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Background: Human neutrophil peptide 1 (HNP-1) inhibits HIV-1 infection by a poorly defined mechanism.

Results: Effects of HNP-1 on individual steps of virus-cell fusion were examined.

Conclusion: HNP-1 interferes with all major steps of HIV-1 entry, from CD4/coreceptor binding to virus uptake and refolding of Env glycoprotein.

Significance: The ability of HNP-1 to block HIV-1 uptake suggests a novel universal mechanism for antiviral activity.

The human neutrophil peptide 1 (HNP-1) is known to block the human immunodeficiency virus type 1 (HIV-1) infection, but the mechanism of inhibition is poorly understood. We examined the effect of HNP-1 on HIV-1 entry and fusion and found that, surprisingly, this α-defensin inhibited multiple steps of virus entry, including: (i) Env binding to CD4 and coreceptors; (ii) refolding of Env into the final 6-helix bundle structure; and (iii) productive HIV-1 uptake but not internalization of endocytic markers. Despite its lectin-like properties, HNP-1 could bind to Env, CD4, and other host proteins in a glycan- and serum-independent manner, whereas the fusion inhibitory activity was greatly attenuated in the presence of human or bovine serum. This demonstrates that binding of α-defensin to molecules involved in HIV-1 fusion is necessary but not sufficient for blocking the virus entry. We therefore propose that oligomeric forms of defensin, which may be disrupted by serum, contribute to the anti-HIV-1 activity perhaps through cross-linking virus and/or host glycoproteins. This notion is supported by the ability of HNP-1 to reduce the mobile fraction of CD4 and coreceptors in the plasma membrane and to precipitate a core subdomain of Env in solution. The ability of HNP-1 to block HIV-1 uptake without interfering with constitutive endocytosis suggests a novel mechanism for broad activity against this and other viruses that enter cells through endocytic pathways.

Mammalian leukocytes and epithelial cells produce defensins, cysteine-rich cationic peptides exhibiting broad antimicrobial and immunomodulatory activities (1–3). These peptides are classified into three subfamilies, α-, β-, and γ-defensins (4–7). All defensins contain three disulfide bonds, which are critical for their anti-microbial activities, including the inhibition of infection by a number of enveloped and nonenveloped viruses (2, 8–14). Mammalian defensins have been reported to inhibit HIV-1 replication in vitro (15–18) and in vivo (19, 20). However, the mechanism underlying the anti-HIV-1 activity of defensins remains controversial. Retrocyclin (a γ-defensin) has been shown to bind to both CD4 and HIV-1 gp120 glycoprotein in a glycan-dependent manner, and this binding is correlated with its anti-HIV-1 activity (21). Based on this lectin-like property of retrocyclin and its ability to reduce the lateral mobility of cell surface glycoproteins, Leikina et al. (22) proposed that γ-defensin acts by reversibly cross-linking the plasma membrane glycoproteins and thus erecting a barrier for virus fusion. Conversely, retrocyclin has been reported to inhibit HIV-1 fusion by specifically binding to the HIV-1 gp41, but not to HIV-2 or SIV gp41, in a glycan-independent manner and preventing the formation of the gp41 6-helix bundle structure (23, 24).

The mechanism of anti-HIV-1 activity of α-defensins, also known as human neutrophil peptides (HNPs),2 is also debated. These defensins have been implicated in inhibition of different steps of the HIV-1 replication cycle, from binding to cognate receptors (4, 25) to post-reverse transcription and even post-integration processes (16, 17, 26). α-Defensins have also been reported to inhibit HIV-1 infection by up-regulating expression and secretion of chemokines (27) and by directly inactivating

2 The abbreviations used are: HNP, human neutrophil peptide; BlaM, β-lactamase; C34 and C52L, peptides derived from the C-terminal heptad repeat domain of HIV-1 gp41; DiI, 1,1′-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate; DMSO, D-(-)-lacto-
the virus in a serum-free medium (17, 26, 28). At the same time, certain human α-defensins (HD5 and HD6) can enhance entry of HIV-1 and unrelated viruses (29–31). HNP-1, -2, and -3 exhibiting lectin-like properties have been reported to bind to CD4 and to HIV-1 gp120 with a relatively high affinity, a feature that appears to correlate with their anti-HIV-1 activity (4). However, HNP-4, which exhibits weak glycan-independent binding to gp120 and CD4, is a more potent inhibitor of HIV-1 infection (32). Thus, the exact steps of HIV-1 replication targeted by α-defensins and the mechanisms of their action are not well understood.

To gain insight into the elusive mechanism of antiviral activity of human defensins, we focused on HIV-1 entry into cells. We have recently provided evidence that HIV enters susceptible cell lines and primary CD4+ T cells via endocytosis and fusion with endosomes (33, 34). We have also dissected key steps of HIV-1 entry and fusion, using respective inhibitors (33–35). Here, by employing imaging, functional and biochemical assays, we examined the effect of an α-defensin, HNP-1, on HIV-1 fusion. Major steps of HIV-1 entry, from binding to CD4 and coreceptors to productive endocytosis and gp41-mediated fusion with endosomes, were analyzed.

Our experiments revealed the striking ability of HNP-1 to interfere with multiple steps of HIV-1 entry. This defensin bound to Env glycoprotein, as well as to CD4 and likely to coreceptors, without inactivating the virus or compromising the cell viability. In addition, HNP-1 down-regulated the expression of CD4 and CXCR4 and blocked weak interactions between Env and CD4 or coreceptors. Moreover, analysis of HIV-1 fusion intermediates downstream of CD4/coreceptor binding showed that HNP-1 also inhibited late steps of fusion, apparently by targeting intermediate conformations of Env. We also found that defensin was able to bind Env and CD4 in a glycan-independent manner and to reduce the mobile fraction of CD4 and coreceptors in the plasma membrane. Perhaps the most unexpected anti-HIV-1 activity of this defensin was the selective inhibition of HIV-1 uptake but not of the overall endocytic activity of a target cell. These findings imply that, through an inherent ability to bind to multiple targets, HNP-1 mounts a powerful defense against HIV-1. However, despite poor inhibitory activity of HNP-1 in the presence of serum, its binding to cellular and virus proteins was not affected by serum. The lack of correlation between the defensin binding and inhibitory activity suggests that, in addition to the ability to engage multiple targets, the antiviral effect may require defensin oligomerization, which could be disrupted by serum.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents—**HEK 293T/17 cells (from ATCC, Manassas, VA) were grown in DMEM supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT), 0.5 mg/ml geneticin (Invitrogen), and penicillin/streptomycin (Sigma). HeLa-derived indicator TZM-bl cells expressing CD4, CXCR4, and CCR5 were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin. HeLa-ADA cells stably expressing Env and Tat from the HIV-1 ADA strain were a gift from Dr. Marc Alizon (Pasteur Institute, France) (36). All media and buffers were obtained from HyClone (Thermo Scientific) or Cellgro (Mediatech Inc., Manassas, VA). The following cell lines and reagents were obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health: indicator TZM-bl cells (donated by Drs. J. C. Kappes and X. Wu (37)); hybridoma cells secreting the anti-HIV-1 gp41 Chessie8 antibody (from Dr. G. Lewis (38)); pBABE-Fusin (donated by Dr. N. Landau (39)); HIV-1 immunoglobulin (HIV-IG) (Dr. Luiz Barbosa from the NHLBI, National Institutes of Health); TAK-779 (40); and anti-CXCR4 antibody 12G5 (donated by Dr. James Hoxie (41)). The 2D7 antibody was purchased from Pharmingen. Human peripheral blood mononuclear cells (PBMCs) were isolated and activated with 10 ng/ml IL-2 (AIDS Reference Reagent Program, National Institutes of Health, from Dr. M. Gately, Hoffmann-La Roche) and 2.5 μg/ml PHA (Sigma), as described previously (33).

The pCAGGS plasmids encoding JRFL or HXB2 Env were provided by Dr. J. Binley (Torrey Pines Institute). The pMDG vector expressing VSV G was provided by Dr. John Young (Salk Institute). The HIV-1-based packaging vector pR8ΔEnv lacking the env gene was from Dr. D. Trono (Geneva, Switzerland). The C52L recombinant peptide was a gift from Dr. Min Lu (Cornell University) (42). The C34 peptide was a gift from Dr. L. Wang (Institute of Human Virology, University of Maryland Baltimore). Anti-human CD4-Alexa Fluor488 conjugate and purified mouse IgG1 and IgG2a, κ isotype controls were obtained from BioLegend (San Diego). AD101 was a kind gift from Dr. J. Strizki (Schering-Plough). Endocytic markers transferrin-Allexa488, dextran-Alexa488 and Dil-low density lipoprotein (Dil-LDL) were from Invitrogen. BMS-806 was synthesized by ChemPacific Corp. (Baltimore, MD), and phorbol 12-myristate 13-acetate (PMA) and AMD3100 were purchased from Sigma. Protein G-horseradish peroxidase (HRP) conjugate was purchased from Bio-Rad, and streptavidin-HRP was from GE Healthcare. The 3,3′,5,5′-tetramethylbenzidine substrate kit for chromogenic detection of the horseradish peroxidase (HRP) activity was purchased from Thermo Scientific. The enhanced luminol-based chemiluminescent (ECL) substrate for the HRP detection on immunoblots was obtained from Pierce. The enzymatic deglycosylation kit was from Prozyme (San Leandro, CA).

**Synthesis and Purification of HNP-1 and Its Derivatives—**The chemical synthesis, oxidative folding, and structural characterization of HNP-1 were described elsewhere (43–45). The six Cys residues in HNP-1 were all replaced by α-aminobutyric acid (Abu), isosteric to Cys, yielding the linear analog Abu-HNP. For biotinylation of HNP-1, an N-terminally acetylated HNP-1 analog, N-acetyl-A11K-HNP-1, was synthesized, folded, and purified and subsequently reacted through its only amino group at Lys-11 with N-hydroxysulfosuccinimide ester-activated biotin as per the instructions provided by the manufacturer (Pierce/Thermo Scientific). Residue 11 was selected for labeling due to its minimal interference with defensin folding and dimerization, as judged by structural analysis of wild type HNP-1. For biotinylation of Abu-HNP, the linear peptide N-acetyl-A11K-Abu-HNP was used. All peptides were purified by reversed-phase HPLC to homogeneity, and their molecular masses were ascertained by electrospray ionization mass spectrometry.
HIV-1 gp41 Coiled Coil and 6-Helix Bundle Formation Assays—The N36 (HIV-1 gp160(546–581)) and C34 (HIV-1 gp160(628–661)) peptides derived from the N- and C-terminal heptad repeat domains of gp41 were synthesized using the O-benzotriazolo-N,N',N'-tetramethyluronium-hexafluorophosphonate activation/N,N'-disopropylethylamine in situ neutralization protocol developed by Kent and co-workers for N-butoxycarbonyl chemistry solid phase peptide synthesis (46). The N36 peptide contains one Lys residue (Lys-574) and was chosen for covalent attachment of succinimidyl ester-activated carboxyfluorescein (FAM) as per the instructions provided by the manufacturer (Invitrogen). An N-terminally acetylated N36 peptide was prepared for site-specific conjugation of FAM at Lys-574. All peptides were purified by reversed-phase HPLC with 0.5 M NaCl (Invitrogen). Peptide concentration was quantified spectroscopically at 280 nm using molar extinction coefficients calculated according to the algorithm of Pace et al. (47).

Size-exclusion chromatographic analysis of N36, C34, and N36-C34 complexes was performed on a Superdex 75 10/300 GL column (GE Healthcare) running PBS, pH 7.4, at a flow rate of 0.5 mL/min. The peptides were prepared in PBS at 10 μM each, from which 100 μL was injected. Gel filtration molecular weight standards were used for calibration. Fluorescence polarization assays were conducted in 384-well plates on a Tecan Infinite M1000 multimode plate reader. The FAM-labeled N36 peptide at 50 nM in PBS was incubated at room temperature for 30 min with a 2-fold dilution series of unlabeled N36 or N36/C34 (0–25 μM, 100 μL total volume per well), and the polarization values were determined at λex = 470 nm and λem = 530 nm. For defensin-induced N36 dissociation assays, 10 μM N36 and 50 nM N-acetyl-N36-FAM in PBS were incubated at room temperature with a 2-fold dilution series of HNP-1 or Abu-HNP (0–100 μM) for 30 min before polarization measurements.

Peptide precipitation induced by defensin was quantified spectroscopically. Briefly, 40 μM soluble C34 or N36/C34 prepared in PBS was incubated at room temperature for 30 min with a 2-fold dilution series of unlabeled N36 or N36/C34 (0–25 μM, 100 μL total volume per well), and the polarization values were determined at λex = 470 nm and λem = 530 nm. For defensin-induced N36 dissociation assays, 10 μM N36 and 50 nM N-acetyl-N36-FAM in PBS were incubated at room temperature with a 2-fold dilution series of HNP-1 or Abu-HNP (0–100 μM) for 30 min before polarization measurements.

Homogenous virus stocks were produced as above, but using 2 μg of the pCAGGS vectors expressing the WSN H1N1 influenza hemagglutinin and neuraminidase or 3 μg of pMDG vector expressing VSV G instead of HIV-1 Env plasmids. After 12 h, the transfection reagent was removed, and cells were further cultivated in phenol red-free media. Cell culture supernatant was collected at 48 h post-transfection, passed through a 0.45-μm filter, aliquoted, and stored at −80 °C. When required, viruses were concentrated by pelleting onto a 20% sucrose cushion. The virus pellet was resuspended in culture medium and stored at −80 °C in aliquots. The infectious titer of virus stocks was determined using TZM-bl cells, as described previously (34).

Virus-Cell and Cell-Cell Fusion Assays—HIV-1 fusion with target cells was measured using the BlaM assay based on the transfer of viral core-incorporated enzyme to the cytosol (48), as described previously (34). Briefly, HIV-1 pseudoviruses with different coreceptor tropism (HXB2, JRFL, and BaL) were bound to TZM-bl cells by centrifugation at 4 °C for 30 min at 1550 × g. PBMCs were resuspended in Hanks’ buffer and allowed to adhere to a poly-l-lysine-coated 96-well plate (2 × 105 cells/well) for 30 min at room temperature and blocked with 10% FBS-supplemented Hanks’ buffer. HXB2 pseudoviruses (4 × 105 IU/well) were pre-bound to PBMCs by centrifugation at 4 °C for 30 min at 1550 × g. After the virus binding step, TZM-bl cells or PBMCs were washed and incubated at 37 °C for 90 min to allow virus entry. Fusion was stopped by placing cells on ice and loading with the BlaM substrate CCF4-AM (Invitrogen). Following the overnight incubation at 12 °C, the BlaM activity was determined from the ratio of blue and green fluorescence signals, using the Synergy HT fluorescence plate reader (Bio-Tek Instruments, Germany).

Fusion between indicator TZM-bl cells and HeLa-ADA cells was measured using Tat-driven luciferase expression by the indicator cells. HeLa-ADA cells were detached from culture plates using a nonenzymatic cell dissociation solution and added to TZM-bl culture. After coculture for 2.5 h at 22 °C to create temperature-arrested stage (TAS) or at 4 °C in control experiments, cells were exposed to fusion inhibitors or left untreated and shifted to 37 °C for 1 h to initiate fusion. Cell fusion was stopped by adding C52L (5 μL), and cells were incubated in the presence of the inhibitory peptide for 12 h at 37 °C. The extent of fusion was evaluated by a luciferase assay, using Steady-Glo system (Promega, Madison, WI).

CD4 and Coreceptor Expression—Surface expression of CD4 and coreceptors was determined by immunofluorescence staining. For detection of CD4 and CCR5 expression, TZM-bl cells were seeded into a 96-well plate (4.5 × 104 cells/well) on the day before the experiment. Cells were washed and pretreated with 5.8 μM HNP-1 (or Abu-HNP) or left untreated. In control experiments, the CD4 and CXCR4 expression was down-regulated by treatment with 0.2 μg/ml PMA for 30–60 min (49, 50), and CCR5 expression was reduced by treatment with CCR5 agonist, regulated on activation normal T cell expressed and secreted (400 nM). After preincubation with or without defensin, cells were washed with HBSS, 10% FBS and placed on ice. Cells were next washed with PBS supplemented with 0.1% sodium azide and 5% calf serum and incubated with primary antibodies to CD4 (OKT4 conjugated with Alexa Fluor488, Bio-
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Legend), CCR5 (2D7) or with isotype-matched antibodies for 1 h at 4 °C. Cells were washed and fixed with 3% PFA. Cells incubated with unconjugated primary antibodies were further incubated with FITC-labeled secondary antibody for 1 h at 4 °C. Cell surface expression of CXCR4 in TZM-bl cells stably expressing this coreceptor (see above) were analyzed by immunofluorescence with allopurinol-conjugated anti-CXCR4 antibodies (12G5). As a negative control for subtracting the background allopurinol signal, we used anti-CD8-allopurinol antibody. The overall fluorescence signal from cells was measured by a plate reader, using a standard fluorescein filter set. Parental HeLa cells lacking CD4 and CCR5 were used as an additional control for expression of these receptors.

HNP-1 Binding to Cells and Proteins—HNP-1 binding to cells was measured using biotinylated HNP-1 (B-HNP) or its linear analog (Abu-B-HNP). TZM-bl or parental HeLa cells were incubated with B-HNP or its linear analog for 10 min on ice, washed, and fixed with 2% PFA. After blocking with 5% FBS in PBS for 30 min, cells were incubated with streptavidin-HRP at 12 °C for 1 h. The amount of cell-associated B-HNP was measured by cell ELISA using a chromogenic HRP substrate.

HNP-1 binding to the tetrameric CD4-IgG2 protein (51) was carried out by incubating 10 µg of native or deglycosylated CD4-IgG2 with B-HNP or Abu-B-HNP for 1 h at 37 °C. Deglycosylation was carried out using the endoglycosidase mixture from the enzymatic deglycosylation kit (Prozyme) consisting of N-glycanase F (24 microunits/µl), O-glycanase (6 µunits/ µl), and sialidase A (24 microunits/µl) in the final mixture. The reaction was allowed to proceed for 24 h at 37 °C. Mouse IgG2a (BioLegend) was used as a negative control. Defensin binding to HIV-1 Env on virus particles was measured using a concentrated HXB2 pseudovirus stock. Viruses were allowed to adhere to polylysine-coated plates in the cold. Where indicated, viruses were enzymatically deglycosylated (using a 3-fold higher concentration of the enzyme mixture compared with the CD4-IgG2 deglycosylation protocol above) for 24 h at 37 °C before attacking the virus to a plate. Immobilized viruses were incubated with B-HNP or Abu-B-HNP in the presence or absence of 10% FBS for 1 h at 37 °C. Unbound defensin was removed by washing with PBS, and viruses were lysed using 2 mM EDTA and 1% Nonidet P-40 in PBS, pH 7.4, buffer for 30 min at 4 °C. The virus-bound B-HNP was immunoprecipitated with streptavidin beads. Immunoprecipitated proteins were separated by SDS-PAGE under denaturing conditions and detected by immunoblotting with the anti-gp41 Chesso8 monoclonal antibody (38).

Internalization of Endocytic Markers and Viruses—To measure cellular uptake of endocytic markers, TZM-bl cells grown on 96-well plates were preincubated with 80 µM dynasore for 30 min or with 200 µM 5-(N,N-dimethyl)amiloride hydrochloride in HBSS for 1 h at 37 °C or left untreated. Cells were washed and incubated on ice for 20 min with Alexa488-conjugated Trf (20 µg/ml) or Dil-labeled LDL (20 µg/ml). Cells were washed once with HBSS and, where indicated, exposed to 5.8 µM of HNP-1 for 5 min on ice prior to shifting to 37 °C for 10 min to allow Trf uptake and 40 min for LDL uptake in the presence or in absence of HNP-1. Uptake of a fluid-phase marker, dextran-Alexa488 (200 µg/ml), was assessed by adding the dextran to cells just before shifting to 37 °C and incubating for 60 min. Cells were then chilled, washed, and treated with Pronase (2 mg/ml) for 10 min on ice to remove noninternalized markers. Cells were harvested by an additional incubation with trypsin/EDTA (3 min at 37 °C), washed, and analyzed by flow cytometry. HIV-1 uptake by cells was measured as described previously (52, 53). Briefly, pH-sensing pseudoparticles were produced by incorporating the Ecliptic pHluorin-ICAM-1 construct (EcpH-ICAM) into the virus membrane and HIV-1 Gag-mCherry into their core. The EcpH quenching at mildly acidic pH permits monitoring of virus uptake and entry into acidic intracellular compartments.

FRAP and FCS Measurements—Fluorescence recovery after photobleaching (FRAP) was used to assess the changes in the lateral mobility of membrane proteins after HNP-1 treatment. HeLa cells grown on glass-bottom Petri dishes were transfected with plasmids encoding CD4-YFP, CCR5-GFP, or CXCR4-GFP. Twenty four hours post-transfection, cells were transferred into Hank’s buffer, mounted on the Zeiss LSM780 laser scanning confocal microscope, and visualized with the ×63/1.4 NA oil immersion objective. Experiments were carried out at room temperature to minimize cell movement. Flat cells expressing low to moderate amounts of fluorescent proteins localized primarily at the plasma membrane were selected for photobleaching. Measurements were performed using the FRAP module available through the Zen software (Carl Zeiss Microimaging). Small rectangular regions of interest along the cell plasma membrane were bleached by a brief exposure to a 488 laser at 100% intensity. We did not use the conventional circular regions or long strips across the cell (54, 55) for photobleaching to avoid the contribution from an intracellular pool of fluorescent proteins. Instead, we selected small rectangular regions (~1 × 2 µm) encompassing the plasma membrane (see below) and parameterized the fluorescence recovery using the mobile fraction and half-time, as determined by the Zen software.

For FCS (56, 57) measurements, we used a Zeiss LSM780 microscope equipped with hybrid GaAsP detectors and a ConfoCaster ×40 (1.2 NA) water immersion objective. CCR5-GFP was excited with a continuous 488 nm argon laser. Zen software controlled the data acquisition and displayed autocorrelation curves. Collected data were processed off-line using the Origin software package (OriginLab, Northampton, MA). The waist (σw) of the excitation beam was calibrated before experiments by measuring the autocorrelation function of different concentrations of fluorescein in solution (supplemental Fig. 1), using a predetermined diffusion coefficient of 300 µm²/s (58, 59). Typical σw values obtained for our setup were 0.25–0.30 µm. Auto-correlation functions were fitted assuming a two-dimensional Brownian diffusion with fast triplet state kinetics and autofluorescent species exhibiting fast three-dimensional diffusion, as described previously (60) and in Equation 1,

\[ G(\tau) = 1 + \frac{1}{N} \left( F_1 + \frac{1}{\tau_{1o}} \left( 1 + \frac{\omega_x}{\omega_x + \tau_{1o}} \right) \right)^{-1} \]

\[ + (1 - F_1) \left( 1 + \frac{\tau}{\tau_{2o}} \left( 1 + \frac{\omega_y}{\omega_y + \tau_{2o}} \right) \right)^{-1} \left( 1 + \left( \frac{\tau_{1o}}{1 - \tau_{1o}} \right)^{-1} \right) + \text{offset} \]  

where \( N \) is the number of particles; \( F_1 \) is the fraction of three-dimensional species; \( \omega_x \) is the waist of the confocal excitation.
volume; $\omega_2$ is the axial radius of the excitation volume; $\tau_{D1}$ is the residence time for three-dimensional diffusing species; $\tau_{D2}$ is the residence time for two-dimensional species; $T$ is the amplitude of the triplet state, and $t$ is the triplet lifetime. Autocorrelation curves were analyzed assuming a Gaussian-Lorentzian excitation volume. The beam was positioned at the periphery of CCR5-expressing cells, and the average autocorrelation function from 10 measurements with an acquisition time of 5 s each was obtained and analyzed. Abnormal individual traces due to photobleaching or cell movement were not taken into account.

RESULTS

Defensin Inhibits HIV-1 Env-mediated Fusion—We examined the ability of the human neutrophil peptide 1 (HNP-1, hereafter also referred to as defensin) to block HIV-1 fusion, using a direct virus-cell fusion assay based on the cytosolic delivery of the 3-[(4,7-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium assay using Promega CellTiter 96® Aqueous One Solution Cell Proliferation Assay according to the manufacturer’s instructions. Unless stated otherwise, data points are means ± S.E. for at least two independent experiments performed in triplicate. B, titration curves of HNP-1 were obtained in the presence of 10% human (HS) or bovine (BS) serum in Hank’s buffer or in serum-free buffer after incubation of cell-bound viruses with defensin at 37 °C for 90 min. The linear analog of HNP-1 (Abu-HNP, filled circles) was used as a control. Data are means ± S.D. for representative experiments. C, HXB2 pseudoviruses were pre-bound to activated PBMCs adhered to a polylysine-coated 96-well plate by centrifugation in the cold. Virus fusion was initiated by shifting to 37 °C and incubating for 90 min in the absence (control) or in the presence of varied concentrations of HNP-1 in a serum-free medium. In control experiments, 2 μM C52L was added to cells to fully block fusion. The extent of fusion was measured by the BlaM assay. Data for 5.8 μM HNP-1 are from a single experiment performed in triplicate. D, time course of HXB2 pseudovirus escape from fusion inhibitors. Viruses were pre-bound to cells in the cold, and their entry was initiated by shifting to 37 °C. Fully inhibitory concentrations of HNP-1 and inhibitors of CD4-binding (BMS-806), coreceptor-binding (AMD3100), and six-helix bundle formation (C52L) were added at indicated time points. Following the addition of fusion inhibitors, cells were loaded with the BlaM substrate, and the extent of virus-cell fusion was quantified after overnight incubation at 12 °C. Larger error bars reflect experimental variability when using different batches of the virus.
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due to defensin binding to serum glycoproteins (32, 62). In full agreement with these studies, we found that the inhibitory activity of HNP-1 was markedly diminished in media containing human or bovine serum (Fig. 1B). The I_{50} value against HXB2 pseudoviruses increased from 1.2 to \sim 10 \mu M in the presence of bovine serum. For this reason, experiments were conducted in the absence of serum, unless indicated otherwise.

Next, we examined the effect of this defensin on HIV-1 fusion with human PBMCs. As with HeLa-derived target cells, pseudoviruses were pre-bound to PBMCs in the cold, and fusion was initiated by raising the temperature in the presence or in the absence of varied concentrations of HNP-1. HIV-1 fusion with PBMCs was inhibited by this defensin (Fig. 1C), albeit at higher concentrations compared with those inhibiting fusion with TZM-bl cells. Even at this high concentration, HNP-1 did not affect cell viability (data not shown).

HIV-1 entry is a multistep process that progresses through CD4 binding, coreceptor engagement, and endocytosis, which culminates in virus-endosome fusion (34, 35). It has been previously reported that \alpha-defensin inhibits Env-receptor interactions by binding to both CD4 and gp120 and, at the same time, by down-modulating the CD4 expression on the cell surface (25). To assess which step(s) of HIV-1 entry are inhibited by defensin, the time course of virus escape from inhibition by defensin was measured and compared with the rate of escape from inhibitors targeting distinct steps of HIV-cell fusion (35). Fusion was stopped at the indicated time points by adding fully inhibitory concentrations of BMS-806 (to assess the receptor binding (63)). The kinetics of CXCR4 binding was determined by adding a high concentration of AMD3100 (64, 65) or T22 (66). Finally, the recombinant C52L peptide derived from the gp41 second heptad repeat domain was used to monitor the progression through late steps of fusion. C52L inhibits Env-mediated fusion by blocking the formation of the final gp41 6-helix bundle structure (42, 67). Because HIV-1 enters HeLa-derived cells by fusing with endosomes (34), escape from the membrane-impermeant C52L should reflect the kinetics of productive endocytosis and not the kinetics of virus fusion at the cell surface (35).

We have previously found that HXB2 binding to CD4 preceded its binding to CXCR4, which in turn was faster than productive endocytosis (escape from C52L, see Fig. 1D) (35). The time course of HXB2 escape from a fully inhibitory concentration of defensin clearly lagged behind the CD4 and coreceptor binding steps and was close to virus escape from C52L. Similar results were obtained for the R5-tropic HIV-1 pseudoviruses (data not shown). Thus, both C52L and defensin appear to inhibit HIV-1 fusion at the stage just prior to productive endocytosis. It must be stressed, however, that the sustained sensitivity to defensin throughout the surface-accessible stages of fusion does not rule out inhibition of earlier steps of this process by defensin. The time-of-addition method reveals only the latest step of fusion blocked by an inhibitor. To assess the effect of defensin on early steps of HIV-1 entry, we employed alternative strategies described below.

HIV-1 Remains Fusion-competent in the Presence of Defensin—The effect of HNP-1 on the ability of HIV-1 to undergo fusion was tested by pre-exposing the cell-bound virus to defensin for different time intervals, washing with a serum-containing medium, and incubating for 90 min at 37 °C to allow fusion (Fig. 2, diagram). Remarkably, HIV-1 retained the ability to fuse with TZM-bl cells in the presence of a fully inhibitory concentration of defensin for at least 1 h at 37 °C. The extent of HXB2 fusion was only slightly reduced following a prolonged incubation with defensin, whereas the BaL fusion even tended to increase within the first 30 min of incubation (Fig. 2, A–C, open circles). In contrast, pretreatment of cell-bound viruses with concanavalin A resulted in a quick loss of fusion activity (data not shown). A small molecule dynamin inhibitor, dynasore, which blocked HIV-1 endocytosis, also irreversibly inactivated the virus (33). Because infectivity assays widely employed to assess the effect of \alpha-defensins on HIV-1 require returning target cells to a complete growth medium, the inhibitory effect of this defensin on virus entry could be grossly underestimated. Our functional experiments provide the first demonstration of reversibility of the HNP-1 block of HIV-1 fusion.

HIV-1 Env Can Bind CD4 and CCR5 in the Presence of Defensin—To identify the defensin-sensitive step(s) of HIV-1 entry, we examined the ability of the virus to engage CD4 and coreceptors and to undergo fusion in the presence of defensin. Viruses were pre-bound to cells in the cold and incubated with a fully inhibitory concentration of defensin for varied times at 37 °C to allow Env to engage the receptor and coreceptor. HNP-1 was removed by washing with FBS-containing medium, and viruses were further incubated with cells for 90 min at 37 °C in the presence of high doses of HIV-1 fusion inhibitors. Any fusion observed under these conditions could only occur as a result of irreversible engagement of CD4 or both CD4 and coreceptor (depending on the fusion inhibitor employed) by HIV-1 Env in the presence of defensin. As in the time-of-addition experiment (Fig. 1D), Env-CD4 binding was assessed by replacing defensin with BMS-806, whereas the formation of ternary Env-CD4-coreceptor complexes was derived from the extent of fusion after addition of AMD3100 or TAK-779.

As expected, fusion was not detected when HNP-1 was replaced with fusion inhibitors before shifting to 37 °C (Fig. 2, t = 0). In some experiments, however, the inhibition of HXB2 fusion upon replacing defensin with BMS-806 was incomplete, consistent with the limited CD4 engagement at 4 °C, which was also apparent in the time-of-addition experiments (Fig. 1D) (35). Upon incubation with defensin at 37 °C, HXB2 particles efficiently engaged CD4, as evidenced by \sim 60% of fusion occurring upon substituting HNP-1 with BMS-806 compared with control experiments using HBSS instead of the fusion inhibitor (Fig. 2A). However, both the small molecule (AMD3100) and peptide (T22) inhibitors of CXCR4 binding blocked fusion, irrespective of the time of preincubation with defensin (Fig. 2A, squares and upright triangles). Thus, HNP-1 allowed partial formation of HXB2-CM4 complexes but blocked the subsequent binding of CXCR4. Overexpressing CXCR4 in TZM-bl cells did not rescue the ability of HIV-1 to engage this coreceptor in the presence of defensin (supplemental Fig. 2). To conclude, defensin blocks HXB2 entry at the coreceptor binding step while permitting partial engagement of CD4.

Next, we examined the defensin-sensitive stage(s) of fusion induced by the CCR5-tropic BaL pseudovirus. In accordance
with a somewhat lower apparent binding affinity of BaL to CD4 compared with HXB2 (35), only ~20% of viruses engaged CD4 in the presence of defensin (Fig. 2B). However, CCR5 binding was relatively efficient, as evidenced by the comparable extents of fusion in the presence of BMS-806 and TAK-779 after a prolonged incubation with defensin (Fig. 2B, triangles versus squares). Note, however, that the CCR5 binding was delayed relative to CD4 binding, which was in contrast to the fast rate of CCR5 engagement observed in the time of inhibitor addition experiments (35). Delayed CCR5 binding could be indicative of competition between the virus and defensin for coreceptor binding.

To further assess whether defensin selectively interferes with Env-CXCR4 interactions, we examined the fusion activity of the chimeric HXB2 Env, in which the gp120 V3-loop was substituted by that of BaL (designated V3BaL) (68). This chimera uses CCR5 for infection and fusion and binds this coreceptor with a higher affinity compared with the HXB2-CXCR4 binding (68, 69). V3BaL engaged both CD4 and CCR5 in the presence of HNP-1 (Fig. 2C). Nearly 60% of viruses that formed functional complexes with CD4 also bound CCR5 (Fig. 2C, triangles versus squares), but defensin markedly slowed down the CCR5 binding step compared with experiments in the absence of HNP-1 (35). Taken together, our findings show that HNP-1
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TABLE 1

Effect of HNP-1 on expression of CD4, CXCR4, and CCR5 on TZM-bl and TZM-CXCR4 cells

Surface expression of CD4 and coreceptors was measured as described under “Experimental Procedures” using a fluorescence plate reader after 45 min (CD4) or 60 min (coreceptors) of HNP-1 pretreatment (5.8 μM) at 37 °C. In control experiments, cells were pretreated with 0.2 μg/ml PMA. Data are means ± S.E. of triplicate measurements.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD4 (% of untreated)</th>
<th>CXCR4 a (% of untreated)</th>
<th>CCR5 (% of untreated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNP-1</td>
<td>28.6 ± 3.3 (n = 3)</td>
<td>22.1 ± 6.8 (n = 6)</td>
<td>93.7 ± 0.8 (n = 3)</td>
</tr>
<tr>
<td>PMA or RANTESb</td>
<td>23.4 ± 6.7 (n = 3)</td>
<td>57.9 ± 5.3 (n = 5)</td>
<td>64.3 ± 0.8 (n = 3)</td>
</tr>
</tbody>
</table>

a Effect of defensin on the CXCR4 expression was determined on TZM-bl cells overexpressing this coreceptor (see supplemental Fig. 2).

b Cells were preincubated with 400 nM regulated on activation normal T cell expressed and secreted (RANTES) for 60 min.

targets both CD4 and coreceptor binding steps. It slows down high affinity interactions (Bal-CCR5 binding), while attenuating or even blocking weak interactions (Bal-CD4 and HXB2-CXCR4 binding).

The block of Bal. fusion, despite its limited binding to CD4 and CCR5, shows that defensin also targets downstream steps of virus entry. Indeed, following a 1-h incubation with HNP-1, all pseudoviruses tested in our experiments remained sensitive to C52L (Fig. 2, A–C, diamonds). The lack of resistance to this gp41 6-helix bundle inhibitor suggests that all fusion-competent viruses remained at the cell surface, accessible to C52L. In other words, defensin appears to prevent HIV-1 endocytosis without significantly inactivating the virus. To gain further insights into the mechanism of HNP-1 action, we assessed its effect on the surface expression of CD4/coreceptors, as well as on the late steps of HIV-1 entry and fusion.

HNP-1 Diminishes Cell Surface Expression of CD4 and CXCR4 but Not CCR5—The reduced or delayed virus binding to CD4 and CCR5 and failure to engage CXCR4 in the presence of defensin (Fig. 2) could be due, at least in part, to induction of CD4 and/or coreceptor endocytosis. For PBMCs and HOS-derived cells engineered to express CD4 and coreceptors, prolonged incubation with HNP-1 or other defensins has been reported to slightly down-modulate the CXCR4 but not CD4 or CCR5 expression (17). In contrast, another study (25) has found that HNP-1 and HNP-2 down-regulate the CD4 coreceptor, but not CCR5 or CXCR4 expression in PM-1 cells. We therefore sought to determine whether HNP-1 reduced the surface levels of CD4 or coreceptors in TZM-bl cells on a time scale relevant to HIV-1 entry.

Attempts to measure expression levels by flow cytometry were not successful, because, surprisingly, defensin strengthened the cell attachment to plates. We thus had difficulty harvesting defensin-treated TZM-bl cells with a nonenzymatic solution without damaging them. For this reason, surface expression of CD4/coreceptors was measured using a fluorescence plate reader. To rule out competition between defensin and antibodies for binding to CD4, we used the monoclonal antibody OKT4 against the membrane-proximal domain of CD4, because binding of this antibody to CD4 is not affected by α-defensins (25). We found that HNP-1, but not Abu-HNP, quickly (half-time ~18 min) decreased the CD4 expression (Fig. 2D and Table 1). The defensin effect on CXCR4 expression was determined using TZM-CXCR4 cells overexpressing this coreceptor. Pretreatment with HNP-1 or PMA (as a positive control (50)) markedly reduced the surface density of this coreceptor (Table 1). Down-regulation of CXCR4 expression may be the reason for the block of HIV-CXCR4 binding by defensin (Fig. 2A). By comparison, the CCR5 expression was not affected by defensin, whereas the CCR5 agonist, regulated on activation normal T cell expressed and secreted (RANTES) for 60 min.

It is striking that HXB2 and, to some extent, Bal. were able to engage a requisite number of CD4 and undergo fusion (Fig. 2, A–C), despite rapid CD4 clearance from the cell surface (Fig. 2D). The HIV-CD4 binding in the presence of defensin likely occurs in kinetic competition with receptor endocytosis. The greater extent of CD4 engagement by HXB2 compared with Bal. is consistent with the faster receptor binding by the former Env, as measured by the rate of virus escape from fusion inhibitors (Fig. 2D) (35). To conclude, defensin inhibits HIV-1 binding to CD4 and CXCR4 through down-regulating their expression and potentially through interfering with Env-coreceptor interactions, as suggested by delayed CCR5 engagement by Env in the presence of HNP-1 (Fig. 2, B and C).

HNP-1 Binds to Viral and Host Glycoproteins in Glycan- and Serum-independent Manner—We measured the defensin binding to cells and viruses using B-HNP. In control experiments, B-HNP inhibited HIV fusion even somewhat more efficiently than wild type defensin, and its activity was grossly diminished in the presence of serum (Fig. 3A). To measure defensin binding to cells, TZM-bl cells were incubated with B-HNP, washed with serum-containing medium to remove unbound peptide, fixed, and incubated with streptavidin-HRP. The amount of cell-associated B-HNP was determined by a cell-based ELISA. Although the fusion inhibitory effect of defensin was strongly attenuated by serum (Figs. 1B and 3A), B-HNP bound to cells equally well in the presence and in the absence of serum (Fig. 3B). Once bound, B-HNP could not be removed from cells by multiple washes with serum-supplemented medium. By comparison, the linear biotinylated defensin (designated Abu-B-HNP) bound poorly to cells even in serum-free medium (Fig. 3C). This demonstrates that the structure of the defensin is essential for binding to cells and that biotinylation does not significantly alter this activity. The overall binding of B-HNP was not affected by expression of CD4 and CCR5, as evidenced by its equal association with TZM-bl and parental HeLa cells (Fig. 3C). The B-HNP-cell binding appeared specific, because it was saturable (Fig. 3C) and could be displaced by an excess of nonbiotinylated HNP-1 but not by Abu-HNP (Fig. 3D).

The fact that the B-HNP binding to cells is strikingly different from its reversible effect on virus fusion (Figs. 1 and 2) implies that this defensin either interacts with cell surface molecules other than CD4 or coreceptors or its binding is not sufficient for inhibition of HIV-1 entry. We therefore tested whether defen-
sin binds to CD4 and gp41 in the presence of serum and whether this binding was glycan-dependent, as has been shown for another defensin, retrocyclin (21, 22). First, we examined binding to the recombinant tetrameric CD4-IgG2 protein containing the N-terminal D1 and D2 domains of CD4 (51), of which the first domain interacts with HIV-1 gp120 (70). Although D1-D2 domains of CD4 are not glycosylated (51), we nonetheless tested the B-HNP binding to both untreated and enzymatically deglycosylated CD4-IgG2. As expected, no significant shift in the mobility of the recombinant protein could be observed following deglycosylation (Fig. 4A). The B-HNP binding to CD4-IgG2 was then assessed by immunoprecipitation with streptavidin-agarose beads followed by immunoblotting. The ability of defensin to effectively immunoprecipitate both untreated and enzymatically deglycosylated CD4-IgG2. As expected, no significant shift in the mobility of the recombinant protein could be observed following deglycosylation (Fig. 4A). The B-HNP binding to CD4-IgG2 was then assessed by immunoprecipitation with streptavidin-agarose beads followed by immunoblotting. The ability of defensin to effectively immunoprecipitate both untreated and enzymatically deglycosylated CD4-IgG2 (Fig. 4A) demonstrated that its binding to the N-terminal domains of CD4 was glycan-independent.

Next, we examined the defensin binding to a native or enzymatically deglycosylated HIV-1 Env on virus particles. The appearance of a lower band (~37 kDa) on Western blots following a prolonged enzymatic treatment implies that a fraction of gp41 was fully deglycosylated (Fig. 4B, lanes 4 and 6). To measure Env-HNP binding, viruses (treated or untreated) were immobilized on microplate wells, incubated with B-HNP, washed, and lysed. Defensin-Env complexes were precipitated with streptavidin beads and subjected to SDS-PAGE. Blotting with an anti-gp41 antibody revealed that HNP-1 efficiently bound glycan-free gp41, as evidenced by similar relative intensities of upper and lower bands in lysate and in immunoprecipitated samples (Fig. 4B, lanes 4 and 6). In control experiments, linear Abu-B-HNP poorly precipitated either native or glycan-free Env (Fig. 4B, lanes 7–9). Interestingly, a combination of gp41 deglycosylation with the defensin pulldown in the presence of serum enhanced the band intensity for both HNP-1 and Abu-HNP samples (Fig. 4B, lanes 6 and 10). This effect could be due to the better binding of the Chessie8 antibody to gp41. Together, these data imply that glycans are not essential for HNP-1 binding to gp41 and CD4 and that serum does not interfere with defensin-gp41 interactions.

The lack of efficient inhibition of HIV-1 fusion by defensins in the presence of serum has been thought to reflect diminished binding to relevant targets. Our results revealed a striking dis-
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HNP-1 Reduces the Mobile Fraction of CD4 and Coreceptors on the Cell Surface—We next asked whether defensin could inhibit HIV-1 fusion by cross-linking the cell surface glycoproteins. Cross-linking would “freeze” glycoproteins on the cell surface and interfere with the virus fusion (22). To test this notion, we measured the mobility of proteins on the cell surface by FRAP (55, 72). Lateral diffusion of cross-linked membrane proteins is slower and/or more restricted than that of free proteins. For these experiments, fluorescently labeled CD4 or coreceptors were individually expressed in HeLa cells by transient transfection. Small rectangular regions along the plasma membrane (Fig. 5A) were photobleached, and fluorescence recovery over time was monitored. The mobile fraction of fluorophores and the half-time of fluorescence recovery (Table 2) were derived from the experimental curves as described previously (54). Because of the CD4 and CXCR4 down-regulation by HNP-1 (Table 1), incubation with defensin was carried out at room temperature (to slow down endocytosis) and was limited to 20–25 min. We found that concanavalin A diminished the rate of fluorescence recovery (Fig. 5B). By contrast, defensin significantly reduced the mobile fraction of CD4 and both coreceptors, consistent with its ability to cross-link a fraction of these glycoproteins, but it did not reduce their lateral diffusion. It is difficult to assess whether the modest reduction of the mobile fraction of CD4 and coreceptors in the absence of significant mobility changes could account for the potent inhibition of HIV-1 fusion by this defensin.

To more quantitatively assess the effect of HNP-1 on mobility of membrane proteins, we employed FCS (56, 57), which measures diffusion coefficients/residence time and the concentration of a fluorophore based on fluctuations of fluorescence intensity in a small confocal volume. The 488 nm laser beam was parked at selected locations on the plasma membrane of TZM-b1 cells transiently expressing CCR5-GFP (Fig. 5C), and the resulting fluorescence signal was acquired and analyzed. The decay of the autocorrelation functions (Fig. 5, D–F) was best fitted with a two-component model (described under “Experimental Procedures”) accounting to fast three-dimensional diffusion in the cytosol (an average residence time of ~200 μs) and much slower lateral diffusion within the plasma membrane (an average residence time of ~30 ms). In agreement with the FRAP data, preincubation with HNP-1 did not significantly alter the lateral mobility of this coreceptor (Fig. 5, D and F). As expected, mild fixation of cells with PFA markedly diminished the CCR5-GFP mobility, as evidenced by a much higher residence time following the fixation step (Fig. 5E). The FCS data are summarized in Table 3. To conclude, both FCS and FRAP measurements showed that HNP-1 did not significantly alter the mobility of CD4 or coreceptors, while reducing the mobile fraction of these proteins. The latter effect is consistent with clustering and/or immobilization of a fraction of CD4 and coreceptor molecules on the cell surface.

Defensin Inhibits HIV-1 Fusion at a Post-coreceptor Binding Stage—Having demonstrated that HNP-1 down-regulates CD4 and CXCR4 expression and slows down Env-CXCR4 binding (Fig. 2, B and C), we sought to determine whether this peptide can also act downstream of the ternary Env-CD4-CXCR4 complex formation, as suggested by the time-of-addition data (Fig. 1D). Toward this goal, a temperature-arrested stage (TAS) was created by pre-binding the virus to cells in the cold and incubating at a temperature just below the threshold for fusion (33, 34). HIV-1 Env-pseudoviruses were bound to TZM-b1 cells in the cold and incubated at 23 or 4 °C (control samples) for 3 h.
The formation of ternary Env-CD4-coreceptor complexes at TAS was ascertained based on the acquisition of resistance to high concentrations of CD4 and coreceptor binding inhibitors (40 μM BMS-806 and 10 μM AMD3100, respectively) upon shifting cells to 37 °C. Under these conditions, any fusion signal (measured by the BlaM assay) must originate from viruses that have formed functional ternary complexes with CD4 and coreceptors.

Fig. 6A shows that preincubation at 23 °C rendered a fraction of viruses resistant to receptor and coreceptor binding inhibitors added prior to raising the temperature. In control experiments, when viruses and cells were preincubated at 4 °C for 3 h, a partial protection against BMS-806 inhibition was observed, consistent with the ability of Env to slowly bind CD4 in the cold (Figs. 1D and 2D) (33–35). The cold-incubated virus remained fully sensitive to AMD3100. By contrast, almost half of the fusion-competent viruses engaged both CD4 and CXCR4 at TAS, as evidenced by considerable fusion in the presence of AMD3100. The nearly complete inhibition of fusion by CS2L added at TAS rules out the possibility that viruses were pro-

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**FIGURE 5. Effect of HNP-1 on the lateral mobility of CD4 and coreceptors.** A, image illustrating the FRAP measurements for HeLa cells transfected with CCR5-GFP. B, CCR5-GFP fluorescence recovery for untreated (black line), as well as HNP-1-treated (red line) and concanavalin A-treated (dotted line) cells. Determination of the mobile fraction and t₁/₂ (shown for untreated cells) is illustrated. C–F, FCS measurements of CCR5-GFP mobility in the plasma membrane. C, confocal image of a TZM-bl cell expressing CCR5-GFP merged with the differential interference contrast channel. The white cross marks the position of the laser beam during one of the FCS measurements. D, normalized autocorrelation curve (green dots) for control cells. The characteristic residence times determined by fitting (solid line) are t₁ = 200 μs and t₂ = 30 ms (assuming a Gaussian volume). E, normalized autocorrelation curve (red dots) for cells pretreated with PFA (0.8%). Optimal fitting (solid line) was obtained for t₁ = 400 μs and t₂ = 403 ms. F, normalized autocorrelation curve (black dots) for cells pretreated with 8.7 μM HNP-1 for 30 min at room temperature. Two characteristic residence times, t₁ = 270 μs and t₂ = 34 ms, were obtained by curve fitting (solid line). Insets to D–F show the curve fitting residuals.
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TABLE 2
Lateral mobility of CD4 and coreceptors in the presence of HNP-1 or concanavalin A (ConA) determined by FRAP

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>M.F.*</th>
<th>t½</th>
<th>ConA</th>
<th>M.F.</th>
<th>t½</th>
<th>HNP-1</th>
<th>M.F.</th>
<th>t½</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4-YFP</td>
<td>54.5 ± 5.2</td>
<td>28.3 ± 4.6</td>
<td>ND</td>
<td>26.7 ± 2.1e</td>
<td>50.0 ± 13.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 4)</td>
<td></td>
<td></td>
<td></td>
<td>(n = 4)</td>
<td></td>
<td></td>
<td>(n = 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR5-GFP</td>
<td>74.6 ± 6.0</td>
<td>18.4 ± 1.1</td>
<td>59.3 ± 4.0</td>
<td>26.1 ± 3.3e</td>
<td>17.5 ± 1.4</td>
<td></td>
<td></td>
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<tr>
<td>(n = 11)</td>
<td></td>
<td></td>
<td></td>
<td>(n = 6)</td>
<td></td>
<td></td>
<td>(n = 22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR4-GFP</td>
<td>49.3 ± 6.2</td>
<td>36.8 ± 4.5</td>
<td>ND</td>
<td>23.7 ± 2.6e</td>
<td>38.8 ± 5.7</td>
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<tr>
<td>(n = 8)</td>
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<td>(n = 13)</td>
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</table>

* M.F. means mobile fraction.
ND means not determined.
Statistically significant difference (p < 0.05) was found. Data are means ± S.E.

TABLE 3
FCS analysis of HNP-1 effect on the CCR5-GFP mobility

A two-component model based on three- and two-dimensional Gaussian diffusion was used to fit the experimental curves. Means ± S.E. are from five independent experiments with TZM-bl cells, whose surface was coated with Fluorescein Membrane Probes (FMP).

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>HNP-1</th>
<th>FCS</th>
<th>PFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>τc (μs) fast component</td>
<td>211 ± 39</td>
<td>249 ± 53</td>
<td>460 ± 100</td>
<td></td>
</tr>
<tr>
<td>τc (ms) slow component</td>
<td>20 ± 9</td>
<td>33 ± 3</td>
<td>244 ± 92</td>
<td></td>
</tr>
<tr>
<td>F1 (%) fast component</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>F2 (%) slow component</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

Collectively, our data imply that defensin not only interferes with both CD4 and/or coreceptor binding steps, but also targets downstream steps of HIV-1 fusion. The ability to inhibit cell-cell fusion from TAS and to bind gp41 (Fig. 4B) suggests that α-defensin may interfere with the gp41 refolding into the final 6-helix bundle in a manner similar to the θ-defensin, reticocyclin (23).

HNP-1 Inhibits Trimerization of the gp41 N-terminal Coiled Coil Domain and Binds to Both the C-terminal Domain and the 6-Helix Bundle Structure—We first examined the ability of defensin to bind to the heptad repeat domains of gp41 that compose the final 6-helix bundle structure. The N36 and C34 peptides derived from the N- and C-terminal heptad repeat domains of gp41, when mixed at a 1:1 molar ratio, form the stable post-fusion core, where three helices of N36 associate into the central coiled coil with hydrophobic grooves on its surface occupied by three helices of C34 in an antiparallel fashion (for review see Ref. 67). Consistent with these structural features, an equal molar (10 μM) mixture of synthetic N36 and C34 peptides eluted predominantly as a hexamer on size-exclusion chromatography (Fig. 7A). By contrast, whereas N36 had a tendency to trimerize by itself, C34 existed only as a monomer at the same concentration in solution (Fig. 7A). Concentration-dependent N36 trimerization and N36/C34 formation of the 6-helix bundle structure were independently verified by fluorescence polarization studies, using a FAM-labeled N36 peptide as a fluorescent indicator (Fig. 7B).

To assess the ability of defensin to interfere with gp41 refolding into the 6-helix bundle structure, we incubated different concentrations of HNP-1 or Abu-HNP with trimeric N36 laced with the fluorescent indicator peptide N-acetyl-N36-FAM. As the HNP-1 concentration increased from 0 to 100 μM, fluorescence polarization decreased in a sigmoidal manner from 320.5 to 254.5 milli-polarizations (Fig. 7C), indicative of an HNP-1-induced dissociation of N36 trimers. By contrast, Abu-HNP caused a marginal decrease in fluorescence polarization of 13 milli-polarizations, suggesting that destabilization of the N36 trimer by defensin was structure-dependent, as is the case with many other functionalities of HNP-1 (13). Importantly, incubation of HNP-1 at different concentrations with either C34 or N36/C34 triggered a defensin dose-dependent aggregation of the virus particle(s), as analyzed by UV-visible spectrophotometry and CD spectroscopy (Fig. 7D and E). The tertiary structure of HNP-1 was also required for the induction of C34 and N36/C34 aggregation in solution as Abu-HNP had no effect on the solubility of C34 and N36/C34 peptides. Taken together, these results strongly indicate that HNP-1 is capable of interacting with both N36 and C34 peptides as well as the N36/C34 bundle structure, thus contributing to the inhibition of fusion, presumably by interfering with the formation of the fusogenic gp41 structure.

Defensin Blocks HIV-1 Uptake without Interfering with Internalization of Endocytic Markers—The sensitivity of HIV-1 to inhibition by C52L after preincubation with defensin (Fig. 2) shows that fusion-competent viruses remained at the cell surface, accessible to the 6-helix bundle inhibitor. This finding implies that HNP-1 can inhibit HIV-1 entry and fusion, at least in part, by blocking the virus endocytosis. To test whether defensin prevented the virus uptake, we employed the fluorescence quenching assay previously developed by our group (52, 53). This assay employs double-labeled pseudoviruses containing HIV-1 Gag-mCherry and carrying a pH-sensitive GFP variant, referred to as EcpH (75), in their membrane. EcpH, which is anchored to the virus membrane by the ICAM-1 transmembrane domain, is virtually nonfluorescent at pH < 6.0 (53, 75). The pH-insensitive mCherry within the virus interior serves as
Defensin Blocks HIV-1 Endocytosis and Key Steps of Fusion

FIGURE 6. HNP-1 inhibits fusion downstream of Env-CD4-coreceptor complex formation. A, a fusion of HXB2 pseudoviruses with TZM-bl cells was monitored after creating a TAS by incubating viruses with TZM-bl cells at 23 or at 4 °C (control samples) for 3 h. Fusion inhibitors (40 μM BMS-806; 10 μM AMD3100; 5.8 μM HNP-1, and 1 μM C52L) were added to cells at TAS and incubated for 5 min prior to raising the temperature to 37 °C for 1 h. The resulting virus-cell fusion was measured by the BlaM assay. Engagement of CD4 and coreceptors at TAS was manifested by resistance of fusion to CD4 and coreceptor binding inhibitors (BMS-806 and AMD3100, respectively). Data are means ± S.E for a representative experiment performed in triplicate. B, HeLa cells constitutively expressing HIV-1 ADA Env and Tat were mixed with indicator TZM-bl cells and preincubated for 2.5 h at 22 °C to create TAS. BMS-806 and AD101 were added to cells together with HNP-1 and C52L at TAS and incubated for 2.5 h at 22 °C to create TAS. AD101-resistant fusion observed after creating TAS was completely blocked by 5.8 μM HNP-1 or 1 μM C52L (last two columns). In these experiments, HNP-1 or C52L were added together with AD101 just prior to raising the temperature from TAS.

Inhibition of HIV-1 uptake prompted us to assess the ability of defensin to interfere with constitutive endocytosis in HeLa-derived cells. Uptake of fluorescently labeled markers of clathrin-dependent endocytosis in HeLa-derived cells. The uptake of a fluid-phase marker, dextran-Alexa488, was measured by flow cytometry. Cells incubated at 4 °C or pretreated with 80 μM dynasore, a small molecule inhibitor of dynamin (76), served as negative controls. We found that Trf and LDL were effectively internalized in the presence of defensin, whereas their uptake was markedly diminished by dynasore (Table 4). In fact, endocytosis of Trf was even somewhat more efficient in the presence of defensin, apparently due to its enhanced binding to cells. We also found that endocytosis of a fluid-phase marker, dextran-Alexa488, was slightly enhanced in the presence of defensin. These results demonstrate the previously unappreciated ability of α-defensin to selectively block HIV-1 and VSV uptake without inhibiting the endocytic activity of the cell.

DISCUSSION

Although the anti-HIV-1 activity of HNP-1 is well documented, the mechanism by which this peptide blocks virus entry and replication remained poorly characterized. Here, we dissected the effects of α-defensin on key steps of HIV-1 entry and fusion and demonstrated that this peptide targets all major steps of the process. Key novel findings of this study include the
ability of HNP-1 to: (i) interfere with functional engagement of CD4 and coreceptors by fusion-competent Env; (ii) reduce the mobile fraction of these receptors in the plasma membrane; (iii) bind to gp41 and host proteins in glycan- and serum-independent manner; (iv) block post-coreceptor binding steps of fusion; and (v) inhibit HIV-1 and VSV uptake, but not constitutive endocytosis. Finally, the major new finding of this study is the lack of correlation between the binding and the fusion inhibitory activity of defensin.

It should be pointed out that another group has not observed significant inhibition of HIV-1 fusion with PBMCs by HNP-1 (17). The lack of effect on fusion is likely due to a lower concentration of defensin used in these studies compared with those employed in our experiments (Fig. 1C). Another study reported strong promotion of HIV-1 infection when the virus was pre-treated with HNP-1 (29). Here, we avoided complications arising from the effects of HNP-1 on the virus attachment step by pre-binding the virus to cells prior to adding defensin.

The observation that HNP-1 binds to the nonglycosylated N-terminal domains of CD4 and to deglycosylated trimeric Env on viruses is in contrast to the previous report of glycan- and serum-dependent binding to monomeric gp120 (4). On the other hand, our data are consistent with the studies showing that α-defensin does not compete with the broadly neutralizing
monoclonal antibody 2G12 for binding to a mannose-rich cluster on gp120 (25). Also, the ability of \( \text{H9251} \)-defensins to compete with antibodies against the N-terminal domain of CD4 (25) lacking sugar residues (51) supports the notion of glycan-independent binding. Interestingly, the retrocyclin-resistant HIV-1 BaL escape mutant acquired three mutations, all of which were substitutions for positively charged amino acids (24). These results indicate that retrocyclin may also interact with Env electrostatically, perhaps in addition to glycan binding. Finally, HNP-1 is a potent ligand of many bacterial toxins that are nonglycosylated.

Although HNP-1 can bind to glycan-free proteins, glycan binding could be essential for cross-linking cell surface glycoproteins in a manner similar to retrocyclin (22). Such cross-linking could be responsible for inhibition of virus endocytosis (Figs. 5 and 8). The ability of HNP-1 to reduce the mobile fraction of CD4 and coreceptors and the lack of correlation between binding to viral/cellular targets and fusion inhibitory activity (Figs. 1–4) are consistent with cross-linking as a prerequisite for anti-HIV-1 activity. We showed that HNP-1 selectively inhibits internalization of HIV-1 Env and VSV pseudotypes, while allowing constitutive endocytosis. Notably, retrocyclin also does not inhibit transferrin uptake (22), but its effect on virus endocytosis has not been explicitly tested. Our results imply that interference with virus internalization could be a major mechanism by which HNP-1 blocks fusion of viruses that enter cells via endocytosis (33, 34). Influenza virus appears to be an exception, because HNP-1 blocks its fusion at a post-inter-
Defensin Blocks HIV-1 Endocytosis and Key Steps of Fusion

**TABLE 4**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Transferrin (of untreated)</th>
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Normalisation step (Fig. 8, E and F, and supplemental Fig. 3C). Similarly, defensins do not inhibit the binding or uptake of nonenveloped viruses but prevent their escape from endosomes, which results in virus accumulation within these compartments (9, 77). The selective block of HIV-1 uptake by α-defensin is proof-of-principle for novel approaches to fight infection. We hypothesize that this unique feature of HNP-1 (and likely of other α-defensins) could potentiate the antiviral activity of the existing fusion inhibitors as well as of neutralizing antibodies.

Although broad anti-microbial activity of defensins is incompatible with specific high affinity interactions with diverse targets, defensins appear to bind glycan moieties of proteins with a relatively high affinity (4). Our data reveal that HNP-1 bound relatively tightly to key players in HIV-1 entry and fusion (Env, CD4, and coreceptors). More strikingly, we found that HNP-1 bound to cellular and virus targets in a glycan- and serum-independent manner, which is in contrast with its markedly attenuated inhibition of HIV-1 fusion in the presence of serum. This novel observation shows that the defensin binding to respective targets is necessary but not sufficient for blocking HIV-1 fusion and that an additional step, perhaps defensin oligomerization (71), must occur to inhibit virus entry/fusion. Our findings are entirely consistent with a growing consensus that the ability of HNP-1 to dimerize, oligomerize, and multimerize upon target binding endows this α-defensin with additional molecular complexity and structural diversity necessary for its functional versatility against a broad array of bacterial, viral, and cellular targets (13).

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18. Deleted in proof
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6381–6388


Supplementary Figure Legends

Suppl. Figure 1. Calibration of the confocal volume by FCS and RICS measurements. (A) The autocorrelation curves were obtained from 4 different fluorescein solutions, 7 nM (blue circles), 3.5 nM (yellow circles), 1.25 nM (green circles) and 0.7 nM (red circles). Curve fitting (solid lines) was performed using a single component model:

\[
G(\tau) = 1 + N_{\text{eff}}^{-1} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + S^2 \frac{\tau}{\tau_D}\right)^{-1/2}
\]

Here, the known parameters, \(N_{\text{eff}}\) (number of particles) and \(\tau_D\) (residence time) (1,2), were used to determine \(S\) (the structural parameter, which equal to waist (\(\omega_0\))/axial radius (\(z\))) by curve fitting. (B) Results for the waist and axial focal radius corresponding to the values obtained by fitting each of the autocorrelation curves in panel A. These measurements produced average values \(\omega_0 = 0.29\ \mu\text{m}\) and \(z = 1.5\ \mu\text{m}\). (C, D) Raster Scan Correlation Spectroscopy (RICS) analysis (3) was applied to a 7 nM fluorescein solution, yielding the same waist radius \(\omega_0 = 0.29\ \mu\text{m}\) and the axial focal radius \(z = 1.5\ \mu\text{m}\). For the RICS analysis, we took 100 images with a scan speed of 4 \(\mu\text{m}/\text{pixel}\) and a pixel size of 60 nm as suggested in (4).

Suppl. Figure 2. Overexpression of CXCR4 on TZM-bl cells and effect of HNP-1 on HXB2 binding to CXCR4. TZM-bl cell clones stably overexpressing CXCR4 were obtained by transduction with a retroviral pBABE vector bearing CXCR4 gene, as described in (5). (A) Cells were detached from culture dishes by non-enzymatic cell dissociation solution, incubated with the APC-conjugated anti-CXCR4 antibody 12G5 (eBioscience, San Diego, CA) for 1 h at 4°C, washed and analyzed by flow cytometry. (B) Analysis of HXB2 fusion with TZM-bl-derived cells overexpressing CXCR4. HXB2 pseudoviruses were pre-bound to cells in the cold and incubated at 37°C in the presence of 5.8 \(\mu\text{M}\) HNP-1. After varied times of incubation, cells were washed with a serum-supplemented Hank’s buffer and overlaid with the same buffer lacking or containing HIV-1 fusion inhibitors, BMS-806, AMD3100 or C52L. Cells were incubated for additional 90 min at 37°C, and the resulting fusion was measured by the BlaM assay.

Suppl. Figure 3. Inhibition of virus endocytosis by HNP-1. (A) A wide-field fluorescence images of TZB-bl cells with bound HXB2 pseudoviruses co-labeled with EcpH-ICAM (green) and Gag-Cherry (red). Viruses were pre-bound to cells in the cold and their internalization was triggered by shifting to 37°C for 60 min in the absence or in the presence of 5.8 \(\mu\text{M}\) of HNP-1 in serum-free medium. (B) Analysis of the EcpH/Cherry signal ratio over time for control cells and cells in the presence of defensin shown in panel A. (C) Images of the influenza virus (PR8 strain) labeled with the membrane dye DiD (red), as described in (6). The TZM-bl cell membrane was labeled with wheat germ agglutinin-Alexa488 (green). Whereas viruses were located peripherally before incubation at 37°C, most particles appear to reside in the cytoplasm, following incubation with or without HNP-1 (8.7 \(\mu\text{M}\)) at 37°C.

Supplemental References:

### Table 1

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### Table 2

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**Suppl. Fig. 1**
Suppl. Fig. 3

A

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B

Green/Red ratio vs. Time (min)

Control vs. +HNP-1

C

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