Retrocyclin-1, a $\theta$-defensin, protects target cells from human immunodeficiency virus, type 1 (HIV-1) by preventing viral entry. To delineate its mechanism, we conducted fusion assays between susceptible target cells and effector cells that expressed HIV-1 Env. Retrocyclin-1 (4 $\mu$m) completely blocked fusion mediated by HIV-1 Env that used CXCR4 or CCR5 but had little effect on cell fusion mediated by HIV-2 and simian immunodeficiency virus Env. Retrocyclin-1 inhibited HIV-1 Env-mediated fusion without impairing the lateral mobility of CD4, and it inhibited the fusion of CD4-deficient cells with cells bearing CD4-independent HIV-1 Env. Thus, it could act without cross-linking membrane proteins or inhibiting gp120-CD4 interactions. Retrocyclin-1 acted late in the HIV-1 Env fusion cascade but prior to 6-helix bundle formation. Surface plasmon resonance experiments revealed that retrocyclin bound the ectodomain of gp41 with high affinity in a glycain-independent manner and that it bound selectively to the gp41 C-terminal heptad repeat. Native-PAGE, enzyme-linked immunosorbent assay, and CD spectroscopic analyses all revealed that retrocyclin-1 prevented 6-helix bundle formation. This mode of action, although novel for an innate effector molecule, resembles the mechanism of peptidic entry inhibitors based on portions of the gp41 sequence.

Three structurally distinct subfamilies of defensins, $\alpha$, $\beta$, and $\theta$, exist in primates (1). $\theta$-Defensins, the smallest of these and the only known cyclic peptides of animal origin, contain only 18 residues (2). Three $\theta$-defensin peptides have been isolated from rhesus macaque leukocytes (3) and bone marrow (4), and intact $\theta$-defensin genes and expressed $\theta$-defensin mRNA transcripts in bone marrow. However, these genes and transcripts harbor a premature stop codon, and neither humans nor their closest primate relatives (chimpanzees and gorillas) produce $\theta$-defensin peptides (5, 6).

Retrocyclin-1 (RC-1), a synthetic cyclic octadecapeptide, represents a $\theta$-defensin peptide that humans could produce if the corresponding gene had not been silenced by mutation. Retrocyclins and other $\theta$-defensins exert broad spectrum antiviral properties in vitro and can protect cells from infection by HIV-1 (6–9), herpes simplex (10), and influenza A viruses (11).

HIV enters a target cell after its gp120/gp41 glycoprotein (Env) binds CD4 (12) and a co-receptor, CCR5 or CXCR4 (13). The ensuing conformational changes result in a 6-helix bundle (6HB) core structure wherein three N-helical regions (N-HR) and three C-helical regions (C-HR) and drive membrane fusion (14–17). Peptides that mimic N-HR or C-HR inhibit fusion by binding their opposite counterpart and preventing 6HB formation (18–26). Other HIV-1 entry inhibitors also exist and act by inhibiting CD4 binding, co-receptor engagement, or affecting membrane organization (27, 28).

Retrocyclin-1 has lectin-like properties and binds glycosylated molecules such as CD4, HIV gp120, and galactosylceramide with high ($K_d$ = 20–200 nm) affinity (29). Under certain conditions, this allows retrocyclins to prevent viral entry by cross-linking cell surface molecules, as recently shown for influenza A (11). Despite its activity against HIV-1, retrocyclin-1 is considerably less effective against HIV-2 or SIV. Because the cross-linked barrier mechanism would not explain such selectivity, we examined the inhibitory effects of retrocyclin-1 on fusion mediated by HIV-1 Env. We found that retrocyclin-1 prevents HIV-1 entry by binding the C-heptad repeat of gp41 in a lectin-independent manner that prevents 6HB formation.

**EXPERIMENTAL PROCEDURES**

**Cell-Cell Fusion Assay**—Dye transfer assays, which involve observing the transfer of fluorescent cytosolic dyes from the target cell type to a non-labeled envelope expressing cell type, were used to quantify the amount on envelope-induced cell-cell fusion. HIV/SIV Env-expressing HeLa cells labeled with CMTMR and target cells labeled with calcein were co-cultured in suspension at 37 °C. Inhibitors were added at the onset of incubation or at various times thereafter. Phase and fluorescent images were collected with a 10X objective lens (30, 31). Normalized results were expressed as percent of the control fusion.

**Binding Studies**—Surface plasmon resonance studies were performed on a Biacore 3000 instrument (Biacore, Uppsala, Sweden) in two modes, standard and competitive (see the supplemental “Methods” section and supplemental Table 3).

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§ The online version of this article (available at http://www.jbc.org) contains supplemental Tables 1–3, supplemental Fig. 1, and supplemental Experimental Procedures.

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3 The abbreviations used are: RC-1, retrocyclin-1; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; 6HB, 6-helix bundle; N-HR, N helical region; C-HR, C helical region; FRAP, fluorescence recovery after photobleaching; sCD4, soluble CD4; BSA, bovine serum albumin.
Retrocyclin Blocks HIV-1 gp41 6-Helix Bundle Formation

Effect on 6-Helix Bundle Formation—Native-PAGE was done as previously described (32). Inhibition of 6HB formation was determined by a modified ELISA using the mAb NC-1 (33, 34). Percent inhibition was calculated as previously described (35). The IC50 was calculated with CalcuSyn software (36), kindly provided by Dr. T. C. Chou (Sloan-Kettering Cancer Center, New York, NY).

CD Spectroscopy—Spectra were obtained on a Jasco 1-715 instrument at 25 °C. Samples, diluted in 10 mM HEPES, pH 7.4, were placed in a 0.1 cm path length CD cell (Hellma, Plainview, NY). The spectra were averaged from four scans, smoothed, and expressed as the mean residue ellipticity [θ]MRE. Additional information about cells, recombinants, and methods is provided in supplemental "Methods and Materials," on-line.

Fluorescence Recovery after Photobleaching (FRAP)—FRAP was performed using a Zeiss LSM 510 (Carl Zeiss, Jena, Germany) confocal laser scanning microscope. HeLa cells were plated on 35-mm glass bottom dishes (MatTek, Ashland, MA) and transfected 24 h prior to confocal analysis with CD4-GFP. These constructs were generous gifts from W. Popik, and have been described previously (37). During FRAP analysis cells were kept at physiological conditions of 37 °C and 5% CO2 in a stage-incubation system ("Incubator S," PeCon GmbH, Erbach, Germany). Retrocyclin was added directly into the medium and incubated for 10 min before commencing FRAP measurements. A 488 nm Ar+ laser line was used for GFP excitation and emission light was collected with a 500–550 bandpass filter. A 40×/1.3 NA oil immersion objective lens was used with a zoom factor of 4. The detector pinhole was opened slightly to acquire an optical section of 2 μm thickness, allowing more light to be collected for better quantification.

Three prebleach images were acquired to determine the rate of non-purposeful photobleaching. Photobleaching was performed by increasing the transmission of the laser to 100% for 20–50 iterations to get as complete a bleach as possible without overbleaching. After photobleaching, 8−10 images were acquired at 1-s intervals. Then the time resolution was changed to 10 s to follow recovery to completion. A total of 20−40 data points was acquired for image analysis.

FRAP analysis was performed using the Medical Imaging Processing, Analysis, and Visualization (MIPAV; CIT/NIH, Bethesda, MD) software package. Data were automatically corrected with background subtraction, and normalization for the non-purposeful photobleaching rate was calculated from the whole cell. Data were analyzed using the one-dimensional FRAP model.

RESULTS

Inhibition of Fusion—Retrocyclin-1 inhibited HIV-1 Env-mediated membrane fusion in a concentration-dependent manner (Fig. 1a), with an IC50 of ~1.5 μM, and complete inhibition at ~4 μM. Retrocyclin-1 inhibited fusion mediated by HIV-1IIIB (X4) and HIV-1BAL (R5) Env but not HIV-2ROD Env (Fig. 1b).

Excluding Potential Membrane Targets—As retrocyclin binds CD4 and glycosphingolipids (29), its inhibition of HIV-1 Env-mediated fusion might reflect interactions with these membrane components. We examined the fusion of CD4-independent 8x Env with various cell lines. The medium contained 10% fetal bovine serum. Retrocyclin was added directly into the medium and incubated at 37 °C for 10 minutes before FRAP measurements began. The mobile fraction and diffusion coefficients are shown as mean ± S.E.

### Table 1

| Retrocyclin | Diffusion coefficient | Mobile fraction | n  |
|------------|-----------------------|-----------------|----|
| 0          | 2.5 ± 0.3             | 94 ± 2          | 26 |
| 10         | 4.6 ± 0.2             | 98 ± 5          | 21 |
| 20         | 3.6 ± 0.4             | 97 ± 1          | 22 |

FIGURE 1. Inhibition of fusion by retrocyclin-1. a, fusion of HIV-1 Env-expressing CV-1 cells with SupT1 target cells was monitored using a dye redistribution assay. The data are normalized to 100% fusion in the absence of inhibitor. b, retrocyclin-1 (RC-1, 4 μM) inhibited fusion mediated by the IIIB and BAL strains of HIV-1 Env but had considerably less effect on Env-mediated fusion mediated by those of the SBL and ROD strains of HIV-2 and the MAC strain of SIV. For comparison, inhibition by cyanovirin-N (CV-N, 1.5 μM) is shown. c, retrocyclin-1 (4 μM) substantially inhibited CD4-independent fusion of CV-1 cells expressing HIV-1IIIB 8x Env (a CD4-independent variant) with 3T3CXCR4 or 3T3CD4/CXCR4 target cells. For comparison, inhibition by cyanovirin-N (1.5 μM) is shown. Retrocyclin-1 also inhibited the fusion of HIV-1IIIB Env-expressing CV-1 cells with glycosphingolipid-deficient GM95CD4/CXCR4 target cells.
Retrocyclin Blocks HIV-1 gp41 6-Helix Bundle Formation

**FIGURE 2.** Retrocyclin-1 inhibits HIV-1 Env-mediated fusion in a late stage of the fusion cascade. a, time of Addition. Inhibitors Leu3a (3 μg/ml), RC-1 (4 μM), and C34 (2 μM) were added at different times after the initial co-culture of HIV-1 Env-expressing CV-1 cells with SupT1 target cells and the fusion was monitored using a dye redistribution assay as described under “Experimental Procedures.” b, soluble CD4-primed cells. Fusion of HIV-1 Env-expressing CV-1 cells (E) with SupT1 target cells (T) was monitored using a dye redistribution assay after pretreatment of targets or effectors. The following conditions were examined: no treatment (E0, T0); co-culture in the presence of inhibitor (E + T + 7); pretreatment with inhibitor of effectors, followed by washing and co-culture with untreated targets (E + W & T0); pretreatment with inhibitor of targets followed by washing and co-culture with untreated effectors (T + W+E0); incubation of effectors for one h at 37 °C with sCD4 (40 μg/ml) followed by washing and incubation with targets (sCD4 E0 T0); incubation of effectors for one h at 37 °C with sCD4 + inhibitor, followed by washing and incubation with targets (sCD4 E + T + 1). The inhibitors were retrocyclin-1 (4 μM) (black bars), sCD4 and C34 (2 μM) (gray bars).

**FIGURE 3.** Binding of retrocyclin-1. Binding to gp41<sub>env</sub> ectodomain, gp120<sub>AV</sub>, and BSA was studied by surface plasmon resonance. Each data point is the mean of values from two separate complete dose-response curves. The biosensor chip contained the following amounts of attached protein: gp41, 2221 relative units (RU); gp120, 5746 RU; BSA, 5605 RU. Each data set has been normalized to show binding to 5000 RU of immobilized protein.

Conclusions

Follows CD4 binding and precedes 6HB formation, N-terminal ectodomain regions are exposed (42), later becoming inaccessible about when membrane fusion occurs (43). Throughout the period of accessibility, HIV-1 Env-mediated fusion can be inhibited by peptides derived from the N-HR or C-HR regions of gp41 (31, 41, 44).

As the time-of-addition studies (Fig. 2a) had implicated N- or C-HR as targets for retrocyclin, we primed HIV-1 Env-expressing cells with 1 μg/ml of soluble CD4 (sCD4) and either retrocyclin-1 or C34 for 1 h at 37 °C before washing and then co-culturing them with target cells. Exposure to this suboptimal sCD4 concentration caused no inhibition, alone or in combination with C34 or retrocyclin-1 (Fig. 2b). However, incubating sCD4-primed cells with retrocyclin-1 or C34 inhibited fusion, lending support to the hypothesis that retrocyclin targets the N-HR and/or C-HR. Fig. 2b also shows that incubating either effectors or targets with RC-1 followed by washing resulted in no fusion inhibition, indicating that the RC-1 binding to targets and unprimed effectors is reversible.

**Binding of Retrocyclin-1 to gp41**—Fig. 3 compares binding of retrocyclin-1 to gp41<sub>HXB2</sub>, gp120<sub>AV</sub>, and bovine serum albumin (BSA). Per unit of mass, gp41 and gp120 bound retrocyclin-1 to an equal extent. However, as the mass of gp120 is ~8 times larger than that of the gp41 ectodomain (~16.4 kDa, exclusive of glycans), 8 times more retrocyclin-1 molecules bound a molecule of gp120 than a molecule of gp41. Supplemental Table 1 shows four experiments comparing binding of retrocyclin-1 to gp120 and gp41. The K<sub>d</sub> of retrocyclin-1 (mean ± S.E., n = 4) for gp41<sub>HXB2</sub> was 67.6 ± 9.1 nM, and the K<sub>d</sub> for gp41<sub>AV</sub> was 33.0 ± 4.7 nM. These differences were significant (p = 0.021, paired t test).

Because retrocyclin-1 has lectin-like properties (29) and the ectodomain of gp41<sub>HXB2</sub> contains N-linked glycosylation sites, we did the experiments shown in supplemental Table 2 to learn whether retrocyclin-1 bound gp41 via its N-linked glycans. By using lecithin, we determined that only high mannose glycans were present in the immobilized gp41 ectodomain. Removing these glycans with endoglycosidase H did not decrease the binding of retrocyclin to gp41, showing that the binding of retrocyclin-1 to gp41 was not carbohydrate-related.

We next used a library of synthetic peptide fragments of HIV-1<sub>MN</sub> gp41 to determine where retrocyclin-1 might bind the gp41 ectodomain. Two C-terminal peptides, gp41<sub>665-685</sub> (EREIDNTSTLIESLEKSS) and gp41<sub>675-685</sub> (DITNLWLYIKI) showed the greatest binding, and gp41<sub>665-674</sub> bound retrocyclin-1 to a lesser extent (supplemental Table 3).
We ran these surface plasmon resonance binding experiments in a competitive mode, using three different biosensors: BSA, gp120LAV, and gp41HXB2. Fig. 3 shows that binding of retrocyclin-1 to immobilized BSA is highly linear with peptide concentration, and its binding to the gp120 and gp41 biosensors is reasonably linear. Consequently, any of these biosensors can be used to assess the concentration of free (unbound) retrocyclin-1 in the presence of a potential binder, as long as the potential binder does not itself bind significantly to the biosensor. Indeed, with only one exception, biosensors containing BSA, gp120LAV, or gp41HXB2 gave similar results (supplemental Table 3).

Fig. 4a shows that retrocyclin-1 bound the C34 domain of HIV-1 with greater affinity than the C34 domains of HIV-2 or SIV. Fig. 4b shows that BSA and gp120 biosensors detected binding between retrocyclin-1 and gp41(633–650) equally well, with an estimated IC50 of ~15 µg/ml (7 µM). Fig. 4c shows that retrocyclin-1 bound more extensively to gp41 (HIV-1HXB2) than to gp36 (HIV-2, Biodesign R5B220). Both recombinant Env glycoproteins had been expressed in yeast, Pichia pastoris. The net anionicity of a gp41 peptide did not fully account for its ability to bind retrocyclin-1, because gp41(651–674) (net charge, −3) bound less well than either gp41(633–650) or gp41(675–685) with respective net charges of −2 and 0. Nor was the net positive charge of retrocyclin-1 sufficient, because retrocyclin-1 that had been reduced and then alkylated with iodoacetamide no longer bound gp41, despite its undiminished net charge of +4 (data not shown). Thus, topological factors involving the placement and accessibility of the charged residues in gp41 and retrocyclin-1 could play key roles in binding.

The gp41 ectodomains from HIV-1 strains MN, HXB2, and IIIB are shown in supplemental Fig. 1. The HXB2 strain used in our surface plasmon resonance experiments and the IIIB strain used in the cell fusion experiments have identical sequences that are similar to the MN sequence used for the gp41 peptide library. gp41(633–650), the peptide with the greatest binding to retrocyclin-1, comprises about half of the C34 sequence.

Retrocyclin-1 Inhibits gp41 6HB Formation—Because retrocyclin-1 bound the C-peptides derived from the HIV-1 gp41 C-HR region, we considered that it might also block C-peptide/N-peptide interactions that form the 6HB of gp41. We tested this (Fig. 5 inset) by a native-PAGE method (32). Neither N36 (lane 1) nor retrocyclin-1 (lane 3) nor a mixture of the two (lane 5) show up as a band, because their net positive charge causes them to migrate up and away from the gel (32). Lane 2, containing C34, shows a band near the bottom of the gel. Lane 4, a mixture of N36 and C34 shows a less intense C34 band and a stronger band higher in the gel that represents the 6HB, as shown by Western blots using the mAb NC-1 (32). Lane 6 (C34 + retrocyclin-1) shows a weaker C34 band, suggesting that C34 interacted with retrocyclin-1 to form a complex with net positive charge. When RC-1 was incubated with C34 before adding N36 (lane 7), the intensity of the 6HB-band was significantly reduced, compared with lane 4, showing that formation of the 6HB was inhibited. Reduced and alkylated retrocyclin-1 (RC-1-RA), which retains the net positive charge of retrocyclin-1 but loses its native conformation, had no significant effect on 6HB formation (lane 8).

To study the effects of retrocyclin-1 on 6HB formation more quantitatively, we modified a previously described sandwich enzyme-linked immunosorbent assay method using mAb NC-1 to improve its sensitivity. As shown in Fig. 5, retrocyclin-1 inhibited gp41 6HB formation in a dose-dependent manner, with an IC50 value of 0.585 ± 0.012 µM. In contrast, reduced and alkylated RC-1 showed no inhibition at up to 100
μM. The ability of retrocyclin-1 to inhibit 6HB formation was also examined by CD spectroscopy (Fig. 6). In the absence of retrocyclin-1, an equimolar mixture of N36 and C34 (12.5 μM) gave a characteristic α-helical spectrum (Fig. 6a), and retrocyclin-1 showed a β-sheet spectrum as reported elsewhere (8). The addition of 5 μM retrocyclin-1 to the N36/C34 mixture reduced its mean residue ellipticity at 222 nm by about half, and larger concentrations of retrocyclin-1 further attenuated the signal (Fig. 6b).

Structural Features—Fig. 7 shows structural representations of C34 and of retrocyclin-1. The arginine residues of retrocyclin-1 appear well positioned to allow at least three of them to interact with accessible anionic carboxyl groups on the exterior helical surface of C34. Whether these hypothesized anionic docking sites suffice to allow retrocyclin-1 to sabotage 6HB formation and the fusion machinery of HIV-1 or whether additional binding interactions also participate awaits further investigation.

DISCUSSION

We used retrocyclin-1, a model peptide, to learn how β-defensins frustrate the molecular machinery used by HIV-1 to enter target cells. We previously reported that the ability of retrocyclin-1 analogues to protect cells from HIV-1 was highly correlated to their ability to bind gp120 and CD4. We have also shown that much of their binding to gp120 and CD4 results from their lectin-like carbohydrate binding properties (29). Yet the present experiments demonstrate that retrocyclin-1 can block HIV-1 entry independently of interactions with gp120 and/or CD4 and independently of glycoprotein binding.

How might this paradox be explained? Other studies, to be presented elsewhere, indicate that the arginine residues of retrocyclin-1 play an essential role in carbohydrate binding. If the same arginines endow β-defensins with their ability to bind the C-terminal heptad repeat of gp41, as depicted in Fig. 7, then the correlation between carbohydrate binding and activity against HIV-1 is readily understood. The ability of gp41(675–685) (DITNWLWYIK) to bind retrocyclin-1 effectively (supplemental Table 3) suggests that the electrostatic interactions suggested by Fig. 7 may be reinforced by additional binding interactions, perhaps involving the isoleucine and cysteine residues of retrocyclin.

The mechanism whereby retrocyclin-1 inhibits HIV-1 entry closely resembles the mechanism of gp41-based peptide inhibitors of HIV-1 mediated fusion (18–26,45). Each of these well characterized HIV-1 inhibitory peptides contains one or more gp41 domains that would normally interact to form 6HBs and drive membrane fusion (41). By binding exposed prefusion sites on gp41, these peptides prevent the formation of the 6-helix, coiled-coil structure and block fusion and downstream entry events (26, 27, 31, 41, 44).

Retrocyclin-1 selectively targeted the C-terminal heptad repeat region of the HIV-1 gp41 ectodomain, and it inhibited 6HB formation between peptides derived from the N- and C-terminal heptad repeat regions of HIV-1 gp41. The finding that retrocyclin-1 had substantially less effect on 6HB formation between the SIV N- and C-peptides is consistent with its lesser effectiveness against this virus. The mechanism described in this report also offers a testable hypothesis to explain why some primary HIV-1 isolates (e.g. those of clade C (9)) are more resistant to retrocyclin-1 than the more uniformly sensitive clade B isolates represented by the gp41 peptides used in this study.
The mechanism of inhibition of HIV-1 entry by retrocyclin-1 revealed in this study is very similar to that exhibited by N- and C-peptide derived from the HIV-1 gp41 N-HR and C-HR, including the only fusion inhibitor approved by the FDA for antiretroviral treatment (T-20 or enfuvirtide). Further confirmation of this mechanism has been provided by recent studies of in vitro selection of HIV-1 resistance to retrocyclin-1 (46). This study reports that passaging HIV-1 under selective pressure by RC-101, a retrocyclin analog, results in a 5–10-fold decrease in viral susceptibility to RC-101. Emergent viral isolates had three amino acid substitutions in their envelope glycoprotein. One was in a CD4-binding region of gp120, and the others were in the HIV-1 gp41 N-HR and C-HR. Each mutation replaced an electroneutral or electronegative residue with one that was positively charged, consistent with the model shown in Fig. 7, which shows anionic docking sites for RC-1 in WT HIV-1 gp41.

The present experiments did not directly test the "cross-linked barrier" mechanism delineated by recent studies with influenza A (11). Those studies, unlike the present ones, were conducted under serum-free conditions. Influenza A infections are initiated on the air side of a respiratory tract interface, potentially a serum-free environment. Those studies, unlike the present ones, were conducted under serum-free conditions. Influenza A infections are initiated on the air side of a respiratory tract interface, potentially a serum-free environment. In serum poor environments, the barrier mechanism (11) and the mechanism defined in this report could co-exist and reinforce each other.

Carbohydrate binding properties may enhance the antiviral properties of 3β-defensins in additional ways. Their ability to bind carbohydrate residues on gp120 and CD4, and to bind numerous target cell membrane constituents (e.g. glycoproteins, glycolipids, and glycosaminoglycans), will establish high local concentrations of 3β-defensins exactly where (and when) its vulnerable prehairpin structure becomes exposed. At present, 3β-defensin peptides are reflections of the past of the human innate immune system. However, based on their small size and intriguing properties, should it become possible to design similar viral inhibitors, 3β-defensins may play a significant role in protecting humans against future infectious challenges.

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