Identification of ⁸¹LGxGxxIxW⁸⁹ and ¹⁷¹EDRW¹⁷⁴ Domains from Human Immunodeficiency Virus Type 1 Vif That Regulate APOBEC3G and APOBEC3F Neutralizing Activity[▽]

Ying Dang, Roderick W. Davis, Ian A. York, and Yong-Hui Zheng*

Departments of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan 48824-4320

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The human cytidine deaminases APOBEC3G (A3G) and APOBEC3F (A3F) potently restrict human immunodeficiency virus type 1 (HIV-1) replication, but they are neutralized by the viral protein Vif. Vif bridges A3G and A3F with a Cullin 5 (Cul5)-based E3 ubiquitin ligase and mediates their proteasomal degradation. This mechanism has been extensively studied, and several Vif domains have been identified that are critical for A3G and A3F neutralization. Here, we identified two additional domains. Via sequence analysis of more than 2,000 different HIV-1 Vif proteins, we identified two highly conserved amino acid sequences, ⁸¹LGxGxxIEW⁸⁹ and ¹⁷¹EDRW¹⁷⁵. Within the ⁸¹LGxGxxIEW⁸⁹ sequence, residues L81, G82, G84, and, to a lesser extent, I87 and W89 play very critical roles in A3G/A3F neutralization. In particular, residues L81 and G82 determine Vif binding to A3F, residue G84 determines Vif binding to both A3G and A3F, and residues ⁸⁶IEW⁸⁹ affect Vif binding to A3F, A3G, and Cul5. Accordingly, this ⁸¹LGxGxxIEW⁸⁹ sequence was designated the ⁸¹LGxGxxIxW⁸⁹ domain. Within the ¹⁷¹EDRW¹⁷⁵ sequence, all residues except N175 are almost equally important for regulation of A3F neutralization, and consistently, they determine Vif binding only to A3F. Accordingly, this domain was designated ¹⁷¹EDRW¹⁷⁴. The LGxGxxIxW domain is also partially conserved in simian immunodeficiency virus Vif from rhesus macaques (SIVmac239) and has a similar activity. Thus, ⁸¹LGxGxxIxW⁸⁹ and ¹⁷¹EDRW¹⁷⁴ are two novel functional domains that are very critical for Vif function. They could become new targets for inhibition of Vif activity during HIV replication.

The function of the lentiviral protein Vif is to neutralize the major host antiretroviral cytidine deaminases that belong to the APOBEC (apolipoprotein B mRNA-editing catalytic polypeptide) family, as recently reviewed by several investigators (18, 29, 31). 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 in humans. They have one or two copies of a cytidine deaminase (CDA) domain with a signature motif (HxEx₂₃₋₂₈PCx₂₋₄C), only one of which normally has deaminase activity.

All seven A3 genes have been shown to inhibit replication of various types of retrovirus via cytidine deamination-dependent or -independent mechanisms. In particular, human A3B, A3DE, A3F, A3G, and A3H inhibit human immunodeficiency virus type 1 (HIV-1) replication, whereas A3A and A3C do not (1, 3, 5, 26, 33, 37). Among these proteins, the expression of human A3G and A3F *in vivo* has been demonstrated, and *in vitro* studies indicate that they have the most potent anti-HIV-1 activity. A3G and A3F share ~50% sequence similarity but have different biochemical properties (32) and different target sequence preferences while catalyzing cytidine deamination of viral cDNAs (13). Expression of human A3B has not been detected (7), and a 29.5-kb deletion spanning from the 3' end of the A3A gene to the 8th exon of

the A3B gene, leading to the complete removal of the A3B gene, has been detected in certain human populations (12). Human A3H is also poorly expressed *in vivo* (20). It was reported that human A3H has four haplotypes (Hap I, II, III, and IV), and only Hap II, which is maintained primarily in African populations, could be stably expressed *in vitro* (19). However, expression of this protein has not been detected in any human populations. Thus, the primary function of HIV-1 Vif is to neutralize A3G, A3F, and, to a lesser extent, A3DE.

Vif hijacks cellular proteasomal machinery to destroy these host cytidine deaminases by protein degradation (15, 27, 30). Vif acts as an adaptor protein that bridges A3 proteins with a Cullin 5-based E3 ubiquitin ligase complex, which includes Cul5, Elongin B (EloB), and Elongin C (EloC) (35). Vif has a BC-box motif (¹⁴⁴SLQYLALA¹⁴⁹) that binds to EloC (16, 36) and an HCCH motif (¹⁰⁸Hx₅Cx₁₇₋₁₈Cx₃₋₅H¹³⁹) that binds to Cul5 (14, 17, 34). On the other hand, Vif also interacts with A3G and A3F. As a consequence of these interactions, A3G and A3F are polyubiquitylated and directed to 26S proteasomes for degradation. In addition, Vif may also inhibit A3 activity independently of proteasomal degradation (10, 11, 24).

Interactions between Vif and A3G/A3F are a key step for their proteasomal degradation, and this mechanism has been extensively studied. First, unique surfaces in A3G and A3F important for Vif interaction were identified, and interestingly, they are located in different regions of the two proteins (9, 23). Second, several discontinuous surfaces on Vif have been found to regulate A3G and/or A3F degradation. The ⁴⁰YRHHY⁴⁴ domain specifically binds to A3G and determines Vif specificity for A3G (22); the ¹¹WxxDRMR¹⁷ and ⁷⁴TGERxW⁷⁹ domains specifically bind to A3F and determine Vif specificity

* Corresponding author. Mailing address: 2215 Biomedical and Physical Sciences, Michigan State University, East Lansing, MI 48824-4320. Phone: (517) 884-5314. Fax: (517) 353-8957. E-mail: zhengy@msu.edu.

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for A3F (8, 22); and the ²¹WxSLVK²⁶, ⁵⁵VxIPLx₄L⁶⁴, and ⁶⁹YxxL⁷² domains determine Vif specificity for both A3G and A3F (2, 6, 8, 21). These results indicate that the mechanism that regulates Vif recognition of A3G and A3F is quite complicated, and understanding this mechanism is critical for pharmaceutical protection of A3G and A3F from Vif-mediated proteasomal degradation.

Based on our current knowledge of these functional domains, it has been thought that Vif interacts with A3G and A3F mainly via its N-terminal region and with Cul5 E3 ubiquitin ligase machinery via its C-terminal region. However, here we identify a new A3G and A3F regulatory domain from the central region and a new A3F regulatory domain from the C-terminal region of HIV-1 Vif. Our results indicate that A3G and A3F interaction surfaces on HIV-1 Vif are structurally complex, and more efforts are required for a complete understanding of this host-pathogen interactive mechanism.

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, and pcDNA3.1-macA3G-V5-6xHis were described previously (6). To express human APOBEC2, its cDNA was cloned into the mammalian expression vector pEF-BOS-3xHA by EcoRI and XbaI digestion. The Vif expression vectors pNL-A1, pNL-A1Δvif, and pNL-A1SIVmacVif were from K. Strebel. The Cul5 expression vector VR-Cul5-HA was from B. Liu and X. Yu. The lysine-free HIV-1 Vif expression vector pNL-A1Vif16K/R and the HIV-1 Vif mutant Y69A were previously described (4, 6). To make other Vif mutants, vif genes were first cloned into the pCR4-TOPO vector (Invitrogen) and mutated with the QuikChange XL site-directed mutagenesis kit (Stratagene). These vif genes were then cloned back into pNL-A1NotI/XbaI/HA by NotI/XbaI digestion. A modified pNL4-3 vector containing a NotI and an XbaI site in the vif open reading frame, pNL4-3NotI/XbaI, was created previously (38). Some of the mutated vif genes were cloned into this vector by NotI and XbaI digestion.

Vif activity assay. Vif activities were measured by their abilities to rescue HIV-1Δvif virus infectivity in the presence of A3G or A3F. Viruses were produced from 293T cells by a standard calcium phosphate transfection. Typically, 20 μg of plasmid DNAs containing 5 μg pNL-LucΔenvΔvif, 5 μg Vif-expression vector, 1 μg vesicular stomatitis virus G-protein (VSV-G) expression vector, and 10 μg A3 expression vector were transfected into 293T cells in a 100-mm culture dish with 20% confluence. The production of HIV-1 was quantified by p24^{Gag} capture enzyme-linked immunosorbent assay (ELISA). Equal numbers of viruses were used to infect GHOST-R3/X4/R5 cells. Thirty-six hours later, the cells were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 3 mM EDTA (lysis buffer). After the nuclei were removed, 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, Hut-78, and PM-1 were cultured in complete RPMI1640 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, the 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 and A3G/A3F binding, 293T cells were cotransfected with A3G-FLAG-hemagglutinin (HA) or A3F-FLAG-HA and Vif expression vectors at a 1:1 ratio. A green fluorescent protein (GFP)-FLAG-HA expression vector was included as a negative control. Twenty-four hours later, the cells were lysed in 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-FLAG antibody M2-conjugated beads (Sigma) for 4 h at 4°C. After extensive washing with phosphate-buffered saline (PBS) containing 500 mM NaCl, the bead-associated proteins were detected by Western blotting. To determine Vif and Cul5 binding, proviral pNL4-3 constructs expressing different Vif mutants were cotransfected with the Cul5-HA expression vector. A human APOBEC2-HA expression vector was included as a negative control.

Twenty-four hours later, the cells were lysed in the same buffer. The cytosolic fraction was isolated and then rocked with anti-HA affinity matrix (Roche) for 4 h at 4°C. After extensive washing with lysis buffer, the 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 GFP, A3F, A3G, or Vif protein. Actin was detected by a polyclonal antibody (C-11) (Santa Cruz Biotechnology). HIV-1 p24^{Gag} and Vif proteins were also detected by two antibodies (no. 3537 and no. 2221) from the NIH AIDS Research and Reference Reagent Program. HRP-conjugated anti-rabbit or anti-mouse immunoglobulin G secondary antibodies were from Pierce. Detection of the HRP-conjugated antibody was performed using an enhanced-chemiluminescence detection kit (Amersham Bioscience).

RESULTS

Identification of two conserved regions in HIV-1 Vif that regulate Vif activity. Previously, we identified a highly conserved ²¹WxSLVK²⁶ domain from lentiviral Vif proteins (6). To further extend this observation, we retrieved 2,683 distinct HIV-1 Vif sequences from GenBank and compared the conservation of each amino acid. Consistently, the previously identified functional domains, including ¹¹WxxDRMR¹⁷, ²¹WxSLVK²⁶, ⁴⁰YRHHY⁴⁴, ⁵⁵VxIPLx₄L⁶⁴, ⁶⁹YxxL⁷², ¹⁰⁸Hx₅Cx₁₇₋₁₈Cx₃₋₅H¹³⁹, ¹⁴⁴SLOYLALA¹⁴⁹, and ¹⁶³LPx₄L¹⁶⁹, were all well conserved (Fig. 1). In addition, we identified two new conserved sequences, ⁸¹LGxGxSIEW⁸⁹ and ¹⁷¹EDRWN¹⁷⁵, for which functions have not been demonstrated. The residues in these two sequences are almost 100% conserved, suggesting that these two regions may have conserved biological functions.

To understand whether they are critical for Vif function, we created two HIV-1 Vif mutants, SIEW/4A, with the four S86, I87, E88, and W89 residues changed to alanines, and EDRWN/5A, with the five E171, D172, R173, W174, and N175 residues changed to alanines, and then determined whether they were still able to neutralize human A3G and A3F. Vif activity was measured by the levels 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, the viruses were poorly infectious, indicating potent antiviral activity of both A3G and A3F, although A3G showed much stronger activity than A3F (Fig. 2A, lanes 2 and 6). The SIEW/4A mutant lost activity toward both A3G and A3F (Fig. 2, lanes 3 and 7), and the EDRWN/5A mutant lost activity toward A3F but not A3G (Fig. 2, lane 4 and 8). We also detected A3G or A3F protein expression in viral producer cells by Western blotting. It was found that the SIEW/4A mutant failed to decrease both A3G and A3F expression, and the EDRWN/5A mutant failed to decrease A3F but not A3G expression (Fig. 2, lanes 3, 4, 7, and 8, bottom). This result is consistent with that from viral infectivity assays. Thus, these results strongly suggested that these two regions play important roles in Vif activity.

Characterization of the ⁸¹LGxGxSIEW⁸⁹ region in HIV-1 Vif. To understand how the ⁸¹LGxGxSIEW⁸⁹ domain regulates Vif activity, we first introduced nine point mutations, L81S, G82D, G84D, V85S, S86A, I87A, E88A, W89A, and R90D, individually into the vif gene to see if they could disrupt Vif function. When these mutants were expressed in viral producer cells in the presence of A3G or A3F, it was found that only the G84D mutant completely lost activity toward A3G, whereas the others still retained almost full activity (Fig. 3A, lane 5).

FIG. 1. Amino acid sequence homology analysis of 2,683 different HIV-1 Vif proteins. The amino acid sequences of 2,683 192-residue HIV-1 Vif proteins were retrieved from GenBank, and unique sequences were aligned. At each position, the most common amino acid was identified and plotted as a percentage of all amino acids at that position. Conserved regions are indicated by boxes. Conserved regions corresponding to known motifs (as described in the text) are indicated. Previously uncharacterized conserved regions are indicated by question marks.

Mutants L81S, G82D, and G84D completely lost activity toward A3F; mutants V85S, I87A, and W89A partially lost activity toward A3F; and mutants S86A, E88A, and R90D retained full activity toward A3F (Fig. 3B, lanes 1 to 11). Because the G84D mutant lost activity toward both A3G and A3F, we introduced a mild mutation at that position to see how Vif activity could be affected. Instead of replacing the noncharged residue (G) with a negatively charged residue (D), it was re-

placed with a similar noncharged residue (A), creating a new mutant, G84A. Although this mutant could still neutralize A3G, it completely failed to neutralize A3F (Fig. 3A, lane 25, and B, lane 25). Thus, Vif protein could not tolerate even a much milder mutation at this position, suggesting that G84 is a critical residue for Vif function.

Since the SIEW/4A mutant completely lost activity toward both A3G and A3F (Fig. 2), this SIEW subdomain was further

FIG. 2. Critical roles of ⁸⁶SIEW⁸⁹ and ¹⁷¹EDRWN¹⁷⁵ residues in Vif function. 293T cells were transfected with HIV-1 reporter construct pNL-LucΔenvΔvif, an indicated HIV-1 Vif expression vector, a VSV-G expression vector, and either a human A3G or A3F 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 levels of viral input (p24^{Gag}). Vif activities are presented relative to the wild-type (WT) Vif protein activity, with infectivity in the presence of wild-type Vif set to 100%. A3G or A3F expression in the presence of various Vif mutants in 293T cells was also determined by Western blotting (bottom). The A3G or A3F protein was detected by an anti-V5 antibody, and Vif proteins were detected by an anti-HA antibody. The standard errors of the mean (SEMs) were calculated from three independent experiments.

characterized by introducing double and triple mutations. For easier understanding, we renamed the SIEW/4A mutant AAAA and named the other mutants similarly by direct use of the amino acid sequence of the four residues with corresponding alanine substitutions. For example, AAEW indicates the mutant with the first 2 residues (SI) mutated to alanines, AIEA indicates the mutant with the first (S) and last (W) residues mutated to alanines, and so on. It was found that, like the AAAA mutant, all mutants bearing triple mutations, such as SAAA, AAEEA, and AIAA, completely lost activity toward both A3G and A3F (Fig. 3A and B, lanes 20, 21, and 22), except that the AAAW mutant was still active toward A3G, but not A3F (Fig. 3A, lane 19). In the case of mutants with double mutations, the levels of retention of activity toward A3G and A3F varied, and the mutants with activity, from high to low, toward A3G were AAEW, AIEA, SIAA, and SAEA, and those with activity, from high to low, toward A3F were AAEW, AIEA, SAEA, and SIAA (Fig. 3A and B, lanes 15 to 18). The activities of mutants AAEW and AIEA were significantly higher than those of the other two, and the SAEA mutant was completely inactive. We also determined A3G or A3F protein expression levels in viral producer cells and found that Vif mutants that failed to rescue viral infectivity also failed to decrease A3G or A3F expression (Fig. 3A and B, bottom). These results indicated that (i) this ⁸¹LGxGxSIEW⁸⁹ region regulates Vif activity toward both A3F and A3G; (ii) residues L81, G82, and G84 play more crucial roles than the others; and

(iii) among the four SIEW residues, residue W89 and, to a lesser extent, residue I87 play more important roles than the other two residues for Vif function. Accordingly, we decided to call this domain ⁸¹LGxGxxIxW⁸⁹.

Characterization of the ¹⁷¹EDRWN¹⁷⁵ region in HIV-1 Vif. To understand how the ¹⁷¹EDRWN¹⁷⁵ residues regulate Vif activity, we created five mutants with a single mutation in this region: E171A, D172A, R173A, W174A, and N175A. Then, their activities against A3G and A3F were tested. Consistently, it was found that, like the EDRWN/5A mutant, all of these single-point mutants retained full activity toward A3G (Fig. 4, lanes 3 to 7). However, all of the mutants except N175 lost activity toward A3F (Fig. 4, lanes 10 to 14), although the E171A and R173A mutants still retained some marginal activity. Western blot analysis confirmed that these four mutants also selectively lost activity to decrease A3F, but not A3G, expression from viral producer cells (Fig. 4, bottom). Thus, we concluded that residues E171, D172, R173, and W174 are almost equally critical for selective regulation of HIV-1 Vif activity toward A3F and grouped them as the ¹⁷¹EDRW¹⁷⁴ domain.

The LGxGxxIxW and EDRW domains in SIVmac239 Vif. To further explore the functions of these two domains, we compared the amino acid sequences of Vif protein from different primate lentiviruses, including HIV-2, simian immunodeficiency virus (SIV) that infects rhesus macaques (SIVmac239), SIV that infects chimpanzees (SIVcpz), and SIVs that infect African green monkeys (AGM) (SIVagm). These two domains

FIG. 3. Mutational analysis of HIV-1 Vif residues 81 to 90. The activities of different HIV-1 Vif proteins with mutations in the amino acid 81-to-90 region were analyzed against human A3G (A) or A3F (B) as described in the legend to Fig. 2. In the case of mutants in the SIEW region (lanes 14 to 22), AAAA indicates the mutant with all 4 of these residues mutated to alanines, AAEW indicates the mutant with the first 2 residues (SI) mutated to alanines, AIEA indicates the mutant with the first (S) and last (W) residues mutated to alanines, and so on. A3G or A3F protein expression levels in viral producer cells were determined by Western blotting as described in the legend to Fig. 2 (bottom). The SEMs were calculated from three independent experiments.

are well conserved in Vif from SIVcpz, are partially conserved in HIV-2 and SIVmac239, and are not conserved in SIVagm Vif proteins. For example, the three critical residues L81, I87, and W89 in the LGxGxxIxW domain are completely conserved in SIVmac239 Vif (L82, I89, and W91), and the two critical residues E171 and R173 in the EDRW domain are only partially conserved in SIVmac239 Vif (Q170 and R172) (Fig. 5A). To determine how they regulate SIVmac239 Vif activity, we created three single-point mutants, L82S, Q170A, and R172A, and a quadruple-point mutant, RITW/4A, with R88, I89, T90, and W91 mutated to alanines. We first determined their activities toward A3G from rhesus macaques (macA3G). It was found that the activity of RITW/4A was completely lost, whereas the others were fully functional (Fig. 5B). Similar

results were also obtained from experiments in which human A3G (huA3G) and A3F (huA3F) were used as targets (Fig. 5C and D). Western blot analysis of A3G or A3F expression from viral producer cells further confirmed these observations (Fig. 5B, C, and D, bottom). The fact that the RITW/4A mutant neutralized A3G and A3F was consistent with our previous results, and the fact that the L82S, Q170A, and R172A mutants still neutralized A3F was inconsistent. Thus, we concluded that the function of the LGxGxxIxW domain is partially conserved whereas that of the EDRW domain is not conserved in SIVmac239 Vif.

The mechanism involved in regulation of HIV-1 Vif activity by the ⁸¹LGxGxxIxW⁸⁹ and ¹⁷¹EDRW¹⁷⁴ domains. One critical question was whether the ⁸¹LGxGxxIxW⁸⁹ and ¹⁷¹EDRW¹⁷⁴

FIG. 4. Mutational analysis of HIV-1 Vif residues 171 to 175. The activities of different HIV-1 Vif proteins with mutations in this region were analyzed against human A3G or A3F, as described in the legend to Fig. 2. A3G or A3F protein expression levels in viral producer cells were determined by Western blotting as described in the legend to Fig. 2 (bottom). The SEMs were calculated from three independent experiments.

domains are involved in the regulation of Vif binding to A3G or A3F, as shown previously for other domains. To address this question, A3F and A3G proteins fused with a FLAG-HA tag were coexpressed with Vif proteins in 293T cells. The proteins were pulled down by Sepharose beads conjugated with anti-FLAG antibodies, and bead-associated proteins were determined by Western blotting. A3G and A3F showed strong binding to wild-type Vif, whereas GFP (as a control protein) did not (Fig. 6A, lanes 1 and 2, and B, lane 1). In addition, A3G showed very weak capability to bind to the Y69A mutant as reported (Fig. 6A, lane 7). These results indicated that this assay is specific for detection of A3 and Vif interactions. When mutants of $^{81}\text{LGxGxxIxW}^{89}$ and $^{171}\text{EDRW}^{174}$ were tested, it was found that A3G bound strongly to the W89A and EDRAWN/5A mutants but bound weakly to the G84D and SIEW/5A mutants (Fig. 6A, lanes 3 to 7); A3F showed reduced capability to bind to the L81S, G82D, G84D, SIEW/4A, and EDRAWN/5A mutants (Fig. 6B, lanes 3 to 7). To understand whether those mutations that affected Vif binding to both A3F and A3G might also affect other Vif activity, we next determined whether these mutants still bound to Cul5 by a similar immunoprecipitation assay. Since human APOBEC2 does not bind to HIV-1 Vif, the human protein was included as a negative control. It was found that wild-type Vif bound to Cul5 (Fig. 6C, lane 6), but not to APOBEC2 (Fig. 6C, lane 10), indicating that this assay is quite specific. In addition, both G84D and EDRAWN/5A mutants strongly bound to Cul5 (Fig. 6C, lanes 7 and 9), whereas the SIEW/4A mutant failed to bind to Cul5 (Fig. 6C, lane 8). Thus, the SIEW/4A mutation affects Vif binding, not only to A3G and A3F, but also to Cul5. In general, these data further support our previous conclusions that the $^{81}\text{LGxGxxIxW}^{89}$ domain regulates Vif activity toward both A3F and A3G whereas the $^{171}\text{EDRW}^{174}$ domain regulates Vif activity toward only A3F, and they highlight the fact that direct protein-protein interaction is critical for

this regulation. However, the $^{81}\text{LGxGxxIxW}^{89}$ domain could potentially regulate Vif binding to A3F, A3G, and Cul5, and the $^{171}\text{EDRW}^{174}$ domain could regulate Vif binding only to A3F, indicating that the $^{81}\text{LGxGxxIxW}^{89}$ domain is functionally more important than the $^{171}\text{EDRW}^{174}$ domain.

Replication of HIV-1 with mutations in the $^{81}\text{LGxGxxIxW}^{89}$ and $^{171}\text{EDRW}^{174}$ domains in human T-cell lines. Next, we evaluated the functions of the two domains in viral replication. We created HIV-1 proviruses bearing a G84D, SIEW/4A, or EDRAWN/5A mutation in the *vif* gene. First, we tested whether these different Vif proteins could reduce A3G or A3F protein levels in virions. Virions were prepared from 293T cells co-transfected with each proviral construct plus an A3G or A3F expression vector and purified via ultracentrifugation. The proteins in the virions were then analyzed by Western blotting. It was found that all the Vif proteins were incorporated into virions (Fig. 7A, lanes 2 to 5). In addition, unlike the EDRAWN/5A mutant, the G84D and SIEW/4A mutants failed to reduce A3G protein levels in virions (Fig. 7A, lanes 8 to 10), and all three mutants failed to reduce A3F protein levels in virions (Fig. 7A, lanes 13 to 15).

Next, we measured viral replication in human T-cell lines. One permissive cell line (CEM-SS) and two nonpermissive cell lines (Hut-78 and PM-1) were selected. 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. As expected, in CEM-SS cells, the wild-type, *vif*-defective, and all three Vif mutant (G84D, SIEW/4A, and EDRAWN/5A) viruses grew equally well (Fig. 7D). In contrast, in Hut-78 and PM-1 cells, only the wild-type virus replicated well, whereas the ΔVif virus showed approximately a 3-log-unit reduction in viral production (Fig. 7B and C). In addition, the G84D and SIEW/4A mutants exhibited growth kinetics very similar to those of the ΔVif virus, and the EDRAWN/5A mutant replicated almost as well as the wild-type

FIG. 5. Identification of a functionally conserved region in the SIVmac239 *Vif* protein. (A) Amino acid sequence alignment of *Vif* proteins from HIV-1, HIV-2, SIVcpz, SIVmac239, and three SIVagm isolates in the 81-to-90 and 171-to-175 regions. Residues in HIV-1 that regulate *Vif* activity toward A3F are shown in blue, and those that regulate *Vif* activity toward both A3G and A3F are shown in red. (B to D) Mutational analysis of SIVmac *Vif* activity toward macA3G (B), human A3G (C), and human A3F (D), as described in the legend to Fig. 2. A3G or A3F protein expression levels in viral producer cells were determined by Western blotting as described in the legend to Fig. 2 (bottom). The SEMs were calculated from three independent experiments.

virus (Fig. 7B and C). These results indicate that the G84D and SIEW/4A mutations completely destroyed *Vif* function and that the EDRWN/5A mutation only partially destroyed *Vif* function. Thus, we confirmed that these two domains play different roles in the HIV-1 replication cycle.

DISCUSSION

In this study, we identified two new *Vif* regulatory domains, ⁸¹LGxGxxIxW⁸⁹ and ¹⁷¹EDRW¹⁷⁴, by comparison of more than 2,000 HIV-1 *Vif* sequences plus functional characterizations. Our functional studies demonstrated that the ⁸¹LGxGxxIxW⁸⁹ domain regulates *Vif* activity toward both

A3G and A3F and the ¹⁷¹EDRW¹⁷⁴ domain regulates *Vif* activity toward A3F (Fig. 2 and 3). These sequences are also conserved in *Vif* from SIVcpz, partially conserved in *Vif* from HIV-2 and SIVmac239, and not conserved in *Vif* from SIVagm (Fig. 5). The ⁸¹LGxGxxIxW⁸⁹ domain is functionally conserved in *Vif* from SIVmac239, because the RITW/4A mutant failed to neutralize A3G and A3F, and the ¹⁷¹EDRW¹⁷⁴ domain is not conserved (Fig. 5). All these results indicate that these two domains, particularly the ⁸¹LGxGxxIxW⁸⁹ domain, are functionally important for *Vif* proteins from HIV-1, HIV-2, SIVcpz, and SIVmac. Interestingly, we found that within the ⁸¹LGxGxxIxW⁸⁹ domain, different residues play different roles. Residue L81 or

FIG. 6. Regulation of HIV-1 Vif binding to human A3G and/or A3F by ⁸¹LGxGxxIxW⁸⁹ and ¹⁷¹EDRW¹⁷⁴ domains. (A and B) Vif binding to A3G or A3F. Different HIV-1 Vif proteins with a C-terminal HA tag expressed from the pNL-A1 vector were coexpressed with A3G (A) or A3F (B) protein with a C-terminal FLAG-HA tag in 293T cells. As a negative control, GFP with a similar FLAG-HA tag was coexpressed with the wild-type Vif protein. The proteins were pulled down by anti-FLAG antibodies and analyzed by Western blotting. The Vif proteins were detected by Vif-specific polyclonal antibody, and GFP, A3F, and A3G were detected by an anti-HA antibody. (C) Vif binding to Cul5. HIV-1 Vif proteins expressed from pNL4-3 with the indicated mutations were coexpressed with Cul5-HA protein in 293T cells. As a negative control, the human APOBEC2 protein with a similar HA tag was coexpressed with the wild-type Vif protein. The proteins were pulled down with anti-HA antibodies and analyzed by Western blotting. The Cul5 and APOBEC2 expression levels were detected by an anti-HA antibody, and Vif expression was detected by a Vif-specific antibody.

G82 alone regulates Vif activity only toward A3F, whereas residue G84 alone regulates Vif activity toward both A3G and A3F (Fig. 3). In addition, the V85, S86, or E88 residue alone failed to change Vif activity, and the I87 or W89 residue alone only moderately regulated Vif activity toward A3G and A3F (Fig. 3A and B, lanes 7 and 9). Notably, the important role of the I87 and W89 residues was amplified if either the S86 or E88 residue, or both, was simultaneously mutated (Fig. 3, lanes 14 to 22). This result is in contrast to that for the ¹⁷¹EDRW¹⁷⁴ domain, where E171, D172, R173, and W174 residues all play almost equal roles in regulation of Vif activity toward A3F (Fig. 4). Our binding results further support these observations: residue L81 or G82 contributed to Vif binding only to A3F, residue G84 contributed to Vif binding to both A3F and A3G, and residues ⁸⁶SIEW⁸⁹ contributed to Vif binding to A3F, A3G, and Cul5 (Fig. 6). Thus, these critical residues in the ⁸¹LGxGxxIxW⁸⁹ domain could be grouped into three units, ⁸¹LG⁸², G⁸⁴, and ⁸⁷IxW⁸⁹. The first unit regulates Vif activity

only toward A3F, whereas the others regulate Vif activity toward both A3G and A3F. Notably, the last unit is the most critical determinant of Vif activity that may directly determine Vif structure, since it affects Vif interaction with both its targets and the E3 ligase machinery. Previously, Simon et al. created a number of Vif mutants with different amino acid deletions (28). Three mutants, $\Delta 7$, with a deletion from residues 73 to 87; $\Delta 22$, with a deletion from 86 to 92; and $\Delta 8$, with a deletion from 86 to 98, all lost activity completely in the nonpermissive H9 cells. All three mutants do not contain an intact ⁸¹LGxGxxIxW⁸⁹ domain. In addition, two other mutants, $\Delta 15$, with a deletion from 169 to 181, and $\Delta 28$, with a deletion from 169 to 190, retained only 20 to 30% activity in H9 cells. These two mutants have completely lost the ¹⁷¹EDRW¹⁷⁴ domain. Thus, their results are consistent with ours, which further highlights the functional importance and functional difference of these two domains.

So far, six A3G and/or A3F regulatory domains have been identified from HIV-1 Vif: ¹¹WxxDRMR¹⁷, ²¹WxSLVK²⁶,

FIG. 7. Regulation of HIV-1 replication in human T cells by the ⁸¹LGxGxxIxW⁸⁹ and ¹⁷¹EDRW¹⁷⁴ domains. (A) Expression of A3G and A3F in different HIV-1 virions. 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. (B to D) Replication of HIV-1 in Hut-78 (B), PM-1 (C), and CEM-SS (D) cell lines. Cells were infected with equal amounts of wild-type, *vif*-defective (ΔVif), or *vif* mutant (G84D, SIEW/4A, or EDRWN/5A) viruses. Viral growth was monitored for 8 days by p24^{Gag} ELISA.

⁴⁰YRHHY⁴⁴, ⁵⁵VxIPLx₄L⁶⁴, ⁶⁹YxxL⁷², and ⁷⁴TGERxW⁷⁹. Among them, one (⁴⁰YRHHY⁴⁴) regulates Vif activity only toward A3G, two (¹¹WxxDRMR¹⁷ and ⁷⁴TGERxW⁷⁹) regulate Vif activity only toward A3F, and three (²¹WxSLVK²⁶, ⁵⁵VxIPLx₄L⁶⁴, and ⁶⁹YxxL⁷²) regulate Vif activity toward both A3G and A3F. All of these domains directly determine Vif binding to A3G and/or A3F. In addition, A3G and A3F use different surfaces to interact with Vif. It was found that in A3G, this surface is located in the N-terminal half from residues 126 to 132, and in A3F, it is in the C-terminal half from 283 to 300 (9, 23). Here, we identified two other A3G and/or A3F regulatory domains in Vif. Mutations in the ⁸¹LGxGxxIxW⁸⁹ domain disrupted Vif binding to A3F, A3G, and Cul5, and mutations in ¹⁷¹EDRW¹⁷⁴ disrupted Vif binding to A3F (Fig. 6). Previously, it was shown that a sequence (¹⁴DRMR¹⁷) in the ¹¹WxxDRMR¹⁷ domain of HIV-1 Vif was responsible for its inability to neutralize AGM A3G (25). When it was replaced with another sequence, SERQ, which is the sequence in AGM Vif, HIV-1 Vif became able to neutralize AGM A3G. However, it is unlikely that the ⁸¹LGxGxxIxW⁸⁹ and ¹⁷¹EDRW¹⁷⁴ domains also determine such species-specific ac-

tivity of Vif, because they are much less conserved in the AGM Vif proteins. Our results further highlight the fact that the assembly of Vif, A3G or A3F, and E3 ligase machinery complexes via direct protein-protein interaction is a critical step for Vif neutralization of these two important antiviral proteins. They have extended the notion that the domains in Vif that regulate its binding to A3G and A3F are different and discontinuous. Since a structural model of Vif-A3G or Vif-A3F interaction is still not established, it is unclear why so many domains are involved and which domain has a more direct effect in these interactions. However, it is predictable that Vif has developed rather complicated mechanisms to recognize these two proteins. A full understanding of these mechanisms is essential for pharmaceutical inhibition of Vif function to prevent HIV-1 infection.

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