

Mutational analysis of the HIV-1 auxiliary protein Vif identifies independent domains important for the physical and functional interaction with HIV-1 reverse transcriptase

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ABSTRACT

The HIV-1 accessory protein Vif plays a dual role: it counteracts the natural restriction factors APOBEC3G and 3F and ensures efficient retrotranscription of the HIV-1 RNA genome. We have previously shown that Vif can act as an auxiliary factor for HIV-1 reverse transcriptase (RT), increasing its rate of association to RNA or DNA templates. Here, by using seven different Vif mutants, we provide *in vitro* evidences that Vif stimulates HIV-1 RT through direct protein–protein interaction, which is mediated by its C-terminal domain. Physical interaction appears to require the proline-rich region comprised between amino acid (aa) 161 and 164 of Vif, whereas the RT stimulatory activity requires, in addition, the extreme C-terminal region (aa 169–192) of the Vif protein. Neither the RNA interaction domain, nor the Zn²⁺-binding domain of Vif are required for its interaction with the viral RT. Pseudotyped HIV-1 lentiviral vectors bearing Vif mutants deleted in the RNA- or RT-binding domains show defects in retrotranscription/integration processes in both permissive and nonpermissive cells. Our results broaden our knowledge on how three important functions of Vif (RNA binding, RT binding and stimulation and Zn²⁺ binding), are coordinated by different domains.

INTRODUCTION

The human immunodeficiency virus (HIV) virion infectivity factor (Vif) is essential for efficient viral replication in natural target cells (1–3). Vif counteracts the action of the cytosine deaminases APOBEC3G (4) and APOBEC3F

(5), that, in the absence of Vif, are incorporated into viral particles and, in the subsequent round of infection, deaminate C to U residues in newly synthesized HIV minus strand DNA, leading to G–A mutation in the HIV proviral DNA (6–10). In infected cells, Vif binds APOBECs and target them to degradation through recruitment of the ubiquitination enzymes ElonginB, C and Cullin 5, thereby preventing APOBECs incorporation into HIV-1 virions (11–15). The analysis of virions produced in nonpermissive cells in the absence of Vif led to conflicting reports, showing that they either have normal viral protein and RNA content (3,16–19), or show abnormal virion morphology (20–22). Nonetheless, a full consensus exists about the observation that Vif-deficient HIV-1 viruses produced in nonpermissive cells, enter the target cells normally but are defective in the production of reverse transcription products (2,3, 23–25). Although the Vif interactions with APOBEC3 ElonginB, C and Cullin 5 proteins are clearly important for virus replication and pathogenesis, Vif seems to have nonessential interactions with other viral proteins. For example, a role of Vif in the reverse transcription process has been postulated. Interestingly, Vif has been detected in HIV virions (17,26,27), binds the viral RNA (28–30), is a component of the reverse transcription complex in HIV-1 infected cells and is required for efficient reverse transcription *in vitro* and *in vivo* (29,31,32). We have previously shown that Vif stimulates the efficiency of HIV-1 reverse transcriptase (RT) *in vitro*, by increasing the rate of association of the enzyme to the primer/template and decreasing the thermodynamic barrier for ternary complex formation (33). However, the mechanistic details of this functional interaction are still unknown. One obvious question is whether this stimulation was dependent on direct protein–protein interaction between Vif and RT and/or on the interaction of Vif with the nucleic acid template. In this work, we characterized the biochemical

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properties of a panel of HIV-1 Vif mutants (Figure 1), showing that Vif physically interacts with HIV-1 RT and stimulates its catalytic activity through two independent domains located at its C-terminus. Moreover, we demonstrate here that neither the RNA binding nor the Zn²⁺-finger domains are required for this interaction. By using pseudotyped lentiviral vectors (LVs), we show that both the RNA-binding and the RT-binding functions are important for efficient retrotranscription/integration of lentivirus, independently from the presence of APOBECs. Our results indicate Vif as the bridging factor in a complex network of potentially competing protein-protein interactions which are mediated by independent functional Vif domains.

MATERIALS AND METHODS

Cells

The HEK-293T cells were propagated in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal calf serum (FCS) (EuroClone Ltd, Paington, UK) and a combination of penicillin-streptomycin and glutamine (PSG). SupT1 and CEMss T-cell lines were grown in RPMI 1640 containing 10% FCS and PSG.

Chemicals

[³H] dTTP (40 Ci/mmol) and [γ -³²P] ATP (3000 Ci/mmol) were from GE Healthcare and unlabeled dNTP's from Roche Molecular Biochemicals. Whatman was the supplier of the GF/C filters. All other reagents were of analytical grade and purchased from Merck or Fluka.

Nucleic acids substrates

The homopolymeric template poly(rA₂₀₀)/oligo(dT₂₀)_{10:1} (Roche Molecular Biochemicals) was prepared as described (33). The 40-mer Poly(rA) oligonucleotide (polyrA₄₀) was purchased by MWG (Florence, Italy).

Mutagenesis

Deletion mutants of HIV-1 were generated by polymerase chain reaction (PCR)-mediated mutagenesis using the WT-Vif Δ LNGFR plasmid as DNA template (34). The gene fragments corresponding to amino acids (aa) 44–192 (Vif Δ N1-43), aa 1–140 [Vif Δ C(141–192)], aa 1–143 [Vif Δ C(144–192)], aa 1–160 [Vif Δ C(161–192)] or aa 1–168 (Vif Δ C169–192) were amplified with specific primers. Chim3 gene encodes a chimeric protein in which a 45-aa-spanning region of F12-Vif (aa 126–170) (34) is embedded in the wt-Vif backbone. Chim3 gene was generated by DNA synthesis (Primm s.r.l, Milano). The PCR-generated and Chim3 mutants were then inserted into pGEX-T4-1 vector for *Escherichia coli* expression and isolation of GST-Vif fusion protein. The recombinant plasmids were constructed by replacing the BamHI–NotI fragment of pGEX with the PCR amplified Vif gene, either full length or the truncated versions. The triple mutant P161A, P162A, P164A, Vif(3P3A) was obtained from Bio-Fab Research Ltd. (Rome, Italy). All the

recombinant proteins were expressed and purified as described below.

Protein expression, purification and western blot analysis

The vector pGEX with the wild type or mutated Vif gene was transformed into BL21 competent cells (Novagen, Madison WI). After growth at 37°C up to optical density of \approx 0.6, the expression of GST-Vif proteins was induced by adding 1 mM of isopropylthio- β -D-galactoside (IPTG). The bacterial cells were lysed by adding lysis buffer (0.25 M Tris-HCl pH 7, Triton X-100 1%, SDS 0.03%, NP-40 0.5%, Tween-20 0.1%, dithiothreitol (DTT) 5 mM, lysozyme 1 μ g/ml) followed by sonication. The supernatants have been conserved and the pellets were resuspended by adding 5 vol of urea buffer (NaPO₄ 0.1 M, 0.01 M Tris-HCl pH 8, NP-40 0.01%, urea 6 M) and sonicated. The supernatant has been inserted into a dialysis membrane (Pierce, Thermo Fischer Scientific) and left overnight at 4°C under magnetic stirring in dialysis buffer (Tris-HCl pH 7, 0.25 M, Triton X-100 1%, SDS 0.03%, NP-40 0.5%, Tween-20 0.1%, DTT 5 mM). The supernatants were applied to equilibrated glutathione-conjugated GSH-Sepharose beads (GE Healthcare) and left shaking overnight at 4°C. Then, the samples were centrifuged and the supernatants were conserved (flow-through). The beads were washed in PBS and then eluted with the Elution Buffer (PBS 1X pH 7.4, GSH 0.03%) for 3 h at 4°C. Eluted fractions containing GST-Vif were stored at –80°C. Samples were analyzed by western blotting with primary antibodies goat anti-GST-rabbit and HRP-anti-Rabbit IgG antibody as the secondary antibody. A chemiluminescence-based system (Pierce Thermo Scientific) was applied to visualize the reacting bands. Proteins were 90% pure as judged by Coomassie staining of SDS-PAGE.

Enzymatic assays

RNA-dependent DNA polymerase RT activity was assayed as described (33). Briefly, a 25- μ l final reaction volume contained TDB buffer [50 mM Tris-HCl pH 8.0, 1 mM DTT, 0.2 mg/ml bovine serum albumin (BSA), 2% glycerol], 10 mM MgCl₂, 0.5 μ g of poly(rA):oligo(dT)_{10:1} (0.3 μ M 3'-OH ends), 10 μ M [³H]dTTP 1 Ci/mmol, 5–10 nM RT and the indicated amounts of recombinant Vif proteins. After incubation at 37°C for indicated time, 20 μ l from each reaction tube were spiked on glass fiber filters GF/C and, immediately, immersed in 5% ice-cold trichloroacetic acid (TCA) (AppliChem GmbH, Darmstadt). Filters were washed three times with 5% TCA and once with ethanol for 5 min, then dried and, finally, added with EcoLume[®] Scintillation cocktail (ICN, Research Products Division, Costa Mesa, CA, USA), to detect the acid-precipitable radioactivity by PerkinElmer[®] Trilux MicroBeta 1450 Counter.

Electrophoretic mobility shift gel assay (EMSA)

Purified recombinant Vif proteins were incubated 5 min at 37°C in the presence of 5'-labeled poly(rA₄₀), in 10 μ l of reaction buffer (50 mM Tris-HCl pH 7.0, 0.25 mg/ml BSA, 1 mM DTT and 1 mM Mg²⁺). Samples were mixed

with nondenaturing gel loading buffer [40% (w/v) sucrose, 0.25% bromophenol blue] and subjected to PAGE on a 5% native gel at 4°C for 2 h at 5 V/cm. Position of the free probe and of the protein–DNA complexes on the gel was visualized by laser scanning densitometry.

Zn⁺⁺-binding assay

Recombinant Vif proteins were incubated overnight at 4°C in the presence of PBS 1X containing 100 mM of the Zn⁺⁺-chelating agent N,N,N,N-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN, Sigma-Aldrich). Next, the proteins were dialyzed against PBS 1X to remove TPEN and immediately applied to a FPLC-HiTrap IMAC FF column (GE Healthcare) previously charged with Zn⁺⁺ according to the manufacturer's protocol and subsequently equilibrated in PBS 1X. Column was washed with PBS 1X and eluted with a linear 0–0.5 M gradient of Imidazole in PBS 1X. Collected fractions were subjected to SDS–PAGE and transferred to a nitrocellulose membrane. Proteins were visualized by immunoblot with anti-GST antibodies. In a control experiment performed with GST only, no GST protein was retained on the column (data not shown).

Pull-down experiments

For the pull-down assays, 5 µg of his-RT and 5 µg of the GST-Vif-wt and GST-Vif-mutant proteins were used. After incubation on ice for 10 minutes, either GSH-Sepharose or Ni-NTA Agarose Beads (QIAGEN) were added, formerly equilibrated in PBS 1X. The proteins were incubated for an hour at 4°C and then centrifuged for 2 min. The supernatants were conserved in order to be analyzed on SDS–PAGE gels. The beads were washed twice in PBS 1X and finally resuspended in Tris–HCl pH 7.5 and Laemmli loading buffer 1X and heated for 5 minutes at 100°C. All samples were centrifuged in order to be electrophoresed in SDS–PAGE gels 12%. The gels were dried, transferred to nitrocellulose membranes and analyzed by western blotting.

Kinetic analysis

Reactions were performed under the conditions described for the HIV-1 RT activity assay. The dependence of the initial velocity of the reaction as a function of variable nucleic acid substrate concentrations (0–300 nM) was measured in the absence or in the presence of fixed increasing amounts (0–40 nM) of the various Vif proteins. Kinetic parameters (k_{cat} , K_m , k_{cat}/K_m) were determined by nonleast squares computer fitting methods utilizing the Michelis–Menten equation. Values used were the means of three independent experiments.

Pseudotyped LVS generation and cellular transduction by LVs

The LVs pΔLNGFR (empty vector) bearing an internal PGK-ΔLNGFR selection marker cassette, and the wt Vif-pΔLNGFR, bearing the WT-Vif under the transcriptional control of the 5' LTR of the vector, were previously described in (34). The VifΔN-ΔLNGFR and

VifΔC-ΔLNGFR LVs were generated by inserting the 447-bp and 423-bp PCR amplicons, respectively, in the ClaI site of the pΔLNGFR empty vector. To amplify the two Vif fragments, the wt-Vif-pΔLNGFR plasmid, as DNA template, and the following primers were used:

for the VifΔN fragment:

Forward: 5'CCA TCG ATG GAT GAG TAC TAA TCC AAA AAT AAG TT-3'

Reverse: 5'CTC CTC TAA TCG ATG CTA GTG TCC ATT CAT TG-3'

for the VifΔC fragment:

Forward: 5'GCA AAG AAT CGA TGG GAT TAT GGA AAA CAG-3'

Reverse: 5'CCA TCG ATG GCT AGT TAT GTC CTG-3'

VSV-G pseudotyped LV stocks were produced by transient co-transfection of 293T cells with the transfer vectors described above, a second-generation packaging construct pCMVΔR8.74 and the pMD.G plasmid encoding the vesicular stomatitis envelope glycoprotein (VSV-G) (34). Viral particles were normalized by RT activity assay and by p24Gag Ag ELISA (COULTER test kit, Beckman Coulter, Fullerton, CA, USA) following manufacturer's instructions. Viral infectivity/integration of the VSV-G pseudotyped LVs was tested by spinoculating SupT1 and CEM A3.01 cells with RT activity-normalized amounts of LVs in the presence of polybrene (8 µg/ml), monitored 7 days after transduction by flow cytometry analysis of ΔLNGFR expression using the anti-human LNGFR monoclonal antibody C40-1457 (BD Pharmingen™), as previously described (34).

HIV-1 infection and RT activity assay

SupT1 and CEMss cells were acutely infected with the molecular clones X4 HIV-1_{NL4-3} and its Vif-deficient (Δ*vif*) derivative, which were produced by transient transfection of the corresponding plasmids in HEK-293T cells. Forty-eight hours after transfection, supernatants were collected, filtered and tested for RT activity. Equal amount of viruses were adsorbed to the cells for 2–5 h at 37°C, then washed out twice with PBS. Cells were eventually resuspended in complete medium and seeded at 0.5–1 × 10⁶/ml in triplicate in 96-well plate. Culture supernatants were harvested every 4 days and stored at –80°C until tested for Mg²⁺-dependent RT-activity assay following standard procedures (34).

RESULTS

HIV-1 Vif physically interacts with the viral reverse transcriptase through its C-terminal domain

We produced recombinant Vif fused to GST (GST-Vif) and recombinant RT fused to a six histidine tag (his-RT). The availability of two different tags allowed us to probe these proteins for physical interaction in pull-down assays. The schematic diagram of the various Vif proteins used is shown in Figure 1, along with their phenotypes, as

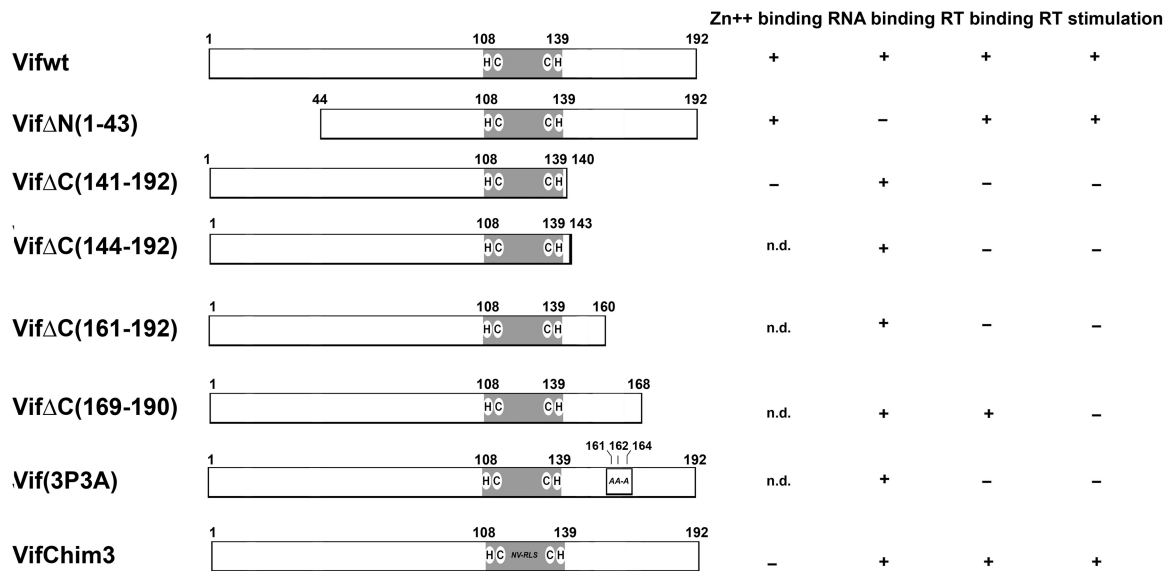


Figure 1. Schematic representation of the different Vif mutants used in this study along with their phenotypes. The Zn⁺⁺-finger domain (aa 108–139) is shaded in gray. The histidines (H) and cysteines (C) essential for Zn⁺⁺ binding are indicated in bold letters. The five unique mutated aa present within the Chim3 Zn⁺⁺-finger are indicated in bold italic letters. The position of the three Ala mutated to Pro residues in the Vif3P3A mutant are indicated. n.d.: not determined. + sign identifies positive interaction, – sign identifies lack of interaction.

revealed in this study. As shown in Figure 2A, full-length HIV-1 Vif was able to pull down HIV-1 RT, thus demonstrating a direct protein–protein interaction between these two proteins. By comparing two Vif mutants, one lacking the first 43 aa at its N-terminal (VifΔN1-43 aa) and one lacking the last 51 aa (VifΔC141–192), for their ability to stimulate the catalytic activity of HIV-1 RT, we have previously hypothesized that the C-terminal domain was important for this function, since the VifΔC141–192 could not stimulate RT activity (33). The VifΔN1-43 and VifΔC141–192 mutant proteins were then tested in pull-down assays for interaction with HIV-1 RT. The VifΔN1-43 mutant was able to physically interact with HIV-1 RT (Figure 2B), whereas the C-terminal deletion in VifΔC141–192 prevented physical interaction between Vif and RT (Figure 2C). Thus, the RT interaction domain of Vif could be assigned to the last 51 aa of the protein. We applied the same type of analysis to the Vif mutant Chim3, derived from the natural mutant F12-Vif (35). The Chim3 mutant contains five unique point mutations in the Zn⁺⁺-finger domain (aa 128–133 *NV-V-RLS*). As shown in Figure 2G, Chim3 was able to physically interact with HIV-1 RT. Next, wt Vif and the mutants Chim3, ΔC and ΔN were tested for their effects on HIV-1 RT activity *in vitro* (Figure 2I). Wild-type Vif, the Chim3 and the ΔN mutants were able to increase the nucleic acid substrate utilization efficiency (k_{cat}/K_m) of HIV-1 RT, whereas ΔC was not. These results identify the C-terminal domain of Vif as essential for the interaction with HIV-1 RT.

The physical interaction of HIV-1 Vif with the viral reverse transcriptase and the stimulation of reverse transcription activity are mediated by two separate domains

In order to further define the domain responsible for the physical and functional interaction between HIV-1

RT and the Vif protein, we constructed three additional Vif mutants carrying progressively shorter deletions at the C-terminus (VifΔC144–192, VifΔC161–192 and VifΔC169–192). Only the VifΔC169–192 mutant was able to physically interact with HIV-1 RT in a pull-down assay (Figure 2F), whereas the other two mutants carrying the aa144–192 and aa161–192 deletions were not (Figure 2D and E). However, when tested in an *in vitro* RT assay, the VifΔC169–192 was found to strongly inhibit the reaction (Figure 2L). These results show that physical interaction between Vif and RT is mediated by the aa 161–168 of Vif, whereas the stimulatory activity requires, in addition, the extreme C-terminal region (aa 168–192) of the protein. In order to further define the interaction domain of Vif with RT, we created a triple mutant P161A, P162A, P164A, called ‘Vif(3P3A)’ and evaluated its ability to physically and functionally interact with HIV-1 RT. As shown in Figure 2H and I, the Vif(3P3A) mutant was not able to pull down the recombinant RT, nor to stimulate the HIV-1 RT activity, indicating that the 161–164 Pro-rich domain was important for the RT–Vif interaction.

RNA binding is not required for the functional interaction of Vif with HIV-1 reverse transcriptase

Both HIV-1 RT and Vif can bind RNA. In order to verify whether the observed stimulation of RT activity by Vif required also interaction of Vif with the RNA template, we tested our panel of mutants for their ability to interact with RNA in EMSAs. As shown in Figure 3, among the mutants tested, only the VifΔN lost its ability to interact with RNA. Since this mutant retained full ability to stimulate HIV-1 RT, we can conclude that the functional interaction between Vif and RT does not require RNA binding. The mutants VifΔC144–192, VifΔC161–192 and Vif 3P3A were also similarly tested

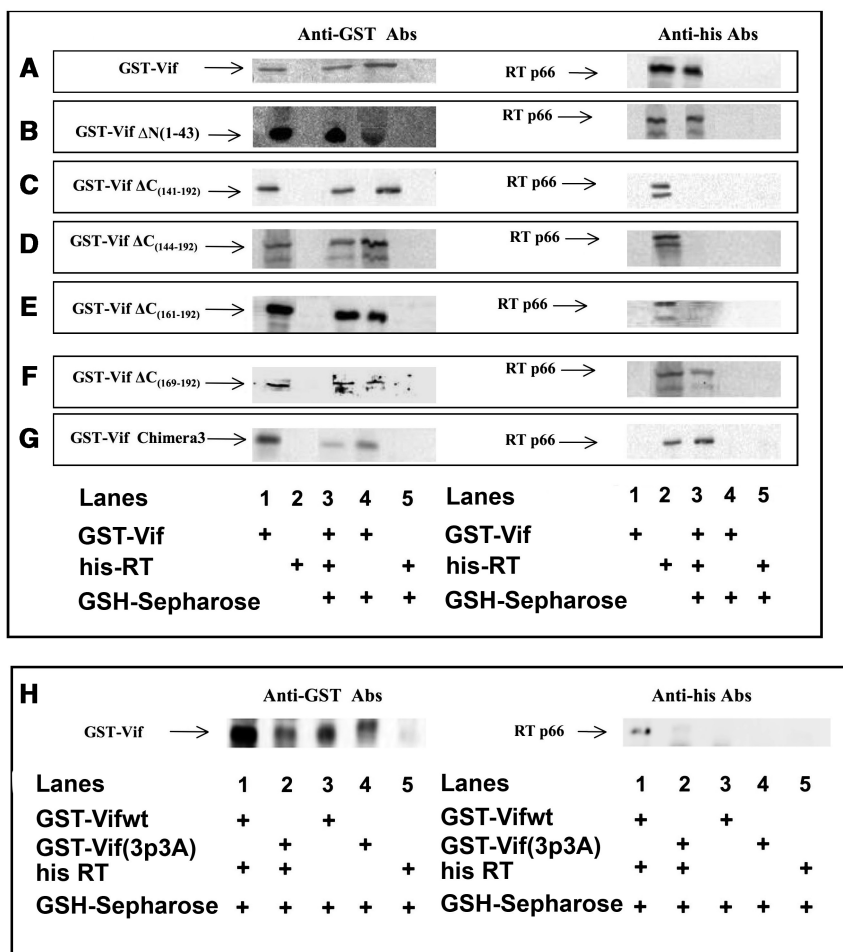


Figure 2. The C-terminal domain of Vif is required for binding and stimulation of the HIV-1 RT activity. (A) Pull-down assay in the presence of GST-Vif wild type (lanes 3 and 4), his-tagged RT (lanes 3 and 5) and GSH-sepharose beads (lanes 3–5). Proteins were visualized with antibodies against the GST- (left part) or the his- (right part) tags, respectively. Lane 1, GST-Vif positive control; lane 2, his-RT positive control. (B) As in (A) but in the presence of GST-VifΔN(1–43). (C) As in (A), but in the presence of GST-VifΔC(141–192). (D) As in (A), but in the presence of GST-VifΔC(144–192). (E) As in (A), but in the presence of GST-VifΔC(161–192). (F) As in (A), but in the presence of GST-VifΔC(169–192). (G) As in (A), but in the presence of GST-VifChim3. (H) Pull down assay in the presence of his-RT and GST-Vifwt or GST-Vif(3p3A). (I) Variation of the nucleic acid substrate utilization efficiency ($k_{cat}/K_{m3'OH}$) of HIV-1 RT as a function of increasing amounts (7.5 nM, 15 nM, 25 nM and 40 nM) of Vif wild type (circles), Chim3 (triangles), VifΔN(1–43) (squares) and VifΔC(141–192) (diamonds). Error bars are \pm SD of three independent replicates. (L) Variation of the velocity of the reaction catalyzed by HIV-1 RT as function of increasing nucleic acid substrate concentrations, in the absence (circles) or in the presence of 80-nM wild-type Vif (circles), 80-nM VifΔC(169–192) or 20-nM VifΔC(169–192). Error bars are \pm SD of three independent replicates.

and found to bind RNA as the wild type (data not shown). The RNA-binding domain of Vif has been previously located in the N-terminal part of the protein (aa 1–64) (32). We show here that deletion of the first 43 aa of Vif is enough to completely abrogate its RNA-binding activity, thus further narrowing down the RNA-binding domain.

The Zn⁺⁺-finger domain of HIV-1 Vif is not required for interaction with RNA or with the viral reverse transcriptase

HIV-1 Vif contains an H-C2-H Zn⁺⁺-finger domain (aa 108–139), which has been shown to be important for protein–protein interactions (35–38). Both the VifΔC and ΔN mutants retained this domain. Conversely, the Chim3

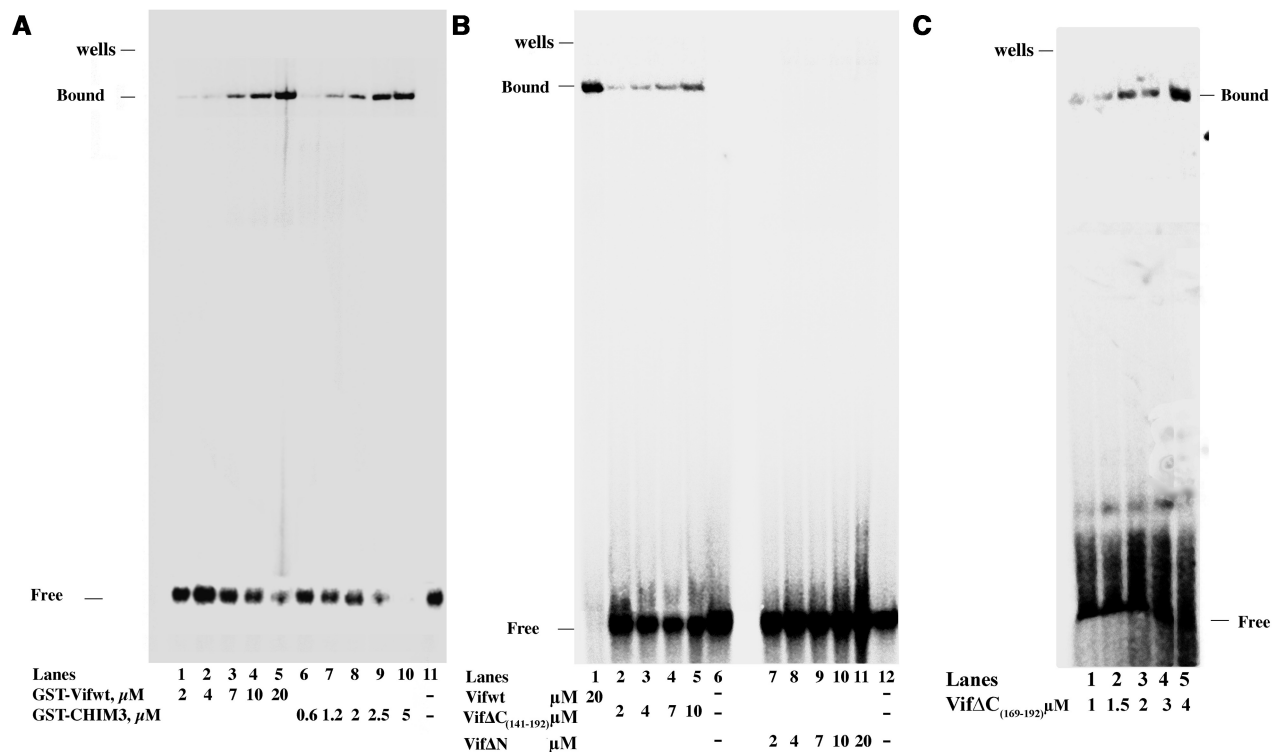


Figure 3. The N-terminal domain of Vif is required for nucleic acid binding. (A) Gel retardation assay in the absence (lane 11) or in the presence of GST-Vif wild type (lanes 1–5) or GST-Chim3 (lanes 6–10) and a 5'-labeled RNA probe (rA40). Protein–RNA complexes were separated from the free probe on a nonreducing polyacrylamide gel. (B) The same as in (A), but in the absence (lanes 6 and 12) or in the presence of GST-Vif wild type (lane 1), GST-Vif $\Delta\text{C}_{(141-192)}$ (lanes 2–5) or GST-Vif $\Delta\text{N}_{(1-43)}$ (lanes 7–11). (C) The same as in (A), but in the presence of GST-Vif $\Delta\text{C}_{(169-192)}$.

mutant had five unique point mutations within this domain. Since the functionality of the Zn^{++} -finger domain strongly relies on proper folding, which might have been affected by the deletions/mutations, we directly verified the ability of the different mutants to bind to a Zn^{++} -based affinity column (HiTrap-IMAC, see 'Materials and Methods' section). Wild-type Vif (Figure 4A) and the ΔN (Figure 4B) mutant were retained on the column, whereas the $\Delta\text{C}_{141-192}$ (Figure 4C) and Chim3 (Figure 4D) mutants were not. The inability of the Vif $\Delta\text{C}_{141-192}$ mutant to bind Zn^{++} is likely due to misfolding of the H-C2-H domain, suggesting that additional amino acids in the close proximity of the His139, on the C-terminal side of the protein, are important for stabilization of this domain. Anyhow, the fact that the Chim3 mutant bearing a nonfunctional Zn^{++} -finger was still able to interact physically and functionally with RT (Figure 2C), demonstrated that the Zn^{++} -finger domain is dispensable for interaction of Vif with HIV-1 RT. In addition, the fact that both Vif $\Delta\text{C}_{141-192}$ and Chim3 mutants were able to interact with RNA (Figure 3), indicated that the Zn^{++} -finger domain is also dispensable for interaction of Vif with the nucleic acid lattice.

The RNA- and reverse transcriptase interacting domains of Vif are important for viral production and proviral integration

In order to understand the contribution of the different domains of Vif, identified in our *in vitro* studies, on the

reverse transcription and integration process of LVs in single round infection of human T cells, we produced recombinant VSV-G-pseudotyped LVs bearing either wt Vif or the mutants $\Delta\text{C}_{141-192}$ and ΔN_{1-43} . LVs in fact are a good surrogate of HIV-1 infection because they perform reverse transcription and integrations processes identically to HIV-1. The different LVs were quantified by both RT activity assay and p24Gag ELISA. As shown in Figure 5A, pseudotyped LVs bearing both ΔC and ΔN mutants showed much lower virus yields (as indicated by the RT activity in the supernatant) than wt Vif. Next, permissive (SupT1) or non permissive (CEM A3.01) cells were single round infected with the different VSV-G-pseudotyped LVs (normalized by RT activity) and the frequency of integration events was measured by FACS analysis of the constitutive expression of the selection marker ΔLNGFR LNGFR carried by the LVs (34). Both permissive (Figure 5B) and nonpermissive (Figure 5C) cells transduced with the ΔC and ΔN mutants, consistently showed lower integration levels than those infected with wt Vif LVs. Pseudotyped LVs carrying no Vif (empty controls) showed even lower integration levels than the ΔC and ΔN mutants, indicating that the effects observed for these two mutants were not due to lack of expression of the mutated Vif proteins. The defective integration noted for LVs carrying no Vif even in permissive cells, indicated the importance of Vif for HIV-1 replication also in permissive cells. To further demonstrate

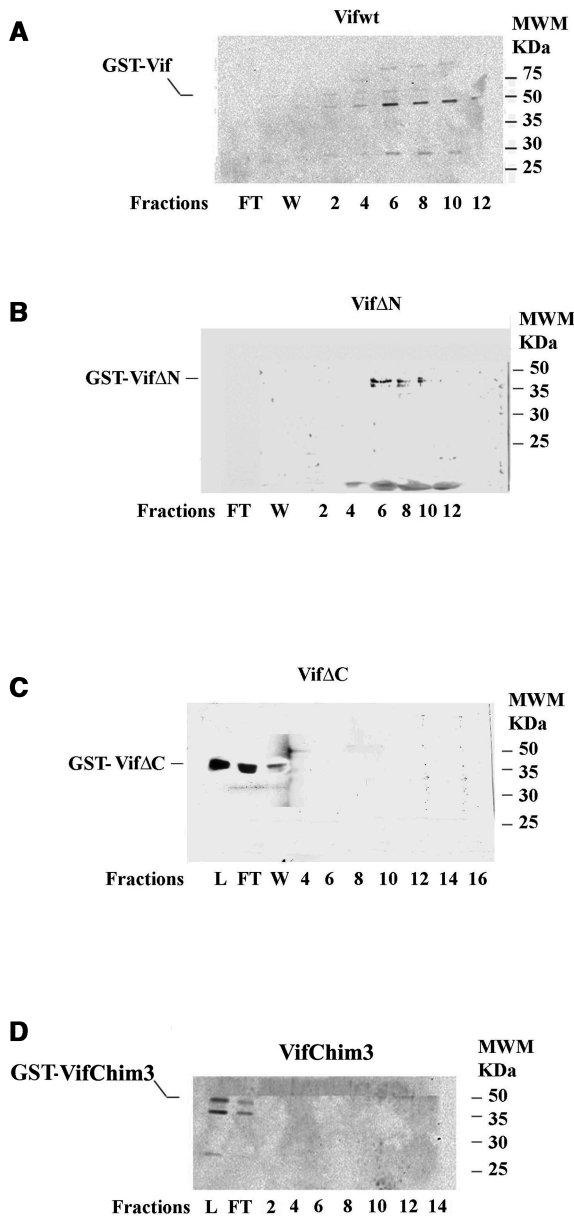


Figure 4. A functional Zn^{++} -finger domain is not required for RNA binding and RT stimulation. (A) Recombinant GST-Vif wild type was eluted from a Zn^{++} -chelating column with a linear gradient of imidazole. Fractions were immunoblotted with antibodies against GST. FT, flow through; W, wash. (B) As in (A), but in the presence of GST-Vif Δ N(1–43). (C) As in (A), but in the presence of GST-Vif Δ C(141–192). (D) As in (A), but in the presence of GST-Chim3Vif.

that HIV-1 Vif bears an APOBEC3G-independent function deriving from its interaction with RT, we infected permissive cells with Vif-proficient and Vif-deficient HIV-1 at two different MOI as indicated (Figure S1). CEMss (panel A and B) and SupT1 (panel C and D) were followed for 24 days after HIV-1 infection. In all conditions, it is manifest that the kinetic of infection of Vif-deficient HIV-1 is delayed and does not reach the peak of viral production at an extent equivalent to that of Vif-proficient HIV-1 (Figure S1). These results are in

agreement with our recent results demonstrating that HIV-1 Vif is involved in the accumulation of retrotranscripts during the early steps of HIV-1 life cycle (35).

DISCUSSION

HIV-1 Vif has been proposed to be required for efficient retrotranscription of the viral RNA genome independently of APOBEC3G. Indeed, analysis of the retrotranscription complexes (RTCs) formed in the absence of Vif, revealed structural and functional alterations (21,31,32). Vif has been detected in HIV virions (17,26,27), binds the viral RNA (28–30), is a component of the reverse transcription complex in HIV-1-infected cells and is required for efficient reverse transcription *in vitro* and *in vivo* (29,31–33). However, the mechanistic and molecular details of this function are poorly known. Here, we provide evidences that Vif stimulates HIV-1 RT through direct protein–protein interaction, which is mediated by its C-terminal domain. Physical interaction appears to require the region comprised between aa 161 and 168, particularly the 161–164 Pro-rich domain, whereas the RT stimulatory activity requires, in addition, the extreme C-terminal region (aa 169–192) of the Vif protein.

It has been proposed that Vif can act as an RNA chaperone, thus stabilizing the right conformation of RTCs and facilitating tRNA primer annealing (39). By producing Vif mutants deleted in their RNA-binding or RT-binding domains, we show here that interaction with RNA is not required for the observed stimulation of the RT activity by Vif. These results suggest that the RNA chaperone and RT stimulation activities of Vif are not interdependent, but can likely act cooperatively to increase the efficiency of the retrotranscription process.

Another domain of Vif important in mediating protein–protein interactions is the H-C2-H Zn^{++} -finger domain (12,36–38). By using a Vif chimeric mutant (Chim3) which bears a nonfunctional Zn^{++} finger domain, we demonstrate here that the ability of Vif to bind Zn^{++} is not required for its functional interaction with RT. In addition, RNA-binding experiments allowed us to identify the aa 1–43 as the region of Vif required for RNA binding, and showed that the Zn^{++} finger domain is not important for interaction with the nucleic acid.

Experiments with VSV-G-pseudotyped LVs bearing either wild-type Vif or the Δ C and Δ N mutants, showed that the absence of either the N-terminal (RNA-binding) or C-terminal (RT-binding) domains can affect viral production and integration, independently from the presence of APOBECs. A marked reduction in integration levels was measured for Δ Vif LVs, even in permissive cells (Figure 5), which correlated with slower replication kinetics of Δ Vif HIV-1 (Figure S1).

A schematic representation of all the protein–protein interaction domains so far identified in the protein Vif is shown in Figure 5D. At least three regions at the N-terminal part (domain Ia, aa 14–17, Ib, aa 40–44 and Ic, aa 52–79) have been shown to mediate the interaction with APOBEC3G-3F (40–42). In the middle part of the protein there is the Zn^{++} -finger (domain II, aa 108–139)

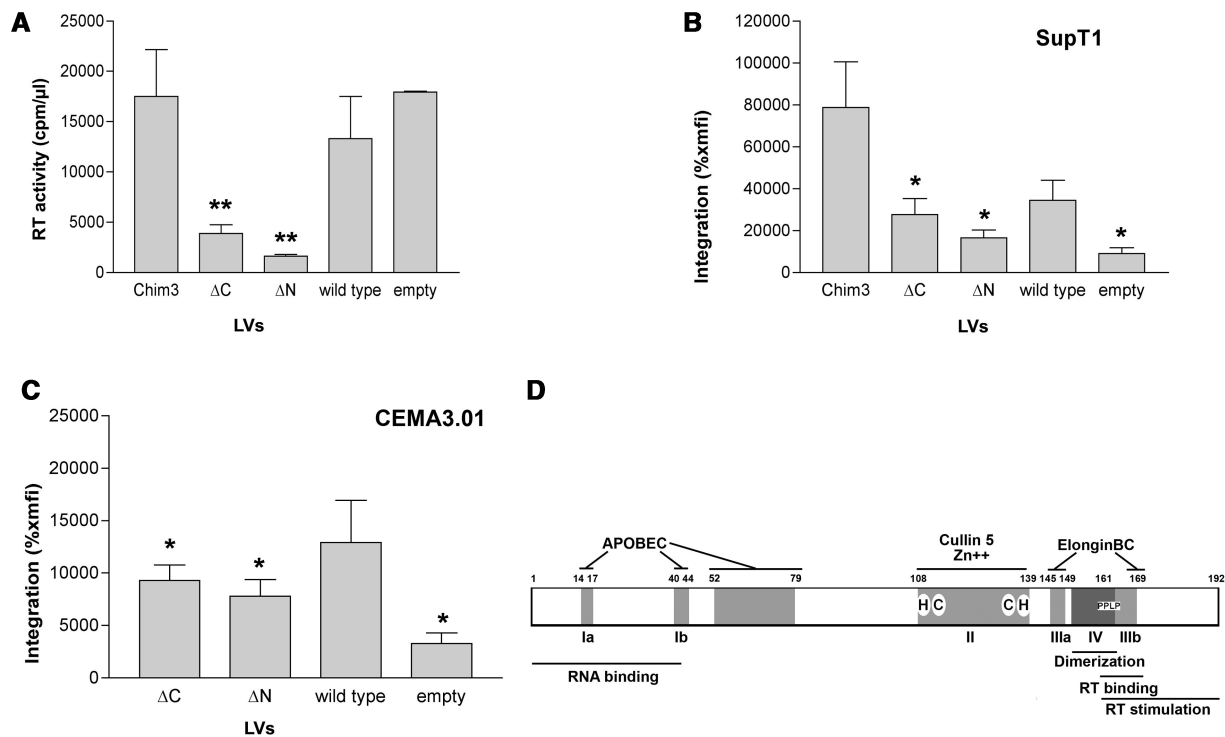


Figure 5. The RNA- and RT-binding domain are important for viral replication and integration. (A) RT activity detected in the supernatant of VSV-G pseudotyped HIV-1-derived LV-producing cells (293T) co-transfected with the transfer vector plasmids carrying no Vif (empty), Vif wild type, Vif Δ N(1–43) or Vif Δ C(141–192) together with the packaging and the VSV-G envelope glycoprotein constructs. (B) Frequencies of integration events, measured as the product of the percent of NGFR⁺ cells and the mfi values, in permissive cells (SupT1) transduced with LVs bearing no Vif (empty), Vif wild type, Vif Δ N(1–43) or Vif Δ C(141–192). (C) The same as in (B), but in nonpermissive cells (CEM A3.01). (D) Schematic representation of the known functional domains of Vif responsible for its interaction with various proteins. In (A–C), values were the means of three independent replicates. Statistical analysis was performed by One-way ANOVA–Tukey multiple test. Statistically significant differences from the reference value (wild type) are indicated by * P <0.1; ** P <0.05.

which has been implicated in mediating direct interaction with Cullin 5. At the C-terminus, the bipartite SOCS Box (domain IIIa, aa 145–149 and IIIb, aa 163–169) has been suggested to mediate the interaction with the ElonginBC complex (12,43). Furthermore, the 151–164-region encompassing the 161–164 Pro-rich domain (domain IV), was shown to be important for Vif dimerization (44).

Our results indicate that the RNA-binding domain overlaps with two of the APOBEC3F/G interaction sites (domain Ia and Ib), whereas the RT interaction domain spans through the dimerization and ElonginBC-binding domains. Homology models of Vif tertiary structure suggested that the Vif C-terminal tail comprising aa 150–192 is likely to be exposed to the solvent, thus representing a possible interaction domain with host proteins (45). Moreover, circular dichroism studies of a synthetic Vif C-terminal domain peptide indicated a high propensity of this domain to assume a disordered structure unless bound to a ligand (46). Thus, a highly regulated order of binding must exist, to ensure that Vif correctly fulfills its various biological functions. HIV-1 viruses lacking Vif can replicate and spread in permissive cells, but several reports indicated that the absence of Vif often reduces the replicative ability of these viruses even in the absence of APOBEC (20,47–50). These observations,

along with the finding that HIV-1 viruses bearing defective Vif proteins are circulating in the human population (51) and that APOBEC proteins can inhibit the viral replication targeting RT activity in a cytidine deaminase-independent manner (52), suggest that much has still to be learned about the Vif-RT-APOBEC axis. Along this line, our results indicate that three important functions of Vif (RNA binding, RT binding and Zn⁺⁺ binding), are independently coordinated by different domains. By selectively targeting each of these domains, either alone or in combination, it would be possible to exploit novel anti-HIV strategies which combine the highest efficacy in suppressing viral proliferation with the lowest cytotoxicity.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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