain in gp120 affect only the CD4-dependent interaction. The observation that anti-V3 loop mAbs also inhibit HIV attachment to CD4+ cells (11) indicates that interaction of the V3 loop with its cell-surface ligands can occur at a step prior to the interaction of gp120 with CD4. In accord with this, several studies have suggested that the V3 loop domain is not necessary for the binding of gp120 to CD4 (12, 13). These observations and the fact that mAbs against the V3 loop block HIV attachment to cells without affecting the potential interaction of gp120 with CD4 (11) indicate that interactions through the
V3 loop and the CD4-binding site of gp120 are two independent events. In accord with this, spontaneous mutations within the V3 loop have been proposed to be responsible for CD4-independent entry of the HIV-1 NDK isolate (14). As the V3 loop is relatively exposed on HIV particles (15) and by virtue of its positively charged residues, it could interact directly with negatively charged components of the cell surface independent of CD4 and chemokine receptors. Potential candidates of such cell-surface components are on the one hand V3 loop binding proteins on the cell surface (discussed in Ref. 16) and on the other hand heparan sulfate proteoglycans that are commonly found on the surface of most vertebrate cell types (10, 17–19).

The pseudopeptide 5[K(CH2N)PR]-TASP referred to as HB-19, which presents pentavalently the K(CH2N)PR tripeptide moiety, is a potent inhibitor of HIV infection of CD4+ cells and preventing the attachment of HIV particles. The fact that HB-19 does not have a significant effect on infection of cells by the simian immunodeficiency virus-mac isolate or by HIV-1 pseudotyped with envelope glycoproteins of other viruses (16, 20). We confirm here that the anti-HIV effect of HB-19 is due to inhibition of the attachment of HIV-1 particles to target cells leading to inhibition of viral entry. The biotin-labeled HB-19 but not the control constructs binds specifically target cells and becomes complexed with the cell-surface-expressed nucleolin. Interestingly, HIV particles can compete with HB-19, both in binding to the cell surface and in complex formation with the cell-surface-expressed nucleolin. On the other hand, no apparent competition occurs with the fibroblast growth factor-2 (FGF-2), known to use heparan sulfates as low affinity receptors (22, 23). This latter observation rules out the possibility that cell-surface heparan sulfate proteoglycans serve as additional targets of HB-19. However, consistent with the implication of heparan sulfates in the HIV attachment process (10, 18, 19), we show that FGF-2 inhibits HIV infection by binding to cells and preventing the attachment of HIV particles. The result of HIV attachment could be inhibited by two different agents that do not compete suggests that the HIV attachment process should be coordinated by two events implicating on the one hand the cell-surface-expressed nucleolin and on the other hand the heparan sulfate proteoglycans.

**EXPERIMENTAL PROCEDURES**

**Materials**—The monoclonal antibody (mAb) specific to human CD4 and reacting with the gp120 binding domain (11) was kindly provided by Dr. Eugene Bomsans (clone CB-T4–2; Eurogenetics, Tessenderlo, Belgium). mAb CC98 directed against human nucleolin (24) was generously provided by Dr. Ning-Hsing Yeh, Graduate School of Microbiology and Immunology, National Yang-Ming Medical College, Shih-Pai, Taiwan, Republic of China. The mAb D3 specific for human nucleolin and used in confocal microscopy studies was kindly provided by Dr. Jau-Shyang Deng, Department of Veterans Affairs, Medical Center, Pittsburgh, PA (25). Polyclonal antibodies were raised in rabbits against a synthetic peptide corresponding to the first 26 amino acid residues of human nucleolin as we had reported (16). The mAb N11-20 directed against the V3 loop of HIV-1 Lai isolate was provided by Dr. Jean-Claude Mazzie, Hybridlab, Institut Pasteur, Paris, France. The mAbs specific to human CXCR4 (12G5) and CCR5 (12D1) were purchased from R & D Systems. The T-tropic HIV-1 Lai was as described before (20). The M-tropic HIV-1 isolates HIV-1 Ba-L (26) and Ada-M (27) were provided by the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, National Institutes of Health. The HIV-1 pseudotyped with VSV envelope glycoproteins was kindly provided by Dr. Olivier Schwartz (Institut Pasteur, Paris). FGF-2 (fibroblast growth factor basic) produced in Escherichia coli was from Sigma. FGF-2 was iodinated (2 x 10^8 Ci/μmol) using the Bolton-Hunter reagent (NEN Life Science Products) by a procedure as recommended by the manufacturer.

**Cells and Virus Preparations**—Human HeLa-CD4-LTR-lacZ cells expressing or not CCR5 were referred to as HeLa P4-C5 and HeLa P4, respectively, and were cultured in Dulbecco’s medium. These HeLa cells were provided by Drs. Olivier Schwartz and Pierre Charneau (Institut Pasteur, Paris, France) and were cultured with 10% fetal bovine serum (FBS) at 37 °C (5% CO2) in medium with 1% bovine serum albumin and 0.02% sodium azide containing 1% 10% FBS. Virus preparations were filtered (0.22-μm pore size, Millipore) before infection of cells. For these experiments, supernatants from HIV-1 pseudotyped with envelope glycoproteins of other viruses (16, 20). We confirm here that the anti-HIV effect of HB-19 is due to inhibition of the attachment of HIV-1 particles to target cells leading to inhibition of viral entry. The biotin-labeled HB-19 but not the control constructs binds specifically target cells and becomes complexed with the cell-surface-expressed nucleolin. Interestingly, HIV particles can compete with HB-19, both in binding to the cell surface and in complex formation with the cell-surface-expressed nucleolin. On the other hand, no apparent competition occurs with the fibroblast growth factor-2 (FGF-2), known to use heparan sulfates as low affinity receptors (22, 23). This latter observation rules out the possibility that cell-surface heparan sulfate proteoglycans serve as additional targets of HB-19. However, consistent with the implication of heparan sulfates in the HIV attachment process (10, 18, 19), we show that FGF-2 inhibits HIV infection by binding to cells and preventing the attachment of HIV particles. The fact that HIV attachment could be inhibited by two different agents that do not compete suggests that the HIV attachment process should be coordinated by two events implicating on the one hand the cell-surface-expressed nucleolin and on the other hand the heparan sulfate proteoglycans.

**Detection of Cell-surface Antigens by FACS Analysis**—The detection of cell-surface antigens was as described before with minor modifications (28). Briefly, HeLa cells (about 10^6) in 6-well plates washed in PBS containing 1% bovine serum albumin and 0.02% sodium azide (PBS/BSA) were incubated (56 °C, 30 min) with 0.22-μm size filters (Millipore) in order to eliminate aggregates and/or cell debris. Such preparations generated concentrated virus that is highly infectious (11).

**Preparation of Nucleus-free Cell Extracts**—HeLa cell monolayers in 150-cm^2 flasks were washed extensively with PBS before adding 1 ml of the lysis buffer E, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM MgCl2, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, aprotinin (1000 units/ml), and 0.5% Triton X-100. After 5 min incubation in buffer E (at room temperature), the flasks were centrifuged at 30,000 x g for 10 min, and the supernatants were stored at −80 °C.

**Peptide Constructs**—The synthesis of 5[K(CH2N)PR]-TASP (herein referred to as HB-19) was as described previously (20, 28). Control peptides were 5[QEQ]-TASP, 5[QCH]-TASP, and 5[KCH]-TASP. For the biotin-labeled compounds, the biotin moiety was introduced during peptide assembly as an Fmoc-N-(9-fluorenylmethoxycarbonyl) Lys–biotin derivative at the carboxyl terminus of the template in HB-19 and 5[QEQ]-TASP and the carboxyl terminus of the K(CH2N)PR monomer. The peptides were obtained at a purity (>95%), and their integrity was controlled by matrix-associated laser desorption ionization-time-of-flight analysis (29). The preparation of the fluoroscein isothiocyanate (FITC, Sigma)-labeled HB-19 was as described before (28). The anti-HIV cyclic peptide TW70 specific for CXCR4 was synthesized as described (30, 31). The TW70 peptide has the amino acid sequence RRWYRKPKPYYCR; the ϕK indicates that the peptide bond is in ϕ configuration has been introduced in the sequence of this peptide in order to stabilize the ϕ-turn in the final structure. The disulfide bridge between the two cysteine residues was generated by air oxidation at pH 8 in water under vigorous stirring for 2 days at room temperature. The final cyclic peptide was more than 95% pure.

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lysis buffer E containing unlabelled HB-19 (50 μM), and the complex formed between cell-surface-expressed nucleolin and the biotin-labeled HB-19 was isolated by purification of the extracts using avidin-agarose (100 μl; ImmunoPure Immobilized Avidin from Pierce) in PBS/EDTA. After 2 h of incubation at 4 °C, the samples were washed extensively with PBS to remove unbound labeled nucleolin. The purified protein was pelleted by centrifugation at 13,000 rpm for 10 min, the electrophoresis sample buffer containing SDS and analyzed by SDS-PAGE. The presence of nucleolin was then revealed by immunoblotting using the monoclonal antibody CC98 as described before (16).

**Assay of HIV Entry in HeLa CD4** Cells—HIV entry was monitored indirectly in HeLa-CD4-LTR-lacZ cells containing the bacterial lacZ gene under the control of HIV-1 LTR promoter. HIV-1 LTR-lacZ entry and replication results in the activation of the HIV-1 LTR leading to the expression of β-galactosidase (32). HeLa-CD4-LTR-lacZ cells expressing recombinant CD4 and constitutively CXCR4 are permissive to infection by T-tropic HIV-1 isolates (33). When they also express recombinant CCR5, such HeLa cells become permissive to M-tropic HIV-1 isolates. These two HeLa cell lines expressing or not CCR5 are referred to as HeLa P4-C5 and HeLa P4, respectively. Cells were plated at 10^6 cells/well in 96-well plates, and at 24 h later cell monolayers were infected with the different HIV isolates (a dose corresponding to 20–40 ng/ml 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 NaHPO_4, 40 mM Na_2HPO_4, 10 mM KCl, 10 mM MgSO_4, 1 mM EDTA, and 50 mM β-mercaptoethanol. After lysis, the reaction mixture containing 10 mM phosphate buffer at pH 7.4, 7.4 mM MgCl_2, 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 10–15-min intervals in a microplate reader using a 570 nm filter. The background value in the β-galactosidase activity in each experiment was measured by including an HIV infection in the presence of AZT (5 μM) that inhibits the HIV reverse transcriptase. The background value of β-galactosidase activity was also monitored in cells in which HIV entry was blocked by the anti-CD4 mAb CB-T4 (5 μg/ml) that reacts with the gp120-binding site in CD4 (11).

**Assay of HIV Particle Attachment to HeLa CD4** Cells—The effect of HB-19 on the amount of HIV-1 Lai particles attached to cells was monitored indirectly (associated with cells) was monitored after 1 h of incubation at 4 °C. These experiments were carried out at 4 °C in order to reduce HIV entry. HIV endpoint titration method has been described before (37). The potential binding of FGF-2 to its high affinity receptor was also investigated by cross-linking of 125I-FGF-2 using 1 mM 3,3'-bis(sulfosuccinimidyl)suberate (Pierce) (37, 38).

**Ligand Blotting and Immunoblotting**—Crude nuclear-free HeLa cell extracts were diluted in 2-fold concentrated electrophoresis sample buffer and analyzed by SDS-PAGE to be electromorphically transferred to 0.22-μm polyvinylidene difluoride films (Bio-Rad). The electroblot blots were saturated with casein-based blocking buffer (Genosys) and washed extensively before incubation with the biotin-labeled HB-19 or the 125I-labeled FGF-2. The biotin was revealed by using streptavidin-horseradish peroxidase complex and light-based enhanced chemiluminescence reagents as provided by the manufacturer (Amer sham Pharmacia Biotech). Immunoblotting to detect nucleolin was carried out using either rabbit polyclonal antibodies (10 μg/ml) raised against the amino-terminal peptide of nucleolin or mAb D3 (0.1 mg/ml). The 125I-labeled protein bands in SDS-PAGE gels were revealed using a PhosphorImager.

**RESULTS**

**HB-19 Inhibits HIV Attachment and Entry in CD4⁺ HeLa Permissive Cell Lines**—HeLa P4-C5 and HeLa P4 cells are recombinant CD4⁺ cell lines permissive to infection by M- and T-tropic HIV-1 isolates, respectively. These cells also contain the bacterial lacZ gene under the control of HIV-1 LTR sequence. HIV entry and replication in such cells result in the activation of the HIV LTR, leading to the expression of the lacZ gene. Consequently, the β-galactosidase activity could be measured in order to monitor HIV entry into cells (see "Experimental Procedures"). One μl of 5(34)[CH₃(NH₂)₃]PR-TASP (herein referred to as HB-19) blocked the T-tropic HIV-1 Lai and the M-tropic HIV-1 Ba-L attachment and entry by more than 90% (Table I). The inhibitory effect of HB-19 in these HeLa cells is dose-dependent, as it has been reported before in other cell lines or in primary T-lymphocytes and macrophages (16, 20, 21). HB-19 does not affect infection by HIV-1 pseudotyped with envelope glycoproteins of Moloney murine leukemia or vesicular stomatitis virus (16, 20),² thus indicating that its inhibitory

| HIV isolate | Peptide construct | Attachment | Entry |
|-------------|------------------|------------|------|
| HIV-1 Lai   | HB-19, 1 μM      | 91%        | No effect<sup>a</sup> |
|             | CP-1, 50 μM      | No effect<sup>b</sup> |
|             | CP-51, 10 μM     | No effect<sup>bc</sup> |
| HIV-1 Ba-L  | HB-19, 1 μM      | 94%        | No effect<sup>a</sup> |
|             | CP-1, 50 μM      | No effect<sup>b</sup> |
|             | CP-51, 10 μM     | No effect<sup>bc</sup> |
| HIV-1 Ada   | HB-19, 1 μM      | No effect<sup>c</sup> |
|             | HB-19, 5 μM      | Enhancing Effect<sup>bc</sup> |
|             | CP-1, 50 μM      | No effect<sup>c</sup> |

<sup>a</sup> No effect, variation less than ±10%.
<sup>b</sup> An enhancing effect of 220%.
<sup>c</sup> An enhancing effect of 250%.
HeLa P4 cells were treated revealed by confocal laser microscopy. Infected monocytes, upon treatment with HIV-1 Ada replication has also been reported by others (39) in for this latter effect remains to be investigated. An enhanced the amount of HIV attachment and entry (Table I). The reason found to resist the inhibitory effect of HB-19, and even higher
attachment and entry process persists for several hours. In
synchronously before addition of HIV, but the inhibitory effect on HIV
resistance of the HIV-1 Ada isolate was conveniently
with other known HIV-1 isolates. Whatever is the case, the
HB-19 isolated from the PBMC and CEM cells results in the formation
of an irreversible complex with the cell-surface-expressed
nucleolin (16, 28). Similarly, incubation of HeLa cells with the
biotin-labeled HB-19 construct resulted in its binding with the
nucleolin (16, 28). A scan of a cross-section toward the middle of the cell monolayer showing the FITC labeling and the corresponding phase contrast are presented.

The specific inhibitory effect of HB-19 with respect to its
structure was demonstrated by the use of its tripeptide moiety
K(CH$_2$N)PR alone (referred to as CP-1) and the control pseudopeptide analogue in which the lysine and arginine residues were modified to arginine and asparagine, respectively, to generate the 5[K(CH$_2$N)PN]-TASP construct (referred to as CP-51). No effect on the attachment and entry of either HIV-1 Lai
and HIV-1 Ba-L was observed by these latter constructs (Table I).

Among different HIV-1 isolates, the M-tropic HIV-1 Ada was
found to resist the inhibitory effect of HB-19, and even higher concentrations consistently resulted in an enhancing effect on the amount of HIV attachment and entry (Table I). The reason for this latter effect remains to be investigated. An enhanced HIV-1 Ada replication has also been reported by others (39) in infected monocytes, upon treatment with $\beta$-chemokines which inhibit infection by other M-tropic HIV-1 isolates. The HIV-1 Ada isolate, therefore, appears to behave differently compared with other known HIV-1 isolates. Whatever is the case, the
HB-19 resistance of the HIV-1 Ada isolate was conveniently
used as a control virus in our experiments described herein.

The Stable Binding of HB-19 to HeLa Cells—The mechanism of the anti-HIV effect of HB-19 was investigated in the HeLa cell lines P4 and P4-C5. By FACS analysis using the biotin-labeled HB-19, we first demonstrated that this inhibitor binds specifically and in a dose-dependent manner the surface of both cell lines studied (as in Ref. 16). The binding is stable, since HeLa cells preincubated with HB-19 could be washed extensively before addition of HIV, but the inhibitory effect on HIV
attachment and entry process persists for several hours. In contrast, no inhibitory effect is observed when HB-19 is added a few hours after incubation of cells with HIV (not shown). Fig. 1 further demonstrates the stable binding of HB-19 to cells by
confocal laser immunofluorescence microscopy. The immuno-

![HB-19B*](image)

**Fig. 1.** The binding of HB-19 to HIV-permissive HeLa P4 cells revealed by confocal laser microscopy. HeLa P4 cells were treated (incubation at 37 °C, 20 min) with the biotin-labeled HB-19 (2 $\mu M$) in culture medium. Cells were then washed with PBS and fixed with PFA and processed for confocal microscopy. The biotin was revealed by streptavidin-FITC complex (Amersham Pharmacia Biotech). A scan of a cross-section toward the middle of the cell monolayer showing the FITC labeling and the corresponding phase contrast are presented.

HB-19 Forms a Stable Complex with the Cell-surface-expressed Nucleolin in HeLa Cells—The specific binding of HB-19 to the surface of PBMC and CEM cells results in the formation of an irreversible complex with the cell-surface-expressed nucleolin (16, 28). Similarly, incubation of HeLa cells with the
biotin-labeled HB-19 construct resulted in its binding with the cell-surface-expressed nucleolin and formed an irreversible complex with it (Fig. 2A). For this purpose, cells were incubated with the biotin-labeled HB-19 under routine experimental conditions used for the HIV entry assay, before washing extensively and preparation of nuclear-free extracts with lysis buffer containing excess unlabeled HB-19. The irreversible complex was then recovered using avidin-agarose. By this procedure, cell-surface nucleolin was isolated without its cytoplasmic proteins during preparation of extracts. Extracts were purified on avidin-agarose to recover complexes formed between cell-surface proteins and the biotin-labeled peptide constructs (16, 28), and the presence of nucleolin was revealed by immunoblotting using mAb CC98 (see “Experimental Procedures”). The numbers on the left show the position of molecular mass (in kDa) protein markers. On the right is the position of nucleolin (p95) and its partial cleavage product (p60). Material extracted from $10^7$ cells was analyzed in each lane. B, the specific binding of HB-19 to the cell-surface-expressed nucleolin. HeLa P4-C5 cells in the culture medium were incubated (37 °C, 30 min) as such (lane 1) or with the control tripeptide monomer K(CH$_2$N)PR (CP-1, 250 $\mu M$; lane 2) or unlabeled HB-19 (50 $\mu M$; lane 3) before the addition of the biotin-labeled HB-19 (2.5 $\mu M$) and further incubation at 37 °C for 30 min. The samples were then processed as in A to recover the biotin-labeled HB-19 coupled to the cell-surface-expressed nucleolin.

**Fig. 2.** Recovery of the cell-surface-expressed nucleolin on HeLa cells by the capacity of HB-19 to bind and form a stable complex with it. A, HeLa P4 cells in the culture medium were incubated (37 °C, 30 min) at different concentrations of the biotin-labeled HB-19 (lanes 0, 0.5, 1, and 2.5 $\mu M$). As controls, HeLa cells were incubated with biotin-labeled control peptides: 50 $\mu M$ K(CH$_2$N)PR (referred to as CP-1) or 10 $\mu M$ 5[QFQ]-TASP (referred to as CP-40). Cells were then washed extensively in PBS containing 1 mM EDTA prior to the preparation of nucleus-free cell extracts. The extraction of cells preincubated with the biotin-labeled HB-19 was performed with lysis buffer E containing 50 $\mu M$ unlabeled HB-19, in order to rule out the possibility that the complex formation could occur with cytoplasmic proteins during preparation of extracts. Extracts were purified on avidin-agarose to recover complexes formed between cell-surface proteins and the biotin-labeled peptide constructs (16, 28), and the presence of nucleolin was revealed by immunoblotting using mAb CC98 (see “Experimental Procedures”). The numbers on the left show the position of molecular mass (in kDa) protein markers. On the right is the position of nucleolin (p95) and its partial cleavage product (p60). Material extracted from $10^7$ cells was analyzed in each lane. B, the specific binding of HB-19 to the cell-surface-expressed nucleolin. HeLa P4-C5 cells in the culture medium were incubated (37 °C, 30 min) as such (lane 1) or with the control tripeptide monomer K(CH$_2$N)PR (CP-1, 250 $\mu M$; lane 2) or unlabeled HB-19 (50 $\mu M$; lane 3) before the addition of the biotin-labeled HB-19 (2.5 $\mu M$) and further incubation at 37 °C for 30 min. The samples were then processed as in A to recover the biotin-labeled HB-19 coupled to the cell-surface-expressed nucleolin.
expected, the biotin-labeled Ke(CH$_2$N)PR tripeptide did not bind the cell-surface-expressed nucleolin, even when it is used in excess (Fig. 2A, lane CP-1). Similarly, no binding was observed with the control biotin-labeled TASP construct 5[KPQ]-TASP (Fig. 2A, lane CP-40). Therefore, the binding of the biotin-labeled HB-19 to cell-surface-expressed nucleolin is specific to the structure of the pentavalently presented tripeptide moiety.

As we demonstrated previously (28) in CEM cells, the binding of the biotin-labeled HB-19 to the HeLa cell-surface-expressed nucleolin was specific since it was reduced drastically in the presence of excess unlabeled HB-19 but not by the control CP-1 pseudopeptide (Fig. 2B, lanes 2 and 3). Nucleolin recovered from the surface of HeLa cells corresponded to less than 20% that found in the nuclear-cytoplasmic fraction. The mechanism of expression of nucleolin on the cell surface remains to be elucidated, since nucleolin does not possess a hydrophobic domain as do conventional membrane-associated proteins. The presence of nucleolin at the cell surface has also been shown by electron microscopy.\(^3\)

Studies on the kinetics of the partial cleavage of the cell-surface nucleolin indicated that cleavage occurs as early as 5 min after the addition of HB-19 (not shown). Such partial cleavage could still be observed at 60 min, but at 6 h post-addition of HB-19, only a trace amount of degraded nucleolin remains detectable (Fig. 3B). The degradation of nucleolin on the cell surface is specific since the detection of other cell-surface antigens such as CD4, CXCR4, CCR5 (monitored by FACS analysis), and the activity of several cell-surface peptidases is not modified in cells treated with 5–10 \(\mu\)M HB-19 (not shown). Furthermore, it should be noted that nucleolin in the cytoplasmic fraction does not appear to be affected (Fig. 3A, panel Cytoplasm). Therefore, despite the continual presence of CD4 and CXCR4/CCR5 on the cell surface, HIV attachment is inhibited in cells that bind HB-19. Although the partial cleavage of nucleolin has been reported before under different experimental conditions (16, 24), this is the first report for the specific cleavage of the cell-surface-expressed nucleolin. It is plausible that the binding of HB-19 to nucleolin changes its conformation making it susceptible to degradation by a cellular protease.

In addition to nucleolin, by using cell extracts we have previously purified the putative HLA class II-associated proteins I and II (PHAP I and PHAP II) as two other V3 loop binding proteins (16). However, at 0.5 to 5 \(\mu\)M concentrations of HB-19 that inhibit the attachment of different HIV isolates to HeLa cells, only nucleolin could be recovered from the cell surface (Fig. 2). Much higher concentrations of HB-19 (20–50 \(\mu\)M) were found to be required for the isolation of PHAP I and PHAP II from intact HeLa cells (not shown) and other cell types (16). Therefore, whether PHAP I and PHAP II are expressed on the cell surface still remains to be confirmed. The high affinity and the specificity of HB-19 to bind cell-surface nucleolin point out that nucleolin is the main target of this pseudopeptide inhibitor of HIV attachment.

Evidence for Cell-surface Expression of Nucleolin Revealed by Confocal Immunofluorescence Laser Microscopy—Nucleolin, which is a major nucleolar protein, has been reported to be found on the cell surface (25, 40, 41 and references therein). By FACS analysis using the mAb D3 specific for nucleolin (25), we could demonstrate the presence of nucleolin along with CD4 and CXCR4 on the HeLa P4 cells studied here (Fig. 4). By FACS analysis, the cell-surface expression of nucleolin has also been shown in primary macrophages and T-lymphocytes (21).

The cell-surface expression of nucleolin was further investigated by using mAb D3 and confocal immunofluorescence microscopy by fixing cells with PFA. By this procedure, a clear signal was observed on the cell surface (not shown). As membrane proteins could cluster into patches when cross-linked by antibodies, HeLa cells were incubated with the mAb D3 at 37 °C for 1 h before PFA fixing and analysis for confocal microscopy. Consistently, addition of mAb D3 to unfixed cells resulted in the redistribution of cell-surface nucleolin into large patches. A cross-section of such cells is shown in Fig. 5A (panel Control Cells) demonstrating the clustering of the cell-surface-expressed nucleolin that was revealed by distinct staining at the periphery of the HeLa cells (that grow as a monolayer). This strong signal at the cell membrane was almost completely lost when cells were treated with a non-ionic detergent used for permeabilization of cells, thus further confirming that the clustering of nucleolin had occurred on the cell surface. No cell-surface staining was observed in HeLa cells incubated with a

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\(^3\) F. Puvion-Dutilleul, unpublished results.
The binding of HB-19 to cells is inhibited by HIV-1 Lai particles. A, the binding of FITC-labeled HB-19 is inhibited by excess unlabeled HB-19 or by HIV-1 Lai particles. HeLa P4 cells in the culture medium were preincubated (4 °C, 30 min) in the absence or presence of 50 μM HB-19 (unlabeled pseudopeptide in excess) or with HIV-1 Lai particles (corresponding to 200 ng/ml of p24). B, the expression of CXCR4 is not modified by incubation of cells in excess unlabeled HB-19 or by HIV-1 Lai particles. The experimental procedure used was as in A. C, the binding of FITC-labeled HB-19 is inhibited by excess unlabeled HB-19 but not by HIV-1 Ada particles. HeLa P4-C5 cells in the culture medium were preincubated (4 °C, 30 min) in the absence or presence of 50 μM HB-19 (unlabeled pseudopeptide in excess) or with HIV-1 Ada particles (corresponding to 200 ng/ml of p24). After these different preincubations, cells were further incubated (4 °C, 30 min) in the presence of either the FITC-labeled HB-19 (1 μM; referred here as HB-19*) or mAb 12G5 (1 μg/ml) specific to CXCR4 (α-CXCR4). Cells were washed and processed for FACS analysis as described (see “Experimental Procedures”). The ordinate gives the relative cell number, whereas the abscissa gives the relative fluorescence intensity. The different samples were as indicated. The peak control of autofluorescence in A and C was obtained by preincubation of cells with unlabeled HB-19 (50 μM). Note, in contrast to unlabeled HB-19, excess control peptide monomer CP-1 (at 250 μM) did not exert any significant effect on the binding of the biotin-labeled HB-19 to cells (not shown).

The cell-surface expression of nucleolin and its reduction following incubation with HB-19 revealed by confocal immunofluorescence microscopy. After 24 h of passaging, HeLa P4 cells were incubated in fresh culture medium in the absence or presence of 2 μM HB-19 for 5 h. The anti-nucleolin mAb D3 (A) and anti-CXCR4 mAb 12G5 (B), each at 5 μg/ml, were added in the different cultures during the last hour, i.e. between 4 and 5 h. Cells were then washed, fixed with PFA, incubated with FITC-labeled anti-mouse IgG, and processed for immunofluorescence confocal microscopy. The different panels show an equivalent layer of a cross-section of HeLa cell monolayers. The clustering of nucleolin and CXCR4 was consistently observed at the cell periphery with the anti-nucleolin mAb D3 and the anti-CXCR4 mAb 12G5, respectively. Note the absence of intracellular nucleolin or CXCR4 staining in intact cells preincubated with the respective monoclonal antibody. Under similar experimental conditions, no cell-surface labeling was observed when mAb LG2–2 specific for histone H2B was used (not shown).

The prolonged incubation of cells with HB-19 led to degradation of the cell-surface-expressed nucleolin (Fig. 3). Consistent with this, by confocal microscopy we show a dramatic reduction of the cell-surface-expressed nucleolin in cells preincubated with HB-19 for 5 h (Fig. 5A, panel Cells + HB-19). In contrast, no apparent effect was detectable for the cell-surface-expressed CXCR4 in HeLa cells preincubated with HB-19 (Fig. 5B), thus demonstrating that the effect of HB-19 is specific on the cell-surface-expressed nucleolin.

The clustering of the cell-surface-expressed CXCR4 and nucleolin by their respective antibodies also occurred at 4 °C (not shown). Consequently, cell-surface-expressed nucleolin behaves as other well-characterized membrane proteins when it is cross-linked with a specific antibody. The Binding of HB-19 to the Cell-surface-expressed Nucleolin Is Inhibited by HIV Particles—The competition between HIV particles and HB-19 to bind target cells was demonstrated by the capacity of HIV-1 Lai particles to inhibit the specific binding of HB-19 to cells (Fig. 6) and reduce the complex formation with the cell-surface-expressed nucleolin (Fig. 7). In these experiments, HIV attachment was carried out at 4 °C in order to block the viral entry process since fusion between viral and cellular membranes requires incubation at physiological temperatures. On the other hand, HIV attachment occurs efficiently at 4 °C, and moreover, the degree of inhibition of this attachment by HB-19 at 4 °C is comparable to that carried out at 37 °C.

The binding of the FITC-labeled HB-19 to the surface of HeLa cells was shown to be specific, since it was prevented by unlabeled HB-19 (Fig. 6, A and C). HIV-1 Lai particles could also interfere with the binding of the FITC-labeled pseudopep-
FGF-2 receptor (44). Interestingly, the binding of 125I-FGF-2 to cell-surface proteins failed (not shown). Scatchard analysis of the 2 mM NaCl-sensitive binding confirmed that the interaction of FGF-2 with HeLa cells is of low affinity type with a calculated Kd of 110 nM. This value is about 1000-fold lower than the Kd reported for the high affinity binding to the FGF-2 receptor. Interestingly, the binding of 125I-FGF-2 was not affected when cells were preincubated with HB-19 (Fig. 8), thus pointing out that the cell-surface target of HB-19 is different from that of FGF-2. Similarly, FGF-2 had no apparent effect on the binding of the FITC-labeled HB-19 receptor (30, 31) inhibiting the cross-linking of the biotin-labeled HB-19 to the cell-surface-expressed nucleolin.

The implication of nucleolin in the mechanism of HIV attachment to CD4+ cells was further demonstrated by the capacity of HIV-1 Lai but not HIV-1 Ada particles to inhibit in a dose-dependent manner the complementing of HB-19 to the cell-surface-expressed nucleolin. Moreover, there was even a slight increase of HB-19 binding to cell-surface-expressed nucleolin in the presence of HIV-1 Ada (Fig. 7). It is of interest to note that the CXCR4-specific anti-HIV peptide TW70 which blocks T-tropic HIV-1 entry (30, 31) did not affect the attachment of HIV-1 Lai particles to cells (see below) nor the binding of HB-19 to cell-surface-expressed nucleolin (not shown).

Inhibition of HIV Entry and Attachment by FGF-2—Heparan sulfates present on the cell surface have been shown to be implicated in the attachment process of HIV particles (10, 18, 19). In order to define the contribution of cell-surface-expressed nucleolin in respect to heparan sulfates, we investigated the anti-HIV effect of FGF-2 that uses heparan sulfates as low affinity receptors (22, 23). The 125I-labeled FGF-2 was shown to bind HeLa P4 cells in a dose-dependent manner (Fig. 8) but failed to achieve saturation consistent with the binding of FGF-2 to its low affinity receptor (44). Indeed, most of the cell-bound 125I-FGF-2 was washed away by 2 mM NaCl treatment (Fig. 8) which disrupts heparan sulfate but not the high affinity receptor-bound FGF-2 (44). The 2 mM NaCl-resistant 125I-FGF-2 binding to cells did not reach a saturation with the increasing dose of FGF-2, thus suggesting that it was mainly due to background nonspecific binding (Fig. 8). HeLa cells therefore, appear to express very low levels, if any, of the high affinity FGF receptors, and several attempts to chemically cross-link the 125I-FGF-2 to cell-surface proteins failed (not shown). Scatchard analysis of the 2 mM NaCl-sensitive binding confirmed that the interaction of FGF-2 with HeLa cells is of low affinity type with a calculated Kd of 110 nM. This value is about 1000-fold lower than the Kd reported for the high affinity binding to the FGF-2 receptor (44). Interestingly, the binding of 125I-FGF-2 was not affected when cells were preincubated with HB-19 (Fig. 8), thus pointing out that the cell-surface target of HB-19 is different from that of FGF-2. Similarly, FGF-2 had no apparent effect on the binding of the FITC-labeled HB-19 receptor (30, 31) inhibiting the cross-linking of the biotin-labeled HB-19 to the cell-surface-expressed nucleolin (not shown).

The effect of FGF-2 on the entry of T-tropic HIV-1 Lai and M-tropic HIV-1 Ba-L was monitored by the 125I-FGF-2 binding to cells. Cells were first preincubated (37 °C, 20 min) with 25 μM HB-19 before further incubation with 30, 60, and 120 nM 125I-FGF-2 as the control. Each point represents the mean of duplicate samples.

The effect of FGF-2 on the entry of T-tropic HIV-1 Lai and M-tropic HIV-1 Ba-L was monitored by the 125I-FGF-2 binding to cells. Cells were first preincubated (37 °C, 20 min) with 25 μM HB-19 before further incubation with 30, 60, and 120 nM 125I-FGF-2 as the control. Each point represents the mean of duplicate samples.
Interestingly, the degree of inhibition of HIV-1 Lai entry in the two cell types (HeLa P4 and HeLa P4-C5 cells) was similar at the different concentrations of FGF-2, thus indicating that expression of CCR5 does not modify its inhibitory efficacy.

In order to study the specificity of FGF-2 with respect to the HIV envelope glycoprotein-mediated viral-entry process, we investigated its inhibitory effect on infection by an HIV-1 pseudotyped with envelope glycoproteins of vesicular stomatitis virus (VSV). At 48 h post-infection, the β-galactosidase activity was measured in cell extracts directly in order to monitor HIV entry. The mean ± S.D. of triplicate samples is shown.

**DISCUSSION**

The results presented here further demonstrate that the HB-19 pseudopeptide is a potent and a specific inhibitor of HIV infection. At concentrations that block HIV attachment to cells, HB-19 binds cells and forms an irreversible complex with the cell-surface-expressed nucleolin (Fig. 2). Consistent with this, HIV particles can prevent the capacity of HB-19 to bind cells and form a stable complex with the cell-surface-expressed nucleolin (Figs. 6 and 7), thus pointing out that nucleolin is implicated in the HIV attachment process. Nucleolin is one of the major RNA-binding proteins of the nucleolus which has been suggested to shuttle between the nucleus and cytoplasm (51, 52). Although its localization has been emphasized to the nucleoli, nucleolin has been reported to be also expressed on the cell surface and serve as a binding protein to different ligands (Refs. 25, 40, and 41 and references therein). Here we have confirmed that nucleolin is expressed on the cell surface, by demonstrating that incubation of intact cells with a specific antibody results in the clustering of the cell-surface-expressed nucleolin (Figs. 4). The role of nucleolin in the HIV attachment and entry processes is under investigation. As nucleolin is characterized as a shuttle protein (51, 52), it might be plausible that interaction of HIV particles with the cell-surface-expressed nucleolin might be functional for chaperoning the viral entry process.
The mean treatment with trypsin to eliminate virus bound on the cell surface of cells incubated with virus were washed with PBS before with cells represented virus particles bound on the surface of cells, a Procedures”). In order to demonstrate that most of the HIV associated bound HIV particles, and the amount of p24 associated with cells was at 4 °C with HIV-1 Lai for 1 h. Cells were then washed extensively with addition to cells at 4 °C as the other samples (11). Cells were incubated at 4 °C with HIV-1 Lai for 1 h. Cells were then washed extensively with culture medium containing 10% fetal calf serum to eliminate free un-bound HIV particles, and the amount of p24 associated with cells was measured as an estimate for the amount of HIV binding ("Experimental Procedures"). In order to demonstrate that most of the HIV associated with cells represented virus particles bound on the surface of cells, a sample of cells incubated with virus were washed with PBS before treatment with trypsin to eliminate virus bound on the cell surface (sample trypsin). The mean ± S.D. of triplicate samples is shown.

Other groups by enzymatic digestion of cell-surface heparan sulfates have suggested the implication of proteoglycans in the HIV attachment process (10, 18, 19). In order to confirm this under physiological conditions, we used the growth factor FGF-2 which is a physiological ligand of heparan sulfates. Indeed, fibroblast growth factors have been shown to exert their effects on target cells by using a dual receptor system, heparan sulfate proteoglycans as low affinity receptors and a family of FGF receptors as the signal-transducing high affinity receptors (22, 23). The binding of FGF with heparan sulfates appears to induce a conformational change and/or formation of the FGF dimer required for interaction with FGF receptors (44). FGF bound to its high affinity receptor is stable and persists after washing cells with 2 M NaCl. On the other hand, the binding of FGF to heparan sulfates is disrupted by 2 M NaCl. Consequently, the amount of cell-associated FGF, sensitive and resistant to 2 M NaCl, is considered as binding to heparan sulfate and the FGF receptors, respectively (37). By such a procedure, we demonstrated here that association of FGF-2 with HeLa P4 cells is mainly due to binding to the low affinity receptors, i.e. the cell-surface-expressed heparan sulfates (Fig. 8). Consistent with previous data on the implication of surface proteoglycans in the HIV attachment process (10, 18, 19), FGF-2 was demonstrated to be a potent inhibitor of HIV attachment and entry in the HeLa cell model studied here (Figs. 9 and 11). These results and the fact that FGF-2 does not compete with HB-19 indicate that FGF-2 and HB-19 have a distinct mode of action in the process of HIV particle attachment to permissive cells. Furthermore, these observations rule out the potential interaction of HB-19 with cell-surface heparan sulfate proteoglycans.

By structure and inhibitory activity relationship studies using analogs of HB-19, previously we had demonstrated that the positively charged side chains of the two basic residues in the tripeptide moiety of HB-19 are essential for the inhibitory structure (20). In addition to this positive charge, the pentavalent presentation of the tripeptide moiety is a complementary determining factor for the anti-HIV activity of HB-19, since the tripeptide moiety alone is not active (Table I), whereas the tetravalent presentation of the tripeptide moiety generates a product with reduced inhibitory activity (20). HB-19 has several biochemical properties that are also manifested by a synthetic V3 loop peptide (16, 20, 28). Indeed, both HB-19 and the V3 loop peptide compete together to bind cells and form a complex with the cell-surface-expressed nucleolin. This interaction is direct as demonstrated by the capacity of HB-19 and the V3 loop peptide to bind nucleolin in ligand blot type experiments and also to purify nucleolin by affinity chromatography using cell extracts. It should be noted that in our direct binding assays, HB-19 and the synthetic V3 loop peptide do not bind CD4 or CXCR4. With partially purified preparations of nucleolin, we could demonstrate that different preparations of recombinant gp120 bind nucleolin with a high affinity comparable to the binding of gp120 to soluble CD4. Such binding is inhibited by HB-19 or by monoclonal antibodies against the V3 loop but not against the CD4-binding domain in gp120. In view of these observations, we suggested that gp120 could bind nucleolin via its V3 loop domain (16). The equilibrium affinity constant $K_a$ value for the binding of a synthetic V3 loop peptide to the nucleolin preparation is $5.1 \times 10^9 \text{M}^{-1}$, whereas that of soluble gp120 is $1.1 \times 10^9 \text{M}^{-1}$ (16). Interestingly, the $K_a$ value for the binding of HB-19 to the nucleolin preparation is $9.6 \times 10^9 \text{M}^{-1}$ (16). This high affinity binding of HB-19 to nucleolin is most probably due to the presence of several stretches of amino acids composed of aspartate and glutamate residues at the amino terminus of nucleolin (53). Such polyanionic domains in nucleolin should provide a well defined structure to account for its specific binding to the HB-19 construct but not to the K$_3$(CH$_2$N)$\psi$(CH$_3$N)PR tripeptide moiety (Fig. 2A). Consequently, the polyanionic domains in nucleolin might be responsible for the interaction of gp120, especially through the basic residues in the V3 loop, and thus provide a potential receptor for the V3 loop to mediate virus attachment. Our hypothesis is that by virtue of the V3 loop domain, nucleolin could interact with gp120 on the surface of HIV particles and thus become implicated in the mechanism of HIV attachment to CD4$^+$ cells. Therefore, agents such as mAb anti-V3 loop or HB-19 which interfere in the interaction of the V3 with the cell-surface-expressed nucleolin block HIV attachment and thus entry.

The V3 loop is relatively exposed on HIV particles (15, 54). It contains several conserved positively charged lysine and arginine residues that account for its net positive charge (55). Consequently, polyanionic molecules that interact with the V3 loop inhibit attachment of HIV particles to cells (56, 57) as is the case with neutralizing monoclonal antibodies specific to the V3 loop (11, 58). In view of this and the ability of chemokines (3, 8, 9) and compounds that interact specifically with chemokine receptors (42, 43) (Figs. 9 and 10) to block HIV entry without affecting HIV attachment, it might be plausible to suggest that the V3 loop by virtue of its positively charged residues could interact directly with negatively charged components of the cell surface independent of CD4 and chemokine receptors. Potential candidates of such cell-surface components are on the one hand heparan sulfate proteoglycans that are commonly found on the surface of most vertebrate cell types (10, 17–19) and on the other hand V3 loop binding proteins such as nucleolin (16, 53). Specificity in the case of HIV attachment could be mediated in part by nucleolin and CD4, since heparan sulfates have been shown to serve as low affinity receptors for different viruses and ligands such as the fibroblast growth factors and...
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chemokines (22, 23, 38, 46–50, 59). Heparan sulfates by providing negatively charged molecules could interact nonspecifically with the V3 loop (18, 19, 56, 57), whereas nucleolin by virtue of the defined structure of its acidic amino acid domains could interact specifically with the V3 loop (16, 53). In accord with this, we show that FGF-2 blocks entry of HIV-1 pseudotyped with the envelope glycoproteins of VSV, whereas HB-19 has no effect on such a pseudotyped virus (Fig. 9). However, it should be noted that there should be cooperativity between heparan sulfates and nucleolin, since HIV attachment could be inhibited by acting independently either on heparan sulfates using FGF-2 or on nucleolin using HB-19. The heparan sulfates may be necessary for the concentration of HIV particles on permissive cells to allow efficient subsequent interaction with nucleolin and CD4. Indeed, interaction of ligands with cell-surface heparan sulfates have often been shown to facilitate a secondary interaction with specific high affinity receptors (22, 23, 59, 60).

The wide spectrum of the specific inhibitory action of HB-19 on different types of HIV isolates, along with its distinct mode of action and stability in serum (16, 20, 21, 28) (results herein),2 make this pseudotpeptide inhibitor a potential drug candidate for HIV-infected individuals.

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