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Peptide inhibitors of HIV-1 protease and viral infection of peripheral blood lymphocytes based on HIV-1 Vif

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ABSTRACT **We recently reported that HIV-1 Vif (virion infectivity factor) inhibits HIV-1 protease *in vitro* and in bacteria, suggesting that it may serve as the basis for the design of new protease inhibitors and treatment for HIV-1 infection. To evaluate this possibility, we synthesized peptide derivatives from the region of Vif, which inhibits protease, and tested their activity on protease. In an assay of cleavage of virion-like particles composed of HIV-1 Gag precursor polyprotein, full-length recombinant Vif, and a peptide consisting of residues 21–65 of Vif, but not a control peptide or BSA, inhibited protease activity. $Vif_{21–65}$ blocked protease at a molar ratio of two to one. We then tested this peptide and a smaller peptide, $Vif_{41–65}$, for their effects on HIV-1 infection of peripheral blood lymphocytes. Both Vif peptides inhibited virus expression below the limit of detection, but control peptides had no effect. To investigate its site of action, $Vif_{21–65}$ was tested for its effect on Gag cleavage by protease during HIV-1 infection. We found that commensurate with its reduction of virus expression, $Vif_{21–65}$ inhibited the cleavage of the polyprotein p55 to mature p24. These results are similar to those obtained by using Ro 31–8959, a protease inhibitor in clinical use. We conclude that Vif-derived peptides inhibit protease during HIV-1 infection and may be useful for the development of new protease inhibitors.**

Gene products required for viral replication are suitable targets for antiviral intervention. In this regard, HIV-1 Vif merits consideration. Vif is essential for productive infection of primary lymphocytes and macrophages by HIV-1 *in vitro* and for pathogenesis in animal models of AIDS (1–4). The predominant view is that Vif acts at the late stages of infection to promote virion processing or assembly (1–4, 6–10). We implicated Vif in the action of HIV-1 protease (PR) by the demonstration that Vif and its N-terminal half (amino acids 1–96), but not its C-terminal half (amino acids 97–192), inhibit cleavage by PR *in vitro* and in bacteria (11). A C-terminal domain in Vif has been shown by others to mediate membrane binding (12) and interaction with the Gag polyprotein (13–14). We have proposed that during HIV-1 infection, Vif blocks premature activation of PR in the cytoplasm and that this inhibition is relieved during particle assembly (11). Extrapolating from this proposal, some fragments from the N terminus of Vif may preserve the PR-inhibitory function but fail to release this inhibition and thereby stably block PR. We report here the identification of peptide derivatives of Vif that inhibit PR-mediated cleavage of Gag *in vitro* and during HIV-1 infection in culture. Like Ro 31–8959 (saquinavir), a protease inhibitor presently in clinical use (15), Vif peptides inhibited HIV-1 protein expression in peripheral blood lymphocytes (PBLs) in culture below the limit of detection. Vif-derived peptides offer an avenue for the development of novel protease inhibitors that use endogenous HIV-1 functions for the treatment of HIV-1 infection.

MATERIALS AND METHODS

Cells and Viruses. PBLs were obtained by venipuncture from HIV-seronegative donors and were isolated by centrifugal elutriation. ADA is an R5 (non-syncytium-inducing, macrophage tropic) HIV-1 strain obtained from H. Gendelman (16). Sendai virus strain Z was purchased from SPAFAS (Preston, CT).

Peptides and Proteins. Vif peptides were based on the sequence of HIV-1/N1T-A *vif* (17), $Vif_{21–65}$ contains residues 21–65 and $Vif_{41–65}$ contains residues 41–65. They were synthesized manually on a preloaded Boc-Val-PAM-resin according to the *in situ* neutralization/HBTU activation protocol for Boc-SPPS (18). After chain assembly, peptides were globally deprotected and cleaved from the resin by treatment with liquid HF 4% p-cresol for 1 h at 0°C. Crude peptides were purified to homogeneity by reverse-phase HPLC and characterized by electrospray mass spectrometry. $Vif_{41–65}$ in addition was purchased from American Peptide (Sunnyvale, CA). Control peptides from human angiotensinogen, β endorphin, or histone 2A were purchased from Sigma. Recombinant Vif was prepared as described (11), and BSA was purchased from Sigma.

Preparation of Virion-Like Particles (VLPs). The entire *gag*-coding region was amplified from HIV-1/NL4-3 by PCR using primers jGMA5 (5′GAAGGAGAGAATTCGGTGCGAGAGCGTCAG3′) and p6R (5′ GCCCCCCTCGAGTTATTGTGACG 3′) and was cloned into the pCITE-4a(+) eukaryotic expression vector from Novagen, generating the intermediate construct pCITE-Gag. The myristoylation signal at the amino terminus of Gag was restored by removing vector sequence 519–629 from pCITE-Gag by amplifying the entire vector by using primers containing the *Nco*I restriction site, CITEL (reverse, 5′-GGTGGTGGCCATGGTATCATCGTG-3′) and CITER (forward, 5′-GGATCCGCCATGGGTGCGAGAGCGTCAG-3′). After amplification, the DNA was digested with *Dpn*I to remove template DNA and with *Nco*I to form cohesive ends, and ligated. The resulting construct was designated pCITEΔ-Gag. Expression of the insert is under the control of the T7 polymerase promoter. H4/CD4 cells were infected with the vaccinia virus vector of T7 polymerase, vTF7–3 (19) at a multiplicity of infection of 10, and were

Abbreviations: PR, protease; PBLs, peripheral blood lymphocytes; $Vif_{21–65}$, peptide of residues 21–65 of N1T-A Vif; $Vif_{41–65}$, peptide of residues 41–65 of N1T-A Vif; VLPs, virion-like particles; CITE, Cap independent translation enhancer; BOC, *tert*-butoxycarbonyl; SPPS, solid-phase peptide synthesis; PAM, 4-hydroxymethylphenylacetic acid.

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transfected 1 h later with pCITEΔ-Gag at 6.5 μg of DNA per 10^6 cells. Cell supernatants were collected 24 h later, clarified by filtration through 0.45 μm filter, and VLPs were isolated by sedimentation at 100,000 × *g* through a 20% sucrose cushion.

Proteolysis Assay. The 250 ng of VLPs were mixed with 50 ng of HIV-1/HXB-2 PR (provided by the AIDS Research and Reference Reagent Repository, Rockville, MD) in a final volume of 20 μl (50 mM Mes, pH 6.0/100 mM $NaCl_2$/2 mM dithithreatol/1 mM EDTA/0.2% nonidet-40), and the mixture was incubated overnight at 37°C. Peptides were added to the reaction mixtures in the systems and amounts indicated in the text. The entire reaction mixture was subjected to electrophoresis in 4–20% polyacrylamide gels, transfer, and staining in Western blots by using anti-Gag mAb AG3.0, which detects the Gag polyprotein p55, processing intermediates, and mature p24 (3, 11).

HIV-1 Infection. PBLs were stimulated with phytohemagglutinin and interleukin-2 (IL-2) in RPMI 1640 containing 10% fetal bovine serum for 3 days before infection; after infection, cells were cultured under the same conditions without phytohemagglutinin. Cells were exposed to 0.1 pg p24 ADA per cell for 1 h at 37°C as a pool, were washed extensively, and then were cultured as indicated in the absence or presence of peptides or the protease inhibitor Ro 31–8959 (15) provided by Roche Products (London, UK). In experiments of viral protein analysis by Western blot, PBLs were infected as described and cultured for 5–6 days in RPMI-IL-2 before exposure to peptides in the presence of Sendai virus.

Treatment of PBLs with Sendai Virus to Facilitate Peptide Internalization. Sendai virus was inactivated by ultraviolet irradiation as described (20). HIV-1/ADA-infected PBLs were treated with inactivated Sendai at 130 hemagglutination units per 10^6 cells in 0.16 M NaCl and 20 mM tricine, pH 7.4 (SV buffer) for 30 min on ice; cells were harvested, washed in ice-cold SV buffer to remove unbound Sendai virus, and incubated in SV buffer containing 1 mM $CaCl_2$ for 1 h at 37°C in the absence or presence of peptides or Ro 31–8959 as indicated in the text. Cells were then harvested and cultured in the absence or presence of peptides or Ro 31–8959 in RPMI-IL-2 for 3 days before evaluation of HIV-1 expression and Gag processing.

Evaluation of HIV-1 Expression. HIV-1 spread after peptide treatment was monitored by measurement of extracellular or intracellular core antigen p24 by using the HIV Ag ELISA kit from Coulter. To test the effects of peptides on the processing of the Gag polyprotein during HIV-1 infection, cell extracts were subjected to electrophoresis in 12% polyacrylamide gels, transfer, and staining in Western blots by using anti-Gag mAb AG3.0.

RESULTS

Effects of Vif Peptides on PR-Mediated Proteolysis *In Vitro*. We have previously shown that Vif and its N-terminal half inhibit PR cleavage of HIV-1 Gag and Pol substrates (11). Because inhibition in the *in vitro* proteolysis assay was achieved at equimolar levels of PR and the N-terminal half of Vif (11) and because PR does not cleave Vif under the conditions of the assay, it is likely that Vif inhibits PR by interaction with PR rather than by interaction or competition with substrate. To better define the region of Vif possessing this activity, we tested $Vif_{21–65}$, a peptide based on residues 21–65 in N1T-A Vif (17). We chose to evaluate this region of Vif because bacterial studies indicated that a truncated Vif protein carrying residues 1–38 failed to inhibit proteolysis (11) and preliminary studies indicate that Vif truncation mutants, which express residues 1–65 or 10–96 inhibit proteolysis (P.S., W. Chao, M. Canki, and D.J.V., unpublished data). To detect PR activity, we established an *in vitro* system for cleavage of VLPs, which are assembled by cells expressing HIV-1 Gag. The extent of PR activity was assessed by detection of Gag substrate and proteolytic products by electrophoresis and Western blot by using the anti-Gag mAb AG3.0. The conditions of the assay were optimized to yield the intermediate cleavage product p41 and the final cleavage product p24 (Fig. 1). When the reaction was conducted in the presence of graded amounts of full-length Vif protein or peptide $Vif_{21–65}$, proteolysis was inhibited. $Vif_{21–65}$ inhibited PR at a twofold molar excess; a 10-fold molar excess of full-length Vif was required in this system for inhibition of PR. There was no inhibition of the reaction by control protein, BSA, or control peptide histone 2A. These results indicate that $Vif_{21–65}$ preserves the ability of Vif protein to inhibit PR activity *in vitro*, raising the possibility that $Vif_{21–65}$ may affect PR activity during HIV-1 replication in living cells. On that basis, we tested the effects of peptides $Vif_{21–65}$ and a smaller peptide from the same region, $Vif_{41–65}$, on HIV-1 virion synthesis and the production of infectious virus by HIV-1-infected PBLs.

Effects of Vif Peptides on HIV-1 Infection and Gag Processing. Human PBLs were infected with the non-syncytium inducing, macrophage-tropic HIV-1 isolate ADA and were cultured immediately after infection in the absence or presence of Vif peptides or control peptide from β endorphin. The PR inhibitor Ro 31–8959 served as a control for inhibition of PR activity and HIV-1 infection (15). Because the multiplicity of infection was low, this system principally reveals the extent of the spread of HIV-1 infection. Virus replication was monitored by measurement of extracellular core antigen p24 by ELISA (Fig. 2*a*). $Vif_{21–65}$, $Vif_{41–65}$, and Ro 31–8959 inhibited HIV-1 infection below the limit of detection by this assay. In dose response tests, at early time points $Vif_{41–65}$ at a concentration of 30 μg/ml, or ≈10 μM, inhibited 90–95% of virus production relative to cultures treated with control peptide from β endorphin (Fig. 2 *b* and *c*). In all cases, cell cultures were >90% viable. These results indicate that Vif peptides can inhibit HIV-1 infection in primary lymphocytes. Similar results of the effects of Vif peptides on HIV-1 infection were obtained in five experiments by using PBLs and in two experiments by using primary macrophages (not shown). A commercially synthesized $Vif_{41–65}$ also was active in the inhibition of HIV-1 infection (not shown).

To determine whether these Vif peptides inhibit HIV-1 infection through their inhibition of PR, we investigated the effects of $Vif_{21–65}$ on PR-mediated processing of Gag during HIV-1 replication in PBLs. To ensure that sufficient Gag was produced to be detected in Western blots, PBLs were infected with HIV-1 and infection was permitted to proceed for 5 or 6 days before culture with inhibitors. Cells were exposed to

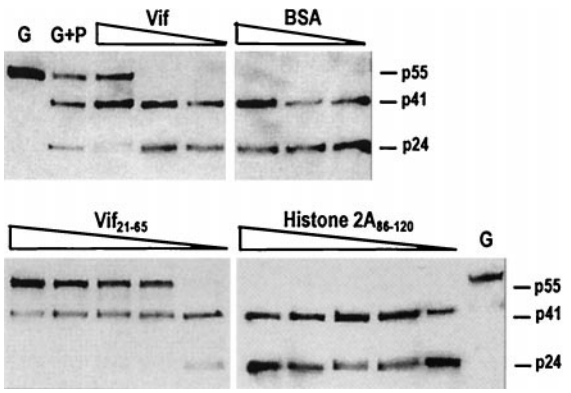

FIG. 1. Inhibition of PR-mediated VLPs cleavage by Vif and Vif peptides *in vitro*. VLPs were purified and subjected to cleavage with HIV-1 PR, electrophoresis, and Western blot staining with anti-Gag. The lanes show VLPs incubated alone (G); VLPs incubated with PR (G + P); VLPs incubated with PR and Vif or BSA at molar ratios of 10, 2.5, and 0.6:1 PR, respectively; and VLPs incubated with PR and $Vif_{21–65}$ or H2A peptides at molar ratios of 20, 10, 5, 2.5, and 1.2:1 PR, respectively. The mobilities of Gag polyprotein p55, intermediate cleavage product p41, and final product p24 are indicated.

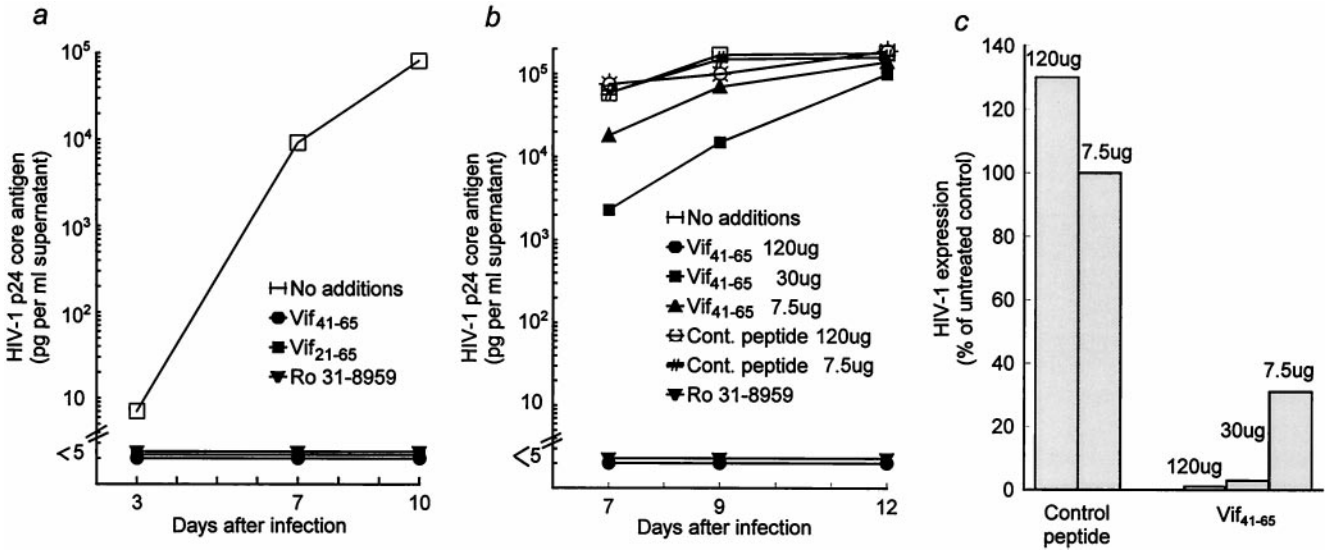

FIG. 2. Inhibition of HIV-1 infection of PBLs by Vif-derived peptides. HIV-1-infected PBLs were cultured with various additions and at the indicated times after infection, cell supernatants were harvested for measurement of viral core antigen p24 levels. (*a*) Culture with no additions to growth medium, 100 μg/ml Vif_{21-65} or Vif_{41-65}, or 1 μM Ro 31–8959. (*b*) Cultured with no additions to growth medium, different concentrations of Vif_{41-65} or β endorphin peptide, or 1 μM Ro 31–8959. (*c*) p24 levels on day 7 after infection as shown in *b* presented relative to the p24 level in cells cultured with no additions to growth medium.

UV-inactivated Sendai virus for 1–2 h to facilitate the entry of macromolecules through membrane pores generated during Sendai virus–cell fusion (21). The cells then were washed and cultured in the absence or presence of peptides or Ro 31–8959. Using fluoresceinated peptides, we previously found that exposure of PBLs to Sendai virus facilitates peptide entry but does not affect HIV-1 infection (not shown). Infected PBLs were evaluated for p24 content by ELISA and for Gag processing by Western blot. Vif_{21-65} reduced the level of intracellular p24 by 70–75% compared with untreated cells or cells exposed to angiotensinongen peptide (Table 1). Analysis of the processing of Gag proteins by Western blot explained this finding. Although the Gag polyprotein was synthesized efficiently in the presence of the Vif peptide, cleavage of p55 to p24 was inhibited (Fig. 3). This result is similar to that obtained by treating cells with the protease inhibitor, Ro 31–8959, in which p24 levels were reduced by 90% and mature p24 was not detectable by Western blot. We conclude that a peptide derived from Vif inhibits HIV-1 infection by its inhibition of PR-mediated processing of Gag.

DISCUSSION

We have found that peptides derived from HIV-1 Vif block HIV-1 PR cleavage of Gag *in vitro* and inhibit both cleavage of Gag by PR and infection by wild-type HIV-1 in PBLs. The peptides were constructed from the region of Vif we had previously identified as inhibiting PR *in vitro* and in bacteria PR (11), and they lacked the essential C-terminal region, which mediates an interaction with Gag or cellular membranes (12–14). Because the relatively large, charged Vif_{21-65} itself has not been modified in any way for tissue culture application, we exposed cells to fusogenic Sendai virus to facilitate peptide

Table 1. Inhibition of p24 production by culture of HIV-1 infected PBL in Vif peptides

Treatment	HIV-1 expression, pg p24 per 10^6 cells	
	Donor 1	Donor 2
None	11,000	26,000
Vif_{21-65}	3,300	6,300
Angiotensinongen peptide	11,000	20,000
Ro 31–8959	1,000	3,900

Five to six days after infection by HIV-1/ADA, PBL were exposed to the indicated additions in presence of Sendai virus or to Sendai virus alone and then were cultured with no additions to the culture medium or the indicated additions for 3 days before collection of cells for assay of intracellular p24 and Western blot.

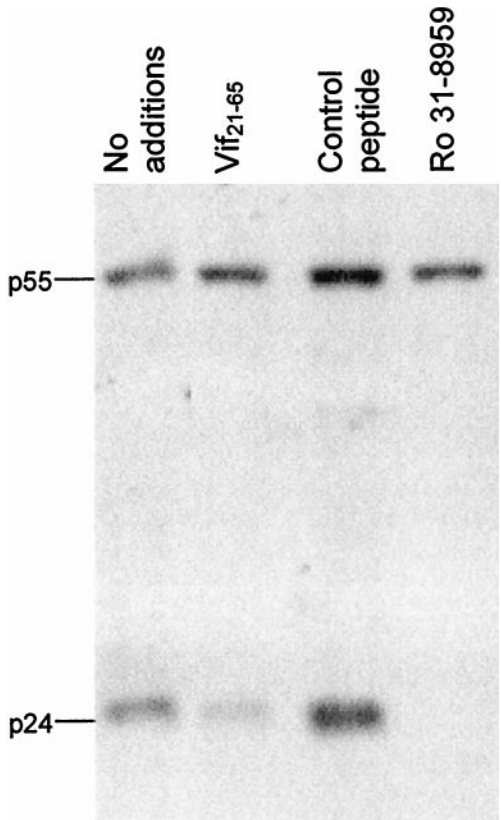

FIG. 3. Inhibition of Gag processing during HIV-1 infection of PBLs by Vif-derived peptides. Extracts were prepared from donor 1 cells from the experiment described in Table 1 and were subjected to electrophoresis and Western blotting by using anti-Gag antibody. The mobilities of the Gag polyprotein p55 and mature p24 are indicated.

entry (21). Studies are in progress to test a larger panel of Vif-derived peptides for inhibition of PR activity and for the kinetic constants of this inhibition to elucidate the mechanism of action of peptide inhibitors on the enzyme. Based on the activity we report here, $Vif_{21–65}$ and $Vif_{41–65}$ represent the first step in the development of an agent to inhibit PR based on the principle of Vif mimicry.

These results also may clarify the role of Vif during HIV-1 infection. We previously proposed that Vif regulates PR activity by inhibiting premature activation of the enzyme in the cytoplasm (11). The inhibitory activity of Vif during HIV-1 replication must be transient, since the overall result of infection in the presence of Vif is the formation of infectious virions containing fully processed proteins (3, 4, 6, 8, 9). However, the net inhibition of PR activity during HIV-1 infection by $Vif_{21–65}$ suggests that a C-terminal domain of Vif absent from this peptide mediates the relief of Vif inhibition of PR. Because a required C-terminal region in Vif binds Gag and cellular membranes (12–14), a model of Vif action can be proposed ascribing specific functions to each domain. The N-terminal domain inhibits Gag-Pol autoproteolysis in the cytoplasm and this inhibition is relieved by dissociation of Vif from Gag-Pol during Vif binding to cellular membranes through the C-terminal domain. The effect of Vif is to constrain PR activity to the assembling virion, permitting orderly and complete processing of Gag and Pol components. The Vif peptides described here preserve the endogenous PR inhibitory capacity of the intact protein and may be useful for the development of new inhibitors of HIV-1 infection. Because HIV-1 has evolved to conserve the Vif-PR interaction, mutants in PR resistant to Vif-based antiviral agents may be less frequent than PR mutants resistant to existing inhibitors.

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