Identification of a Critical T(Q/D/E)_x5ADx₂(I/L) Motif from Primate Lentivirus Vif Proteins That Regulate APOBEC3G and APOBEC3F Neutralizing Activity[▽]

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Primate lentiviruses are unique in that they produce several accessory proteins to help in the establishment of productive viral infection. The major function of these proteins is to clear host resistance factors that inhibit viral replication. Vif is one of these proteins. It functions as an adaptor that binds to the cytidine deaminases APOBEC3G (A3G) and APOBEC3F (A3F) and bridges them to a cullin 5 (Cul5) and elongin (Elo) B/C E3 ubiquitin ligase complex for proteasomal degradation. So far, 11 discontinuous domains in Vif have been identified that regulate this degradation process. Here we report another domain, T(Q/D/E)_x5ADx₂(I/L), which is located at residues 96 to 107 in the human immunodeficiency virus type 1 (HIV-1) Vif protein. This domain is conserved not only in all HIV-1 subtypes but also in other primate lentiviruses, including HIV-2 and simian immunodeficiency virus (SIV), which infects rhesus macaques (SIVmac) and African green monkeys (SIVagm). Mutations of the critical residues in this motif seriously disrupted Vif's neutralizing activity toward both A3G and A3F. This motif regulates Vif interaction not only with A3G and A3F but also with Cul5. When this motif was inactivated in the HIV-1 genome, Vif failed to exclude A3G and A3F from virions, resulting in abortive HIV replication in nonpermissive human T cells. Thus, T(Q/D/E)_x5ADx₂(I/L) is a critical functional motif that directly supports the adaptor function of Vif and is an attractive target for inhibition of Vif function.

Vif is a small viral protein that has 192 amino acids and is expressed by most lentiviruses, except for equine infectious anemia virus. It was first discovered in human immunodeficiency virus type 1 (HIV-1) (13, 14, 31), and its function in HIV-1 infection has been studied extensively (9, 34). Infection of human T-cell lines with *vif*-defective (Δ Vif) HIV-1 identified two different cell types, namely, permissive cells that can be infected by Δ Vif HIV-1 and nonpermissive cells, which are resistant to Δ Vif HIV-1 (10, 36). Genomic complementation analysis indicated that these nonpermissive cells express a Vif-sensitive dominant viral inhibitor(s) (17, 27). The first inhibitor identified was APOBEC3G (A3G) (25), which belongs to the APOBEC (*apolipoprotein B* mRNA-editing catalytic polypeptide) family. In humans, this family consists of APOBEC1; activation-induced deaminase (AID); APOBEC2; a subgroup of APOBEC3 (A3) proteins, including A3A, A3B, A3C, A3DE, A3F, A3G, and A3H; and APOBEC4. All seven A3 genes have been shown to inhibit replication of various types of retrovirus by cytidine deamination-dependent and -independent mechanisms, as reviewed recently (21, 30, 35). In particular, human A3B, A3DE, A3F, A3G, and A3H inhibit HIV-1 replication, whereas A3A and A3C do not (2, 5, 6, 25, 39, 46). Among these, the protein expression of A3G and A3F in human primary tissues has been demonstrated, and *in vitro* studies indicate that these proteins have the most potent anti-HIV-1 activities. A3G and A3F share ~50% sequence simi-

larity but have different biochemical properties (38) and different target sequence preferences while catalyzing cytidine deamination of viral cDNAs (15).

Vif hijacks the cellular proteasomal machinery to destroy A3G and A3F by the protein degradation pathway (18, 26, 33, 46). Vif acts as an adaptor protein that bridges A3 proteins to a cullin 5 (Cul5)-based E3 ubiquitin ligase complex, which includes Cul5, EloB, and EloC (44). These interactions trigger the polyubiquitylation of Vif, A3G, and A3F and direct them to 26S proteasomes for degradation. Thus, Vif binding to A3G or A3F as well as to Cul5/EloBC is a critical step for A3G and A3F degradation. Although A3G and A3F share a high level of homology, different surfaces are used for Vif interaction. Vif binds to the N-terminal region of A3G, from residues 126 to 132, and to the C-terminal region of A3F, from residues 283 to 300 (12, 24). In addition, 11 discontinuous motifs in the Vif protein have been identified as regulating Vif interactions with A3G, A3F, or the Cul5/EloBC E3 ligase complex. Three motifs determine Vif interaction with the E3 ligase. The ¹⁰⁸Hx₅Cx₁₇₋₁₈Cx₃₋₅H¹³⁹ motif, also called the HCCH zinc finger, binds to Cul5 (16, 20, 41); the ¹⁴⁴SLQYLA¹⁴⁹ motif, which is also called the BC box, binds to EloC (19, 45); and the ¹⁶¹PPLPx₄L¹⁶⁹ motif, which is also called the Cul box, binds to Cul5 (32, 45). The ¹⁶¹PPLP¹⁶⁴ subdomain has multiple activities, which not only determine Vif dimerization (43) but also regulate Vif binding to A3G (8, 37) and EloB (1). The other 8 motifs regulate the interaction between Vif and A3G/A3F. The ²¹WxSLVK²⁶ (3, 7) and ⁴⁰YRHHY⁴⁴ (23) motifs regulate Vif binding to A3G; the ¹¹Wx₂DRMR¹⁷ (23), ⁷⁴TGERxW⁷⁹ (11), and ¹⁷¹EDRW¹⁷⁴ (4) motifs regulate Vif binding to A3F; and the ⁵⁵VxIPLx₄L⁶⁴ (11), ⁶⁹YxxL⁷² (22), and ⁸¹LGxGx₂LxW⁸⁹ (4) motifs regulate Vif

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FIG. 1. Identification of a highly conserved Vif sequence. (A) Amino acid sequence comparison of a region from residues 81 to 107 in HIV-1 Vif with those in other primate lentivirus Vif proteins. Residues with high conservation are shown in red, those with low conservation are shown in blue, and those that are not conserved are shown in black. (B) Conservation of the T(Q/D/E)xxPxxADxx(I/L) motif in primate lentivirus Vif proteins. Vif sequences from the indicated virus strains were identified in GenBank and analyzed for the presence of this motif.

binding to both A3G and A3F. The ⁸¹LGxGxxIxW⁸⁹ motif also regulates Vif binding to Cul5 (4). Thus, HIV has developed rather complicated mechanisms to assemble a protein degradation complex to neutralize these two critical host factors. A full understanding of these mechanisms is essential for pharmaceutical inhibition of Vif function to prevent HIV-1 infection. Here we report another functional motif from a previously uncharacterized region of HIV-1 Vif that regulates Vif interactions with A3G, A3F, and the Cul5/EloBC E3 ligase complex. Since this Vif region is the only one left uncharacterized, this is a significant step toward a complete understanding of this important host-pathogen interaction.

MATERIALS AND METHODS

Plasmids. The HIV-1 proviral constructs pNL4-3Δvif, pNL-LucΔvif, and pNL-LucΔenvΔvif and the mammalian expression vectors pcDNA3.1-huA3F-V5-6xHis, pcDNA3.1-huA3F-FLAG-HA, pcDNA3.1-huA3G-V5-6xHis, pcDNA3.1-huA3G-FLAG-HA, pcDNA3.1-GFP-FLAG-HA, pcDNA3.1-aggA3F-V5-6xHis, and pcDNA3.1-aggA3G-V5-6xHis were described before (4, 6, 38). The Vif expression vectors pNL-A1, pNL-A1Δvif, pNL-A1SIVmacVif, and pNL-A1SIVagmVif were obtained from K. Strebel. The Cul5 expression vector VR-Cul5-HA was obtained from X. Yu. For HIV-1 Vif mutant expression, vif genes were first cloned into the pCR4-TOPO vector (Invitrogen) and then mutated by use of a QuikChange XL site-directed mutagenesis kit (Stratagene). These vif genes were then cloned back into pNL-A1NotI/XbaI/HA by NotI and XbaI digestion. A modified pNL4-3 vector containing NotI and XbaI sites in the vif open reading frame, pNL4-3NotI/XbaI, was created before (47). Some of these mutated vif genes were cloned into this vector by NotI and XbaI digestion.

Vif activity assay. Vif activities were measured by the ability to rescue HIV-1 Δvif infectivity in the presence of A3G or A3F. Viruses were produced from 293T cells by a standard calcium phosphate transfection procedure. Typically, 20 μg of plasmid DNA, including 5 μg pNL-LucΔenvΔvif, 5 μg Vif expression vector, 1 μg vesicular stomatitis virus glycoprotein (VSV-G) expression vector, and 10 μg A3 expression vector, was transfected into 293T cells in a 100-mm culture dish at 20% confluence. The production of HIV-1 was quantified by p24^{Gag} capture enzyme-linked immunosorbent assay (ELISA). Equal amounts of viruses were used to infect GHOST-R3/X4/R5 cells. Thirty-six hours later, cells were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 3 mM EDTA

(lysis buffer). After removal of the nuclei, the cytosolic fraction was used to determine luciferase activity, using a luciferase assay kit (Promega).

HIV-1 growth curve. The human T-cell lines CEM-SS and H9 were cultured in complete RPMI 1640 culture medium containing 10% fetal bovine serum, 10 μg/ml ampicillin, and 50 μg/ml streptomycin. Viruses were produced from 293T cells after transfection with pNL4-3 expressing wild-type or mutant vif genes. A total of 2 × 10⁵ T cells were infected with HIV-1 containing 150 ng p24^{Gag} for 2 h at 37°C. After being washed, cells were resuspended in a 48-well culture plate, and culture supernatants were collected every other day for ELISA measurement of viral p24^{Gag}.

Immunoprecipitation. To determine Vif binding to A3G, A3F, or Cul5, 293T cells were transfected with pNL4-3 expressing different vif genes in the presence of A3G-FLAG-HA, A3F-FLAG-HA, or Cul5-HA expression vector at a 6:1 ratio by a standard calcium phosphate transfection protocol. Twenty-four hours later, cells were lysed in a lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.6, 3 mM EDTA, 1% Triton X-100). The cytosolic fraction was isolated and then rocked with anti-hemagglutinin (anti-HA) affinity matrix (Roche) for 4 h at 4°C. After being washed extensively with lysis buffer, bead-associated proteins were detected by Western blotting.

Western blotting. Horseradish peroxidase (HRP)-conjugated anti-HA antibody (Roche) or HRP-conjugated anti-V5 antibody (Invitrogen) was used to directly detect the expression of A3F or A3G (anti-V5) or of Vif protein (anti-HA). Actin was detected by a polyclonal antibody (C-11; Santa Cruz Biotechnology). HIV-1 p24^{Gag} and Vif proteins were detected by two antibodies (no. 3537 and 2221) from the NIH AIDS Research and Reference Reagent Program. HRP-conjugated anti-rabbit and anti-mouse immunoglobulin G secondary antibodies were from Pierce. Detection of HRP-conjugated antibody was performed using an enhanced chemiluminescence detection kit (Amersham Biosciences).

RESULTS

Identification of a highly conserved amino acid sequence from an uncharacterized region of primate lentivirus Vif proteins. Searching for conserved residues by use of sequence alignment is a very useful approach for identification of functional motifs within a protein. So far, 11 functional motifs have been identified in HIV-1 Vif proteins, and all residues within these domains are highly conserved. Among the 192 HIV-1 Vif residues, there was one region, residues 91 to 107, next to the ⁸¹LGxGxxIxW⁸⁹ domain, that remained uncharacterized

(Fig. 1A). We aligned the amino acid sequences of this region among different Vif proteins from HIV-1, HIV-2, simian immunodeficiency virus (SIV) from chimpanzees (SIVcpz), SIVmac239, and SIVagm strains. We identified six almost completely conserved residues. These residues are summarized as the consensus sequence T(Q/D/E)_x₂Px₂ADx₂(I/L) (Fig. 1A). It is present in 98% of 2,638 Vif sequences from HIV-1, 96.3% of 27 Vif sequences from HIV-2, 79.8% of 109 Vif sequences from SIVcpz, 87.5% of 32 Vif sequences from SIVmac, and 100% of 6 Vif sequences from SIVagm (Fig. 1B). Thus, this is a highly conserved sequence among primate lentivirus Vif proteins and therefore may have an important biological function.

Function of the ⁹⁶T(Q/D/E)_x₂Px₂ADx₂(I/L)¹⁰⁷ sequence in HIV-1. To understand the function of the T(Q/D/E)_x₂Px₂ADx₂(I/L) sequence, we created a large number of HIV-1 Vif mutants bearing single amino acid mutations in the region of residues 91 to 107. Initially, 17 mutants were created, including K91D, R92D, R93D, Y94A, S95A, T96A, Q97A, V98S, D99R, P100S, D101R, L102S, A103D, D104R, Q105A, L106S, and I107A mutants. Next, we determined whether these mutants were able to neutralize human A3G and A3F. The D99R mutant was poorly expressed and was not included in this experiment. Vif activity was measured by the level of enhancement of ΔVif HIV-1 infectivity in the presence of A3G or A3F expression in viral producer cells, using a single-round replication assay. In the absence of Vif, viruses were poorly infectious, indicating potent antiviral activities of both A3G and A3F, although A3G showed a much stronger activity than that of A3F (Fig. 2A, lane 1, and B, lane 1). Almost all of the mutants neutralized A3G and A3F as well as wild-type Vif did, except for two mutants, the A103D and D104R mutants. The A103D and D104R mutants completely lost activity toward both A3G and A3F (Fig. 2A and B, lanes 14 and 15). We also detected A3G or A3F protein expression in viral producer cells in the presence of these mutants by Western blotting. It was found that all of the Vif mutants were expressed well (Fig. 2A and B, lower panels). Importantly, unlike the other mutants, both the A103D and D104R mutants failed to decrease both A3G and A3F expression (Fig. 2A and B, lanes 14 and 15, lower panels), which is consistent with the viral infectivity assay results. Thus, we identified A103 and D104 as critical residues for Vif activity.

Since residues T96, Q97, P100, and I107 are highly conserved, we wondered why T96A, T97A, P100S, and I107 mutations did not disrupt Vif activity. Since the T96A, Q97A, P100S, and I107A mutations are relatively conservative substitutions, they may not be significant enough to change the Vif phenotype. Previously, we and others found that charges are important for Vif function (3, 7). Accordingly, we changed residue T96 to either D (negatively charged) or R (positively charged), residue Q97 to R, and residue I107 to R, creating another four mutants, the T96D, T96R, Q97R, and I107R mutants. Since both P and S are polar residues, we changed P100 to the nonpolar amino acid A, creating the P100A mutant. In addition, since the D99R mutant was not expressed, we created a D99K mutant to see how residue D99 contributes to Vif activity. When these mutants were expressed in viral producer cells in the presence of A3G or A3F, we found that the T96D, T96R, and I107R mutants lost the ability to neutralize

both A3G and A3F, the Q97R mutant selectively lost activity toward A3F, and the D99K and P100A mutants were still active against both proteins (Fig. 2A and B, lanes 19 to 26). We also determined A3G and A3F protein expression in viral producer cells and found that Vif mutants that failed to neutralize A3G and A3F also failed to decrease their expression levels, as determined by Western blotting (Fig. 2A and B, lanes 19 to 26, lower panels). Thus, we identified T96, Q97, and I107 as other critical residues for Vif function. Since residue P100 did not seem to be essential from these experiments, we summarized this conserved sequence as the functional motif ⁹⁶T(Q/D/E)_x₅ADx₂(I/L)¹⁰⁷.

Further characterization of residues A103 and D104 in HIV-1. In the A103D and D104R mutants, either the uncharged nonpolar residue A was changed to negatively charged D or the negatively charged D residue was changed to positively charged R. These mutations may have changed the overall charges on the Vif protein, resulting in a loss of Vif function. To further confirm that charges affect Vif function, we introduced additional mutations at these two positions. We changed the uncharged residue A103 to positively charged R or uncharged S, V, and Y residues, with differences in polarity, creating another four mutants, the A103R, A103S, A103V, and A103Y mutants; and we changed the negatively charged residue D104 to uncharged A, S, and Y residues, creating another three mutants, the D104A, D104S, and D104Y mutants. When their activities were determined, we found that the A103R, A103Y, and D104Y mutants had completely lost activity against both A3G and A3F; the D104S mutant had partially lost activity against both A3G and A3F; the D104A mutant had selectively lost partial activity toward A3G; and the A103S and A103V mutants were still active against both A3G and A3F (Fig. 3A and B). These results were further confirmed by Western blotting of A3G or A3F protein expression in the presence of these mutants in viral producer cells (Fig. 3A and B, lower panels). Taken together, these results confirm that Vif activity is significantly affected by the charges on the protein. In addition, the side chains of the amino acids at these two positions may also affect Vif activity.

Function of T(Q/D/E)_x₅ADx₂(I/L) motif in SIVs. To understand whether the T(Q/D/E)_x₅ADx₂(I/L) motif regulates Vif activity in SIVs, we introduced single or double mutations in this motif into SIVmac and SIVagm Vif proteins. Five SIVmac239 Vif mutants were created, including TD/RR (T98 and D99 were changed to arginines), P102A, A105D, D106R, and L109R mutants; five SIVagm Vif mutants were also created, including TE/RR (T99 and E100 were changed to arginines), P103A, A106D, D107R, and I110R mutants. The SIVmac239 Vif activity was determined against human A3G (huA3G) and huA3F, and SIVagm Vif activity was determined against African green monkey A3G (agmA3G) and agmA3F. It was found that for SIVmac, the TD/RR, A105D, D106R, and L109R Vif mutants completely lost activity toward both A3G and A3F, and the P102A mutant slightly lost activity toward huA3G and huA3F (Fig. 4A and B). Similarly, for SIVagm, the TE/RR, A106D, D107R, and I110R mutants almost completely lost activity toward both agmA3G and agmA3F, and the P103A mutant slightly lost activity toward agmA3F but not agmA3G (Fig. 4C and D, lanes 4). These results were further confirmed by Western blotting when A3G

FIG. 2. Mutational analysis of HIV-1 Vif residues 91 to 107. 293T cells were transfected with the HIV-1 reporter construct pNL-Luc Δ env Δ vif, pNL-A1 expressing the indicated HIV-1 Vif-HA protein, a VSV-G expression vector, and a human A3G (gray bars) (A) or A3F (white bars) (B) expression vector. The infectivity of the virus produced from the transfected cells was determined by infection of GHOST cells and quantitation of luciferase enzyme produced in the GHOST cells, after normalization by level of viral input (p24^{Gag}). Vif activities are presented relative to the wild-type Vif protein activity, with infectivity in the presence of wild-type (WT) Vif set to 100%. A3G or A3F expression in the presence of various Vif mutants in 293T cells was also determined by Western blotting (lower panels). The A3G and A3F proteins were detected by an anti-V5 antibody, and Vif proteins were detected by an anti-HA antibody. The standard errors of the means (SEM) were calculated for three independent experiments.

FIG. 3. Further characterization of HIV-1 Vif residues A103 and D104. HIV-1 Vif mutants expressed from pNL-A1 and bearing the indicated mutations at amino acid positions 103 and 104 were analyzed for neutralizing activity toward A3G (A) and A3F (B), as described in the legend to Fig. 2. The SEM were calculated for three independent experiments.

or A3F protein expression was determined in the presence of these mutants in viral producer cells (Fig. 4A to D, lower panels). Taken together, we concluded that the T(Q/D/E)_xADx₂(I/L) motif is also critical and plays a similar function in SIVs.

Regulation of Vif interaction with A3G, A3F, and Cul5 by the T(Q/D/E)_xADx₂(I/L) motif. Since the T(Q/D/E)_xADx₂(I/L) motif is critical for Vif activity, we wondered whether it acts through regulation of Vif interaction with A3G, A3F, or Cul5, as shown previously for the other motifs. In order to test these interactions by immunoprecipitation, 11 *vif* mutant genes, including T96D, T96R, Q97R, P100A, A103D, A103R, A103Y, D104A, D104R, D104Y, and I107R mutants, were cloned back into the proviral HIV-1 construct pNL4-3 from the HIV-1 subgenomic expression vector pNL-A1, so these Vif proteins no longer expressed the HA tag. The G84D mutant was included as a control because we have previously demonstrated that this mutant fails to interact with A3G and A3F but not Cul5 (4). A3G, A3F, and Cul5 proteins fused with an HA tag were coexpressed with the wild-type or mutant Vif proteins in 293T cells. Proteins were pulled down by Sepharose beads conjugated with anti-HA antibodies, and bead-associated proteins were determined by Western blotting. It was found that all of these Vif proteins, except for the T96D and P100A mutants, completely lost their interaction with A3G and A3F (Fig. 5A and B). The P100A mutant still interacted with A3G and A3F, but at reduced levels, and the T96D mutant showed further decreased levels of such interaction (Fig. 5A and B, lanes 2 and 5). In addition, like the G84D mutant, the T96D, Q97R, P100A, A103D, D104A, D104Y, and I107R mutants still interacted with Cul5, whereas the T96R, A103R, A103Y, and D104R mutants showed much-reduced interac-

tions with Cul5 (Fig. 5C). Overall, we concluded that the T(Q/D/E)_xADx₂(I/L) motif determines Vif interaction with A3G, A3F, and Cul5.

Regulation of HIV-1 in human T-cell lines by T(Q/D/E)_xADx₂(I/L) motif. To further confirm the importance of the T(Q/D/E)_xADx₂(I/L) motif, seven HIV-1 mutants bearing a mutation in this motif were produced, and their replication in permissive and nonpermissive human T-cell lines was compared to that of the wild-type virus. These HIV-1 mutants were T96D, T96R, Q97R, P100A, A103R, D104Y, and I107R mutants. All of the mutations except for P100A impaired Vif's ability to neutralize A3G and A3F and disrupted Vif binding to A3G, A3F, and/or Cul5 in our previous experiments. First, we tested whether these different Vif proteins were encapsidated into virions and whether they failed to exclude A3G or A3F protein from virions. Virions were prepared from 293T cells cotransfected with each proviral construct plus an A3G or A3F expression vector and were purified via ultracentrifugation. Proteins in virions were then analyzed by Western blotting. It was found that all of these Vif mutant proteins were encapsidated as efficiently as the wild-type protein (Fig. 6A and B, middle panels). In addition, all mutants except the P100A mutant failed to reduce A3G and A3F protein levels in virions (Fig. 6A and B, top panels). In this experiment, the P100A mutant was as active as the wild-type Vif protein (Fig. 6A and B, lanes 2, 6, and 8).

Next, we compared viral replication in permissive CEM-SS cells versus nonpermissive H9 cells. These cells were infected with equal amounts of each virus, and viral replication was determined by measuring the p24^{Gag} concentration in the supernatant, using an ELISA kit. In CEM-SS cells, the wild-type and ΔVif viruses and all seven Vif mutant viruses, including the

FIG. 4. Mutational analysis of the T(Q/D/E) $_5$ AD $_2$ (I/L) motif in SIVmac Vif and SIVagm Vif proteins. 293T cells were transfected with the HIV-1 reporter construct pNL-Luc Δ env Δ vif and a VSV-G expression vector plus the indicated SIVmac expression vector and a human A3G expression vector (A), the indicated SIVmac expression vector and a human A3F expression vector (B), the indicated SIVagm expression vector and an agmA3G expression vector (C), or the indicated SIVagm expression vector and an agmA3F expression vector (D). Viral infectivity as well as protein expression of A3G, A3F, and Vif in 293T cells was analyzed as described in the legend to Fig. 2. The SEM were calculated for three independent experiments.

T96D, T96R, Q97R, P100A, A103R, D104Y, and I107R mutants, grew equally well (Fig. 6D). In contrast, in H9 cells, only the wild-type and P100A mutant virus replicated well, whereas the Δ Vif virus and other mutant viruses showed approximately two- to three-log reductions in viral production (Fig. 6C). Among these particular mutants, the Q97R and T96D mutants replicated slightly better but still showed an $\sim$ 100-fold reduction of viral particle production compared to the wild-type and P100A mutant viruses. The I107R, D104Y,

A103R, and T96R mutants replicated as badly as the Δ Vif virus. Thus, we have confirmed that the T(Q/D/E) $_5$ AD $_2$ (I/L) motif plays a critical role in the regulation of HIV-1 replication in nonpermissive cells.

DISCUSSION

In this report, we identify a functional motif, T(Q/D/E) $_5$ AD $_2$ (I/L), in primate lentivirus Vif proteins. Sequence

FIG. 5. Regulation of protein interactions by the T(Q/D/E)_xADx₂(I/L) motif. Vif binding to A3G (A), A3F (B), or Cul5 (C) was determined by immunoprecipitation. HIV-1 Vif proteins expressed from pNL4-3 with the indicated mutations were coexpressed with A3G-HA, A3F-HA, or Cul5-HA protein in 293T cells. Proteins were pulled down with anti-HA antibodies and analyzed by Western blotting. A3G, A3F, and Cul5 expression was detected by an anti-HA antibody, and Vif expression was detected by a Vif-specific antibody.

analysis indicated that it is highly conserved in HIV-1, HIV-2, and SIVs. Our biological analyses demonstrate that this motif critically determines Vif neutralizing activity against A3G and A3F and that its integrity is essential for HIV-1 replication in nonpermissive human T cells.

Among the 192 amino acids of HIV-1 Vif, 106 are conserved in 95% of >2,000 HIV-1 isolates (data not shown). The Vif N terminus contains 16 of these conserved residues, and this region overlaps with the C terminus of the viral polymerase (Pol) protein. Since the *pol* gene is essential for viral replication, the conservation of these 16 residues probably reflects that of the Pol protein. Nonetheless, the ¹¹Wx₂DRMR¹⁷ motif, which regulates Vif activity against A3F, is located in this region. The Vif C terminus contains 4 of these conserved residues (P177, R184, M189, and G191), and this region overlaps with the N terminus of the Vpr protein. Among the remaining 86 of the conserved residues, 64 are located inside the 11 functional motifs, and the other 14 are distributed outside these motifs. Among these 14 residues, 3 (H28, S32, and W38) are between ²¹WxSLVK²⁶ and ⁴⁰YRHHY⁴⁴, 4 (P49, S52, S53, and E54) are between ⁴⁰YRHHY⁴⁴ and ⁵⁵VxIPLx₄L⁶⁴, 1 (T68) is between ⁵⁵VxIPLx₄L⁶⁴ and ⁶⁹YxxL⁷², 2 (V142 and G143) are between ¹⁰⁸Hx₆Cx₁₇₋₁₈Cx₃₋₅H¹³⁹ and ¹⁴⁴SLQYLA¹⁴⁹, and 4 (L150, A152, L153, and P156) are between ¹⁴⁴SLQYLA¹⁴⁹ and ¹⁶¹PPLPx₄L¹⁶⁹. Residues S32, W38, and L150 have previously been found to have a strong impact, whereas residues H28, P49, S52, S53, E54, and P156 were found to have no impact on Vif activity (23, 28, 29, 42). The role of residues T68, N142, N143, A152, and L153 in Vif activity has not been studied. Y94, T96, Q97, P100, A103, D104, L106, and I107 are the

remaining 8 of those 86 conserved residues, and they are located in the newly identified T(Q/D/E)_xADx₂(I/L) motif.

Among these 8 residues, we found that mutation of Y94, P100, and L106 did not change Vif activity (Fig. 2), and Y94 and L106 are less conserved among primate lentivirus Vif proteins (Fig. 1). In contrast, any mutation in T96, Q97, A103, D104, or I107 seriously disrupted Vif activity. However, not all types of mutation led to disruption of Vif activity. T96A, Q97A, and I107A mutations did not disrupt Vif function, while T96D, T96R, Q97R, and I107R mutations did (Fig. 2). In addition, at position 103, A103D and A103R mutations, but not A103S and A103V mutations, were able to disrupt Vif activity; at position 104, D104R, D104A, D104S, and D104Y mutations were all able to disrupt Vif activity (Fig. 2 and 3). Previously, Yamashita et al. also reported that the T96A mutation did not change Vif activity against A3G and A3F, while the D104A mutation did (42). Arginine (R) is positively charged, and aspartate (D) and glutamate (E) are negatively charged residues. The T(Q/D/E)_xADx₂(I/L) motif contains two negatively charged residues. Any increase or decrease of positively or negatively charged residues in this motif resulted in disruption of Vif activity. Previously, we and others have demonstrated that the ²¹WxSLVK²⁶ motif is also very sensitive to charge changes (3, 7). Taken together, these results suggest that charge is an important factor for regulation of Vif activity.

Charges could be important for Vif and A3G/A3F interactions. Previously, we reported that changes in the charge of HIV-1 Vif residues 22, 26, and 84 severely disrupted Vif interaction with A3G and/or A3F (4, 7), indicating that

FIG. 6. Regulation of HIV-1 replication in nonpermissive human T cells by the T(Q/D/E)₅ADx₂(I/L) motif. Encapsidation of A3G (A) or A3F (B) into HIV-1 virions bearing *vif* mutations was determined. Virions were produced from 293T cells, and protein expression was measured by Western blotting. A3G and A3F were detected by an anti-V5 antibody, Vif was detected by a polyclonal antibody, and Gag was detected by a monoclonal antibody. Replication of these viruses was determined in H9 (C) and CEM-SS (D) cells by inoculating equal amounts of wild-type (WT), *vif*-defective (ΔVif), and different *vif* mutant (T96D, T96R, Q97R, P100A, A103R, D104Y, and I107R) viruses. Viral growth was monitored for 10 days by p24^{Gag} ELISA.

these associations could be mediated by electrostatic interactions between these proteins. Consistently, T96D, T96R, Q97R, A103D, A103R, D104A, D104R, D104Y, and I107R mutants in the T(Q/D/E)₅ADx₂(I/L) motif all failed to interact with A3G and A3F (Fig. 5A and B). However, it

seems that Vif and Cul5 binding is much less dependent on electrostatic interaction. Although the T96R, A103R, A103Y, and D104R mutants showed reduced binding, the T96D, Q97R, A103D, D104A, D104Y, and I107R mutants still bound to Cul5 (Fig. 5C). Thus, Vif may have developed

FIG. 7. (A) Schematic diagram of known functional motifs in HIV-1 Vif protein. Numbers indicate amino acid positions. Motifs that regulate A3F binding are in green, those that regulate A3G binding are in blue, those that regulate both A3G and A3F binding are in red, and those that regulate Cul5/EloBC binding are in pink. (B) Schematic representation of the process of Vif interacting with Cul5/EloBC and A3G or A3F to promote A3G and A3F protein degradation. Vif surfaces that recognize Cul5/EloBC are indicated in pink, those that recognize A3F are in green, and those that recognize A3G are in blue. The surface that recognizes both A3G and A3F is indicated in red.

different mechanisms to interact with these two different types of host proteins.

Although Vif is a small viral protein, it has evolved 12 distinct functional motifs to target A3G and A3F for proteasomal degradation, as summarized in Fig. 7A. Structural studies have demonstrated that the HCCH, SLOYLEA, and PPLPx₄L motifs directly interact with Cul5, EloC, and EloB, respectively (1, 40). All 9 other motifs have been found to regulate Vif interactions with A3G and/or A3F, but we do not know which of these motifs bind directly to A3G and/or A3F. Since these motifs are discontinuous and separated from each other, it is more reasonable to speculate that not all of them are directly involved in binding to A3G and/or A3F. Instead, most of them may only determine Vif structure and constitute unique surfaces that are specific for A3G and/or A3F interactions (Fig. 7B). In particular, there may exist three distinct surfaces on Vif proteins for A3G and A3F interactions, including one that recognizes both A3G and A3F and two that recognize either A3G or A3F but not both. Disruption of any of these motifs may damage these surfaces, which indirectly disrupts interactions between Vif and A3G or A3F. Thus, our results further highlight that the assembly of Vif, A3G or A3F, and Cul5/EloBC E3 ligase complexes is complex and extensively regulated. A precise understanding of the mechanisms of binding and regulation is essential for pharmaceutical design of Vif inhibitors as part of anti-HIV therapy.

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