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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Running Title: Vif T[Q/D/E]x₅ADx₂[I/L] motif

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Abstract

Primate lentiviruses are unique in that they produce several accessory proteins to help establishment of productive viral infection. Their major function is to clear host resistant factors that inhibit viral replication. Vif is one of these proteins. It functions as an adaptor that binds to 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, eleven Vif discontinuous domains have been identified that regulate this degradation process. Here, we report another domain, T[Q/D/E]x₅ADₓ₂[I/L], which is located between residues 96 to 107 in the human immunodeficiency virus type (HIV)-1 Vif protein. This domain is not only conserved in all HIV-1 subtypes, but also in other primate lentiviruses, including HIV-2 and simian immunodeficiency virus (SIV) that infects rhesus macaques (MAC) (SIVmac) and African green monkeys (AGM) (SIVagm). Mutations of the critical residues in this motif seriously disrupted Vif neutralizing activity to both A3G and A3F. This motif regulates Vif interaction not only to A3G and A3F, but also to 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 non-permissive 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.

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Introduction

Vif is a small viral protein that has 192 amino acids and that is expressed by most lentiviruses, except for equine infectious anemia virus. It was first discovered in HIV-1 (13, 14, 31), and its function has been extensively studied in HIV-1 infection (9, 34). Infection of human T cell lines with vif-defective (ΔVif) HIV-1 identified two different cell types, which are called permissive cells that can be infected by ΔVif HIV-1, and non-permissive cells that are resistant to ΔVif HIV-1 (10, 36). Genomic complementation analysis indicated that these non-permissive 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. 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. 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 them, the protein expression of A3G and A3F in human primary tissues 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 (38) and different target sequence preferences while catalyzing cytidine deamination of viral cDNAs (15). Vif hijacks cellular proteasomal machinery to destroy A3G and A3F by protein degradation pathway (18, 26, 33, 46). Vif acts as an adaptor protein that bridges A3 proteins to a Cullin-5-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 Cul5/EloBC is a critical step for A3G and A3F degradation. Although A3G and A3F share high homology, different
surfaces are used for Vif interaction. Vif binds to the N-terminal half region of A3G from residues 126 to 132, and the C-terminal half region of A3F from 283 to 300 (12, 24). In addition, eleven discontinuous motifs in the Vif protein have been identified to regulate Vif interactions with A3G, A3F, or the Cul5/EloBC E3 ligase complex. Three motifs determine Vif interaction with the E3 ligase. A $^{108}$Hx$_5$Cx$_{17,18}$Cx$_{3,5}$H$^{139}$ motif, which is also called HCCH zinc finger, binds to Cul5 (16, 20, 41); a $^{144}$SLQYLA$^{149}$ motif, which is also called BC-box, binds to EloC (19, 45); and a $^{161}$PPLPx$_4$L$^{169}$ motif, which is also called Cul box, binds to Cul5 (32, 45). The $^{161}$PPLP$^{164}$ subdomain has multiple activities, which not only determines Vif dimerization (43), but also regulates Vif binding to A3G (8, 37) and EloB (1). The other 8 motifs regulate the interaction between Vif and A3G/A3F. The $^{21}$WxSLVK$^{26}$ (3, 7) and $^{40}$YRHHY$^{44}$ (23) motifs regulate Vif binding to A3G; the $^{11}$Wx$_2$DRMR$^{17}$ (23), $^{74}$TGERxW$^{79}$ (11), and $^{171}$EDRW$^{174}$ (4) motifs regulate Vif binding to A3F; and $^{50}$VxPLx$_4$L$^{64}$ (11), $^{69}$YxxL$^{72}$ (22), and $^{81}$LGxGx$_2$IxW$^{89}$ (4) motifs regulate Vif binding to both A3G and A3F. The $^{81}$LGxGx$_2$IxW$^{89}$ 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 fully 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 Cul5/EloBC E3 ligase complex. Since this Vif region is the only one left uncharacterized, this is a significant step towards 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 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-agmA3F-V5-6xHis, and pcDNA3.1-agmA3G-V5-6xHis were described before (4, 6, 38). The Vif expression vectors pNL-A1, pNL-A1Δvif, pNL-A1SIV/macVif, and pNL-A1SIVagmVif were from K. Strebel. The Cul5 expression vector VR-Cul5-HA was from X.Yu. To make HIV-1 Vif mutant expression, vif genes were first cloned into pCR4-TOPO vector (Invitrogen), and mutated by 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 XbaI site 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 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 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 p24Gag capture enzyme-linked immunosorbent assay (ELISA). Equal amount of viruses were used to infect GHOST-R3/X4/R5 cells. Thirty-six hours later, cells were lysed in 50mM Tris-HCl, pH 7.4, 150mM NaCl, 1% Triton X-100, 3mM EDTA (lysis buffer). After removing the nuclei, the cytosolic fraction was used to determine luciferase activity using a luciferase assay kit (Promega).

HIV-1 growth curve. Human T-cell lines CEM-SS and H9 were cultured in the complete RPMI1640 culture media 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 x 10^5 T cells were infected with HIV-1 containing 150 ng p24Gag for 2 hours at 37 °C. After washing, cells were re-suspended in a 48-well culture plate, and culture supernatants were collected every other day for ELISA measurement of viral p24Gag.

**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 vectors at a 6:1 ratio by a standard calcium phosphate transfection protocol. Twenty-four hours later, cells were lysed in a buffer (150 mM NaCl, 20 mM Tris-HCl, pH7.6, 3mM EDTA, 1% TritonX-100). 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, 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) were used to directly detect the expression of A3F, A3G, or Vif proteins. Actin was detected by a polyclonal antibody (C-11) (Santa Cruz Biotechnology). HIV-1 p24Gag 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 –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 a highly conserved amino acid sequence from an uncharacterized region of primate lentivirus Vif proteins. To search for conserved residues by sequence alignment is a very useful approach for identification of functional motifs within a protein. So far, eleven 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 from residues 91 to 107, which is next to the $^{81}$LGxGxxIxW$^{89}$ domain, remained uncharacterized (Fig. 1A). We aligned amino acid sequence of this region among different Vif proteins from HIV-1, HIV-2, SIV from chimpanzees (SIVcpz), SIVmac239, and SIVagm strains. We identified six almost completely conserved residues. These residues are summarized as a consensus sequence $^{96}$T[Q/D/E]xxPxxADxx[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 $^{96}$T[Q/D/E]xxPxxADxx[I/L]$^{107}$ sequence in HIV-1. To understand its function, we created a large number of HIV-1 Vif mutants bearing a single amino acid mutation from 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 L107A. After that, we determined whether these mutants were able to neutralize human A3G and A3F. Mutant D99R was poorly expressed and was not included in this experiment. Vif activity was measured by the levels of enhancement of $\Delta$Vif 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 a potent antiviral activity of both A3G and A3F, although A3G showed much stronger activity than A3F (Fig. 2A, lane 1; Fig. 2B, lane...
1). Almost all these mutants neutralized A3G and A3F as well as the wild-type Vif, except two mutants A103D and D104R. A103D and D104R completely lost activity to both A3G and A3F (Fig. 2A and 2B, lanes 14 and 15). We also detected A3G or A3F protein expressions in viral producer cells in the presence of these mutants by Western blotting. It was found that all these Vif mutants were expressed well (Fig. 2A and 2B, lower panels). Importantly, unlike the other mutants, both A103D and D104R failed to decrease both A3G and A3F expression (Fig. 2A or 2B, lanes 14 and 15, lower panels), which is consistent with viral infectivity assay result. 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 Vif phenotype. Previously, others and we 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, and creating another four mutants T96D, T96R, Q97R, and I107R. 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 mutants T96D, T96R, and I107R lost activity to neutralize both A3G and A3F, Q97R selectively lost activity to AF, and mutants D99K and P100A were still active against both proteins (Fig. 2A and 2B, lanes 19 to 26). We also determined A3G or A3F protein expressions in viral producer cells, and found that Vif mutants that failed to neutralize A3G and A3F also failed to decrease their expressions, as determined by Western blotting (Fig. 2A and 2B, lanes 19 to 26, lower panels). Thus, we identified T96, Q97, and I107 as other critical residues for Vif function. Since residue P100 does not seem to be essential from these experiments, we summarized this conserved sequence as a functional motif.
Further characterization of the 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 was changed to positively charged R. These mutations may have changed the overall charges on Vif protein, resulting in 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 with differences in polarity, creating another four mutants A103R, A103S, A103V, and A103Y; we changed the negatively charged residue D104 to uncharged A, S, and Y, creating another three mutants D104A, D104S, and D104Y. When their activity was determined, we found that mutants A103R, A103Y, and D104Y completely lost activity against both A3G and A3F; mutant D104S partially lost activity against both A3G and A3F; mutant D104A selectively lost partial activity to A3G; and mutants A103S and A103V were still active against to both A3G and A3F (Fig. 3A and 3B). These results were further confirmed by Western blotting analysis of AG or A3F protein expression in the presence of these mutants in viral producer cells (Fig. 3A and 3B, lower panels). Taken together, these results confirm that Vif activity is significantly affected by charges on the proteins. In addition, the side-chain of amino acid at these two positions many also affect Vif activity.

Function of T[Q/D/E]x5ADx2[I/L] motif in SIVs. To understand whether this motif regulates Vif activity in SIVs, we introduced single or double mutations in this motif in 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; and another five SIVagm Vif mutants were also created including TE/RR (T99 and E100 were changed to arginines), P103A, A106D, D107R, and I110R. The SIVmac239 Vif activity was determined against human (hu) A3G and A3F, and SIVagm Vif activity was determined against AGM A3G and A3F. It was found that in SIVmac, Vif mutants TD/RR, A105D, D106R, and L109R completely lost activity to
both A3G and A3F, and mutant P102A slightly lost activity to huA3G and huA3F (Fig. 4A and 4B); and similarly in SIVagm, mutants TE/RR, A106D, D107R, and I110R almost completely lost activity to agmA3G and agmA3F, but not agmA3G (Fig. 4B and 4D, lane 4). These results were further confirmed by Western blotting when A3G or A3F protein expression was determined in the presence of these mutants in viral producer cells (Fig. 4A, 4B, 4C, 4D, 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 this motif is critical for Vif activity, we wondered whether it acts through regulation of Vif interaction with A3G, A3F, or Cul5, as shown previously by the other motifs. In order to test these interactions by immunoprecipitation, eleven vif mutant genes including T96D, T96R, Q97R, P100A, A103D, A103R, A103Y, D104A, D104R, D104Y, and I107R, were cloned back to the proviral HIV-1 construct pNL4-3 from the HIV-1 subgenomic expression vector pNL-A1, so these Vif proteins do not express the HA tag anymore. The G84D mutations were included as a control because we have previously demonstrated that this mutant failed to interact with A3G and A3F but not Cul5 (4). After that, A3G, A3F, or Cul5 proteins fused with a HA tag were co-expressed 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 these Vif proteins except T96D and P100A mutants completely lost interaction with A3G and A3F (Fig. 5A and 5B). Mutant P100A still interacted with A3G and A3F, but at reduced levels; and mutant T96D showed further decreased levels of such interaction (Fig. 5A and 5B, lanes 2 and 5). In addition, like the G84D mutant, mutants T96D, Q97R, P100A, A103D, D104A, D104Y, and I107 still interacted with Cul5, whereas mutants T96R, A103R, A103Y, and D104R showed much reduced interaction with Cul5 (Fig. 5C). Overall, we conclude that this motif determines Vif interaction with all A3G, A3F, and Cul5.
Regulation of HIV-1 in human T cell lines by T\[Q/D/E\]x5ADx2\[I/L\] motif. To further confirm the importance of this motif, seven HIV-1 mutants bearing a mutation in this motif were produced, and their replication in permissive and non-permissive human T cell lines were compared to the wild-type virus. These HIV-1 mutants were T96D, T96R, Q97R, P100A, A103R, D104Y, and I107R. All these mutations except P100A impaired Vif 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 proteins from virions. Virions were prepared from 293T cells co-transfected with each proviral construct plus an A3G or A3F expression vector, and purified via ultra-centrifugation. Proteins in virions were then analyzed by Western blotting. It was found that all these Vif mutant proteins were encapsidated as efficiently as the wild-type protein (Fig. 6A and 6B, middle panels). In addition, all mutants except P100A failed to reduce A3G and A3F protein levels in virions (Fig. 6A and 6B, top panel). In this experiment, mutant P100A was as active as the wild-type Vif protein (Fig. 6A and 6B, lane 2, 6, and 8).

Next, we compared viral replication in the permissive CEM-SS versus non-permissive H9 cells. These cells were infected with equal amounts of each virus and viral replication was determined by measuring p24Gag concentration in the supernatant using an ELISA kit. In CEM-SS cells, the wild-type, ΔVif, and all seven Vif mutant viruses including T96D, T96R, Q97R, P100A, A103R, D104Y, and I107R grew equally well (Fig. 6D). In contrast, in H9 cells, only the wild-type and P100A mutant virus replicated well, whereas the ΔVif and other mutant viruses showed approximately a two to three-log reduction in viral production (Fig. 6C). Among these particular mutants, Q97R and T96D replicated slightly better, but still showed ~100-fold reduction of producing viral particles when compared to the wild-type and P100A mutant. I107R, D104Y, A103R, and T96R replicated as badly as the ΔVif virus. Thus, we have confirmed that this motif plays a critical role in the regulation of HIV-1 replication in non-permissive cells.
Discussion

In this report, we identify another functional motif \(T[Q/D/E]x_5ADx_2[I/L]\) from primate lentivirus Vif proteins. Sequence 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 its integrity is essential for HIV-1 replication in non-permissive human T cells.

Among the 192 amino acids of HIV-1 Vif, 106 are conserved in 95% of more than 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 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 \(^{11}Wx_2DRMR^{17}\) 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, G191), and this region overlaps with the N-terminus of Vpr protein. Among the rest of 86 conserved residues, 64 are located inside of the eleven functional motifs, and the other 14 are distributed outside of these motifs. Among these 14 residues, 3 (H28, S32, and W38) are between \(^{21}WxSLVK^{26}\) and \(^{40}YRHHY^{44}\), 4 (P49, S52, S53, and E54) are between \(^{40}YRHHY^{44}\) and \(^{56}VxIPLx_4L^{64}\), 1 (T68) is between \(^{56}VxIPLx_4L^{64}\) and \(^{69}YxxL^{72}\), 2 (V142, G143) are between \(^{108}Hx_6Cx_{17-18}Cx_3H^{139}\) and \(^{144}SLQYLA^{149}\), and 4 (L150, A152, L153, and P156) are between \(^{144}SLQYLA^{149}\) and \(^{161}PPLPx_4L^{169}\). 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 \(T[Q/D/E]x_5ADx_2[I/L]\) 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, and I107 seriously disrupted Vif activity. However, not all types of mutation led to disruption of Vif activity. Mutations of T96A, Q97A, or I107A did not disrupt Vif function, while T96D, T96R, Q97R, and I107R did so (Fig. 2). In addition, at position 103, mutations of A103D and A103R but not A103S and A103V were able to disrupt Vif activity; at position 104, mutations of D104R, D104A, D104S, and D104Y 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]xADx[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, others and we have demonstrated that the WxSLVK 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 residue 22, 26, and 84 severely disrupted Vif interaction with A3G and/or A3F (4, 7), indicating that these associations could be mediated by electrostatic interactions between these proteins. Consistently, in this T[Q/D/E]xADx[I/L] motif, mutants T96D, T96R, Q97R, A103D, A103R, D104A, D104R, D104Y, and I107R all failed to interact with A3G and A3F (Fig. 5A and 5B). However, it seems that the Vif and Cul5 binding is much less dependent on electrostatic interaction. Although mutants T96R, A103R, A103Y, and D104R showed a reduced binding, mutants T96D, Q97R, A103D, D104A, D104Y, and I107 still bound to Cul5 (Fig. 5C). Thus, Vif may have developed different mechanisms to interact with these two different types of host proteins.

Although Vif is a small viral protein, it has evolved twelve distinct functional motifs to target A3G and A3F for proteasomal degradation, as summarized in Fig. 7A. Structural studies have...
demonstrated that the HCCH, SLQYLA, and PPLPxL motifs directly interact with Cul5, EloC, and EloB, respectively (1, 40). All the other 9 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, one that recognizes both A3G and A3F, and two that recognizes either A3G or A3F but not both. Disruption of any 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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References


Figure legends

Figure 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 other primate lentivirus Vif proteins. Residues with high conservation is shown in red, low conservation in blue, and not conserved in dark. 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.

Figure 2. Mutational analysis of HIV-1 Vif 91-107 residues. 293T cells were transfected with HIV-1 reporter construct pNL-LucΔenvΔvif, pNL-A1 expressing HIV-1 Vif-HA protein as indicated, a VSV-G expression vector, and either a human A3G (black bar) (A) or A3F (open bar) (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 levels of viral input (p24Gag). Vif activities are presented in 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 or 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 from three independent experiments.

Figure 3. Further characterization of HIV-1 Vif residues A103 and D104. HIV-1 Vif mutants expressed from pNL-A1 bearing the indicated mutations at amino acid positions 103 and 104 were analyzed for their activity to neutralize A3G (A) and A3F (B), as in Figure 2. The standard errors of the means (SEM) were calculated from three independent experiments.
Figure 4. Mutational analysis of the T[Q/D/E]x5ADx2[I/L] motif in SIVmacVif and SIVagmVif proteins. 293T cells were transfected with HIV-1 reporter construct pNL-LucΔenvΔvif and a VSV-G expression vector plus an indicated SIVmac expression vector and a human A3G expression vector (A), an indicated SIVmac expression vector and a human A3F expression vector (B), an indicated SIVagm expression vector and an AGM A3G expression vector (C), or an indicated SIVagm expression vector and an AGM A3G expression vector (D). Viral infectivity as well as protein expressions of A3G, A3F, and Vif in 293T cells was analyzed as in Fig. 2. The SEMs were calculated from three independent experiments.

Figure 5. Regulation of protein interactions by the T[Q/D/E]x5ADx2[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 co-expressed 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.

Figure 6. Regulation of HIV-1 replication in non-permissive human T cells by the T[Q/D/E]x5ADx2[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 an equal amount of wild-type (WT), vif-defective (Δvif), or different vif mutant (T96D, T96R, Q97R, P100A, A103R, D104Y, and I107R) viruses. Viral growth was monitored for 10 days by p24Gag ELISA.
Figure 7. (A) 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, A3G binding in blue, both A3G and A3F binding in red, and Cul5/EloBC binding in pink. (B) A schematic representation of the process that Vif interacts with Cul5/EloBC and A3G or A3F to promote A3G and A3F protein degradation. Vif surfaces that recognize Cul5/EloBC are indicated in pink, A3F in green, and A3G in blue. The surface that recognizes both A3G and A3F is indicated in red.
A: L8xGxxINV \( \rightarrow \) I9xS[Q/D/E]xxPxxADxx[IL] motif in primate lentivirus Vif proteins

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B: T[Q/D/E]xxPxxADxx[IL] motif in primate lentivirus Vif proteins
Fig. 6
A B
C D
7 8 9 10 11 1 2 3 4 5 6
Vif
A3G
p24
Gag

Days post-infection
CEM-SS
WT
"9LI
T96D
Q97R
P100A
A103R
D104Y
I107R
T96R

Days post-infection

0.1
1
10
100
1000
0 2 4 6 8 10
H9
Fig. 7

A

B

N-Wx_DRMR-A3F-A3G-21
WxSLVK-A3G-26
-YRHHY-A3G-26
-YxPLx_L-A3F/A3G-64
-YxxL-A3F-74
-TGERxW-A3F/A3G-74
-LGxGx_LxW-A3F/A3G-96
-TQxxADxI-A3F/A3G-96
-HCCH-Cul5-107
-SLQYLA-EloB-149
-PPLPx_L-EloC-149
-EDRW-171
-Cul5-161
-EloB-A3G-169
-A3F-174

A3G

Vif

Cul5/EloBC

A3F

Vif

Cul5/EloBC