Human immunodeficiency virus type 1 Vif binds the viral protease by interaction with its N-terminal region

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The retroviral Gag and Gag–Pol precursors are processed by the viral protease (PR), which is translated as part of the large polyprotein (Oroszlan & Luftig, 1990; Vogt, 1996; Tomasselli & Heinrikson, 2000). Proteolytic processing is a key step in virus maturation: without specific cleavage of precursors, the virion is not infectious (Crawford & Goff, 1985; Kohl et al., 1988; Wills et al., 1989). The retroviral PR is an aspartic dimeric protease in which the sequences at the termini of the enzyme form a hydrophobic interface between the monomers in the homodimeric structure of the active enzyme (Pearl & Taylor, 1987). The interactions of the four terminal regions of the two PR monomers strongly stabilize the dimeric form of PR, this being a prerequisite for enzyme activity. The dimerization of PR probably occurs when the enzyme is part of the Gag–Pol precursor, resulting in autoprocessing of the polyproteins and production of the structural and catalytic mature viral proteins (Co et al., 1994; Crawford & Goff, 1985; Kaplan & Swanstrom, 1991a; Kohl et al., 1988; Kotler et al., 1992; Oroszlan & Luftig, 1990; Zybarth et al., 1994). However, retrovirus precursors synthesized in vertebrate host cell cytoplasm are devoid of proteolytic activity until virus assembly and/or particle release. Human immunodeficiency virus type 1 (HIV-1) is exceptional, however, since its PR seems to be active both in the cytoplasm (Kaplan & Swanstrom, 1991b) and at the cell membrane (Kaplan et al., 1994); thus, its regulation is more complex than that of the simple retroviruses.

HIV-1, like other lentiviruses, expresses six auxiliary genes, tat, rev, vif, vpr, vpu and nef, in addition to the canonical retroviral genes gag, pol and env. Vif (virion infectivity factor) is synthesized in the late phase of infection (Garrett et al., 1991) and is predominantly cell associated (Camaur & Trono, 1996; Goncalves et al., 1994). Recent reports demonstrated that Vif is associated with viral core structures (Liu et al., 1995) and specifically packed into HIV particles either by interaction with viral genomic RNA (Khan et al., 2001) or by interaction with Gag and Gag–Pol precursors (Bardy et al., 2001). Sova et al. (2001) demonstrated that Vif incorporates mainly into particles containing unprocessed Gag molecules but is largely absent from mature virions.

There is a wide consensus that Vif is absolutely essential for production of infectious particles in HIV-infected peripheral blood lymphocytes (PBLs) and macrophages (Aldrovandi & Zack, 1996; Chowdhury et al., 1996; Courcouil et al., 1995; Gabuzda et al., 1992; Sakai et al., 1993; Strebel et al., 1987; von Schwedler et al., 1993). While the phenotype of the vif gene is quite clear, the mechanism and location of Vif activity is still controversial. Simm et al. (1995) showed abnormally processed viral proteins in PBLs infected with ∆vif HIV-1. However, other reports demonstrated that the protein composition of ∆vif and wild-type HIV-1 particles released from restrictive cells is indistinguishable (Bouyac et al., 1997b;
Fouchier et al., 1996; Khan et al., 2001). A direct effect of Vif on autoprocessing of viral precursors was shown in bacterial cells and cell-free systems (Kotler et al., 1997). In addition, Vif peptides derived from aa 30 to 65 and 78 to 98 bind PR and inhibit both PR-mediated hydrolysis of synthetic peptides in vitro and production of infectious virus from HIV-1-infected human cells (Baraz et al., 1998; Friedler et al., 1999; Potash et al., 1998). Inhibition of the autoprocessing of viral precursors due to interaction with Vif was also shown by Bardy et al. (2001). The experiments described in this report were addressed to determine whether Vif binds PR and to map the interaction sites of Vif with PR.

ELISA microwells were coated for 18 h at 4 °C with 200 μL containing 0.2 μM BSA, thyroglobulin or recombinant HIV-1 Vif, purified as described by Yang et al. (1996). The wells were aspirated and blocked with low-fat milk for 1 h at room temperature. Following washes with PBS containing 0.05% Tween 20, increasing concentrations of HIV-1 and avian sarcoma leukaemia virus (ASLV) PRs, purified as described previously (Baraz et al., 1998; Kotler et al., 1988), in 200 μL of 0.1 M NaCl in 50 mM sodium phosphate buffer (pH 7.4) were added to each well and incubated for 2 h at room temperature. The amount of PRs that specifically bound to Vif was determined by anti-HIV-1 or anti-ASLV PR polyclonal sera. Fig. 1(A) shows that the PR of HIV-1 but not that of ASLV bound to Vif in a dose-dependent manner. There was no binding of PRs to thyroglobulin or BSA, which were used as negative controls (Fig. 1A). In a reciprocal experiment (Fig. 1B), the ELISA microwells were coated with HIV-1 wild-type PR and active site-mutated PR<sup>D35I</sup>, ASLV PR, thyroglobulin or BSA, and increased amounts of Vif protein were added to the plate. The Vif protein bound to either PRs or control proteins was quantified by an anti-HIV-1 Vif polyclonal serum. Wild-type and mutated HIV-1 PRs bound equal amounts of Vif, indicating that alteration of catalytic residues in the active site of PR does not affect the binding of Vif to the enzyme. ASLV PR bound only small amounts of Vif compared to HIV-1 PR, while thyroglobulin- or BSA-coated microwells did not bind Vif, demonstrating the specificity of Vif binding to HIV-1 PR. The binding of Vif to PR was confirmed further by using CNBr-activated beads and immunoprecipitation with specific anti-PR or anti-Vif sera (data not shown).

In order to determine the regions in the PR molecule that interact with Vif, we measured by ELISA the binding of Vif to linear peptides derived from five conserved regions of the HIV-1 PR molecule (Fontenot et al., 1992). Microtitre plates were coated for 18 h at 4 °C with 50 μM of PR-derived peptides diluted in 0.1 M Tris·HCl, pH 8.8. All peptides bound equally to the surface of the microwells, as determined by a novel ELISA method (data not shown) (Steinitz & Baraz, 2000). The wells were blocked and washed, as described above, and the Vif molecules bound to the PR-derived peptides were detected by polyclonal anti-Vif sera. Peptide PR<sub>1–9</sub> exhibited the most intensive binding of Vif protein (Fig. 2A). Peptides PR<sub>21–32</sub>, PR<sub>47–56</sub> and PR<sub>78–88</sub> bound Vif but not as efficiently as PR<sub>1–9</sub>. PR<sub>94–99</sub>, a peptide of 6 aa derived from the most intensive binding of Vif protein (Fig. 2A). Peptides PR<sub>21–32</sub>, PR<sub>47–56</sub> and PR<sub>78–88</sub> bound Vif but not as efficiently as PR<sub>1–9</sub>. PR<sub>94–99</sub>, a peptide of 6 aa derived from the C terminus of the PR molecule, as well as the RikA peptide (RGDLFAVDTC-NH<sub>2</sub>), used as a negative control, did not bind Vif. In order to assure that PR<sub>94–99</sub> peptide did not bind Vif due to its small size, an additional C-terminal peptide, PR<sub>89–99</sub>, was synthesized. Fig. 2(A) shows that even the elongated C-terminal peptide did not bind Vif.

We then assessed whether the PR-derived peptides compete with the binding of Vif to PR. To this end, Vif was preincubated with increased concentrations of PR-derived peptides for 18 h at 4 °C before adding to PR-coated ELISA wells. Fig. 2(B) demonstrates that 75 and 150 μM of PR<sub>1–9</sub> reduce Vif to PR binding by about 75 and 98%, respectively. The other PR-derived peptides and the control RikA peptide do not significantly decrease the binding of Vif to PR. These results suggest that Vif binds specifically to the conserved N-terminal region of PR, which is represented by peptide PR<sub>1–9</sub>. To verify that Vif binds to the PR terminus, we constructed chimeric HIV-1<sup>ASLV</sup> PR in which the termini of HIV-1 PR, aa 1–4 (Pro–Gln–Ile–Thr) and aa 96–99 (Thr–Leu–Asn–Phe), were replaced by PCR with the ASLV PR termini, aa 1–4.
Blocking the binding of Vif to PR by PR-derived peptides. The experiment was performed as described in (A) but the wells were coated with 90 nM PR. Vif (100 nM) was added after preincubation with the indicated amounts of PR-derived peptides for 18 h at 4°C. Each point (A405) represents binding minus non-specific binding of Vif to uncoated wells blocked with milk.

Fig. 3. Blocking the binding of PR to Vif by Vif-derived peptides. The wells were coated with 0–2 µM Vif and 40 nM PR was added to the wells after preincubation with the indicated amounts of Vif-derived peptides for 18 h at 4°C. Following 2 h of incubation at room temperature, the plates were washed and the PR bound to Vif was determined by ELISA using anti-PR serum.

In order to identify the regions in the whole Vif molecule that interact with PR, we have determined which of the Vif-derived synthetic peptides interfere with the binding of PR to Vif (Baraz et al., 1998). Purified Vif was coupled to the ELISA microwells and HIV-1 PR was then added, following preincubation with Vif78–92 and Vif88–98 peptides, which were found previously to be PR inhibitors. As a control, we used a peptide derived from the C terminus of Vif (Vif170–191), which is essential for Vif function (Goncalves et al., 1995) and for binding of Vif to Pr55Gag (Bouyac et al., 1997a; Huvent et al., 1998). The results of this experiment clearly show that preincubation of PR with Vif88–98 and Vif78–92 prevents the binding of PR to Vif in a dose-dependent manner. It was demonstrated that a concentration of 32 µM of the Vif88–98 peptide is sufficient to reduce the binding of PR to Vif by more than 50%. Vif78–92 also blocks binding but not as efficiently as Vif88–98. However, Vif17–29 and Vif170–191 do not interfere with Vif to PR binding (Fig. 3). Taken together, these results strongly suggest that the aa 78–98 region in Vif interacts with the N terminus of HIV-1 PR (aa 1–9).

Here we report that HIV-1 Vif directly interacts with the viral PR in cell-free systems. The HIV-1 Vif to PR binding is specific, since Vif does not interact with non-viral proteins and ASLV PR. The competition experiments specifically allocate the site of interaction to the central part of the Vif molecule (aa 78–98) and to the N terminus of PR (aa 1–9). The finding that chimeric HIV-1ASLV PR does not bind HIV-1 Vif supports this conclusion. The N- and C-termini of the two PR monomers establish a four-strand β-sheet structure, which is essential to the formation of the active homodimer enzyme (Vogt, 1996). Thus, our results suggest that Vif interferes with the dimerization of PR (Babe et al., 1991, 1992; Schramm et al., 1996, 1999). This suggestion is further supported by the following findings: (i) Vif88–98 specifically inhibits PR as a non-substrate-based inhibitor (Friedler et al., 1999); (ii) HIV-1 Vif-derived peptide Vif88–98 inhibits HIV-2 PR but not ASLV PR and pepsin (Blumenzweig et al., 2002; Friedler et al., 1999). These results are compatible with the sequence similarity between the PR termini of HIV-1 and HIV-2 (PQITL3…CTLNF9 and PQSL5…MCLNL9, respectively). In this regard it should be noted that the Δvif HIV-2 and Δvif HIV-1 phenotypes could be complemented by the reciprocal Vif molecules (Reddy et al.,
1995; Simon et al., 1995); and (iii) a stretch of 5 aa in the central part of Vif is quite similar to the ‘consensus sequence’ for dimerization inhibitors derived from the PR molecule (Schramm et al., 1996) as shown below:

Consensus sequence: \( ^{84}T\ Y/\ Y \ D/E/N \ L/Y/\ W^{99} \)

Vif: \( ^{84}V\ S\ I\ E\ W^{99} \)

It is unknown yet whether the interaction site of Vif bound to PR is limited solely to PR1–9 and Vif78–98. Others and we have demonstrated that an additional region in the Vif molecule, namely aa 30–65, is involved in PR inhibition and that peptides derived from both regions inhibit PR (Baraz et al., 1998; Friedler et al., 1999; Potash et al., 1998). Thus, it is plausible that the interaction of Vif with PR involves two discontinuous regions of Vif and that the mechanism of inhibition is more complex.

In accordance with our results, Bardy et al. (2001) demonstrated that Vif inhibits the PR-mediated processing of Gag precursors in vitro. Vif is encapsidated more efficiently into virus-like particles containing Gag–Pol rather than Gag precursors. However, these authors could not find significant interaction between Vif and PR in vivo or in vitro. This discrepancy can be explained by the differences in the applied systems.

The time window and the location of Vif activity are not yet known. Previously, we suggested that Vif is active at the late phase of infection and is responsible for the delay of PR activation when it is part of the Gag–Pol precursor. This lag period may prevent PR from digesting cellular proteins, assures the migration of viral structural components to the site of virion assembly at an appropriate molar ratio and allows the organization of precursors in a pattern required for maturation. However, others and we were unable to show that the presence or absence of Vif caused a measurable effect on the protein composition of viruses produced by restrictive or permissive host cells (Bouyac et al., 1997b; Fouchier et al., 1996; Khan et al., 2001). Now that it is clear that Vif and PR are present in the mature virions (Khan et al., 2001) and that PR is also present in the preintegration complex (Karageorgos et al., 1993), it is plausible that Vif regulates PR in the virion and/or at the early stage of infection. The notion that \( \Delta vif \) virions released from restrictive cells exhibit less stable cores than wild-type cores (Ohagen & Gabuzda, 2000) supports this suggestion. Experiments designed to assess the relevance of this model in HIV-1-infected cells are now being carried out in our laboratory.

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