Identification of Specific Determinants of Human APOBEC3F, APOBEC3C, and APOBEC3DE and African Green Monkey APOBEC3F That Interact with HIV-1 Vif

Jessica L. Smith and Vinay K. Pathak*

Viral Mutation Section, HIV Drug Resistance Program, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, Maryland 21702

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Human APOBEC3F (hA3F) and human APOBEC3G (hA3G) are potent anti-human immunodeficiency virus (anti-HIV) host factors that suppress viral replication by hypermutating the viral genome, inhibiting reverse transcription, and hindering integration. To overcome hA3F and hA3G, HIV-1 encodes Vif, which binds and targets these host proteins for proteasomal degradation. Previously, we reported that the hA3F-Vif interactions that lead to hA3F degradation are located in the region comprising amino acids 283 to 300. We have now performed mutational analysis of this region and found that the 289EFLARH294 amino acids contribute to hA3F-Vif binding and are critical for A3F’s sensitivity to Vif. Mutants in which E289 is mutated significantly increase hA3F’s ability to inhibit viral infectivity in the presence of Vif, and coimmunoprecipitation assays show that binding of Vif to the E289K mutant is decreased. We examined the role of the EFLARH sequence in other A3 proteins, including human A3C (hA3C), human A3DE (hA3DE), African green monkey A3F (agmA3F), and rhesus macaque A3F (rhA3F). hA3C, hA3DE, and agmA3F were all susceptible to degradation induced by HIV-1 Vif, while rhA3F was not. Mutagenesis of the glutamate in the EFLARH sites of hA3C, hA3DE, and agmA3F decreases the susceptibilities of these proteins to Vif-induced degradation. Together, these results indicate that the EFLARH region in A3F, hA3C, hA3D, and agmA3F interacts with HIV-1 Vif and that this interaction plays a role in the Vif-mediated proteasomal degradation of these A3 proteins. These studies identify a conserved region in 3 of 7 human A3 proteins that is critical for degradation mediated by HIV-1 Vif and provide structural insights into the hA3F-Vif interactions that could facilitate the development of a novel class of anti-HIV agents.

Human cytidine deaminases APOBEC3F (hA3F) and APOBEC3G (hA3G) are potent cellular defense proteins that counter Vif-deficient human immunodeficiency virus type 1 (HIV-1) (27, 46, 56, 71). hA3F and hA3G inhibit HIV-1 by cytidine deamination resulting in G-to-A hypermutation of the viral genome (18, 24, 31, 51, 63, 67), inhibition of viral DNA synthesis (1, 3, 14, 15, 20, 22, 26, 29, 34, 35, 40, 60), and inhibition of viral DNA integration and provirus formation (29, 34, 35).

To counter these cellular antiviral factors, the HIV-1 Vif protein acts as a scaffold between a cellular E3 ubiquitin ligase complex (consisting of cullin 5, elongin B, elongin C, and RING finger protein 1) and hA3F/hA3G (64), leading to their polyubiquitination and degradation (7, 28, 33, 37, 47, 50, 64). Because hA3F and hA3G are potent antiviral proteins, identification of the regions in Vif and hA3F/ hA3G that are important for interactions between these proteins could be used to design inhibitors to block hA3F/ hA3G degradation.

Several domains in Vif have been identified as being important for its mechanism of inducing hA3G/hA3F ubiquitination and degradation, including an 144SLQYLA149 domain which interacts with elongin C and an 109H-X7-C-X17,18-C-X3,5-H139 domain which interacts with cullin 5 (2, 30, 36, 38, 65). We previously identified distinct regions in Vif that are required for interactions with hA3F (14DRMR27) or hA3G (40YRH¥44) (43). Other studies (recently reviewed in reference 49) also demonstrated that certain residues in Vif are required for hA3F or hA3G interactions, but not both, whereas other regions seem to be important for interactions with both hA3F and hA3G (6, 8, 10, 11, 19, 39, 42, 43, 48, 52, 58).

Within the APOBEC3 proteins, two distinct regions in hA3G and hA3F that interact with Vif have been identified (44). In hA3G, we found that amino acids 126 to 132 are important for Vif binding. These results are consistent with other studies in which amino acids 128 to 130 were identified as being critical for Vif interactions (21). In hA3F, we showed that amino acids 283 to 300 are important for binding to Vif as well as Vif-induced degradation (44). Here, we have extended our analysis of the hA3F protein to show that this region can be further narrowed down to include only 289EFLARH294. While it has previously been shown that HIV-1 Vif-induced degradation of A3G can be countered by a single amino acid change in hA3G(D128K) (4, 32, 45, 57), such a single amino acid change has not been identified for hA3F. Here, we show that a single mutation in hA3F(E289K) can decrease binding of hA3F to Vif and prevent its Vif-induced degradation. Furthermore, we found that the analogous residues in two other human A3 proteins and African green monkey A3F (agmA3F) are also important for their Vif-induced degradation.
The pFLAG-A3F (43) plasmid and the pcDNA-HVif (44) plasmid were described previously (43); in the G binder Vif mutant, four alanine substitutions replaced 46YRHH44. All APOBEC3 mutants were generated by site-directed mutagenesis using a QuiChange II site-directed mutagenesis kit (Stratagene). Plasmids expressing N-terminally myc-tagged agmA3F and rhesus macaque A3F (rhA3F) were kindly provided by T. Hatzizis and Guang Yu (45). To generate plasmids that express human A3C (hA3C) and human A3DE (hA3DE) proteins fused to the FLAG epitope, clones pFLAG-A3C and pFLAG-A3DE were constructed using pcDNA3.1-APOBEC3C-V5-6xHis and pcDNA3.1-APOBEC3D-V5-6xHis, respectively, which were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from B. Matija Peterlin and Yong-Hui Zheng (45). The primers used for hA3C amplification were 5'-GATCCGGCGCGCTATGAATCCACAGATCAGAAATCC-3' (FWD-FLAG-A3C) and 5'-GATCTCTAGATCATGGACGACTCCCGTAGCC-3' (REV-FLAG-A3C). For amplification of hA3DE, 5'-GATCCGGCGCGCTATGAATCCACAGATCAGAAATCC-3' (FWD-FLAG-A3DE) and 5'-GATCTCTAGATCATGGACGACTCCCGTAGCC-3' (REV-FLAG-A3DE) were used. The modified human embryonic kidney 293T cell line (59) and the HeLa-derived HIV-1 reporter cell line TZM-bll (55), which has an HIV-1 Tat-driven firefly luciferase gene, were maintained in complete medium (CM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, and 1% glutamine. Virus production and titration. For virus production, 293T cells were seeded at 4 × 10^5 cells per well in six-well plates 1 day prior to transfection with the following plasmids: 3.33 μg of the HIV-1 vector genome pHDV-EGFP (53), 0.67 μg of vesicular stomatitis virus glycoprotein expression plasmid pHCMV-G (61), 0.34 μg of wild-type Vif (44) or mutant pFLAG-A3C, and 4.5 μg of either WT or mutant pcDNA-HVif. To maintain equal amounts of DNA, pcDNA3.1-noMCS (43) was used when needed. To test the antiviral function of hA3C, 0.34 μg of WT or mutant pcDNA-HVif, and 1.2 μg pGreen Lantern-1 (pGL) were added to the DNA-DMEM samples dropwise with gentle vortexing. Following a 20-min incubation of the DNA-PEI-DMEM samples at room temperature, the samples were added to the cells after the CM was removed. The cells were incubated at 37°C for 3 h to 4 h, and the DNA-PEI-DMEM samples were aspirated and replaced with CM (1 ml). After 48 h, the virus-containing supernatant was filtered through a 0.45-μm filter and quantified using a p24 CA enzyme-linked immunosorbent assay (PerkinElmer). TZM-bll cells were seeded at 4 × 10^5 cells per well in 96-well plates 1 day prior to infection. Virus samples were diluted with CM to have equivalent concentrations of p24 CA (50 ng/ml), and the samples (100 μl) were used to infect the TZM-bll cells. After incubation at 37°C for 3 to 4 h, another 100 μl of CM was added to each well. The culture medium was removed 72 h later and replaced with 100 μl of DMEM without phenol red and 100 μl of Brefersite Luciferase solution (PerkinElmer). Luciferase enzyme activity was measured using a LUMiStar Galaxy luminometer.

Co-IP assays. Commmunoprecipitation (co-IP) assays were done as previously described (43, 44). 293T cells were seeded at 4 × 10^5 cells per 100-mm-diameter dish, and 24 h later, the cells transfected with 6 μg of WT or mutant pFLAG-A3F, 9 μg of either WT or mutant pcDNA-HVif, and 1.2 μg pGreen Lantern-1 (pGL), which expresses green fluorescent protein from a cytomegalovirus immediate-early promoter (Gibco/BRL), as a positive control for transfections. To maintain equal amounts of DNA, pcDNA3.1-noMCS was used when needed. PEI was used for transfections as described above, except that 2.5 ml of PEI-DMEM was added to 2.5 ml of DNA-DMEM. After 48 h, the cells were harvested and samples for co-IP were prepared as previously described (44). Western blotting was used to analyze the eluted complexes as well as the input cell lysates. The APOBEC3 proteins were detected using a rabbit anti-FLAG polyclonal antibody (Sigma) at a 1:5,000 dilution, followed by a horseradish peroxidase-labeled goat anti-mouse secondary antibody at a 1:10,000 dilution. Protein bands were visualized using a Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer).

APOBEC3 degradation. Western blot assays were performed to determine WT or mutant A3F protein levels in the presence of excess Vif. 293T cells were seeded at 4 × 10^5 cells per 100-mm-diameter dish, and 24 h later, the cells were transfected with 0.8 μg of WT or mutant pFLAG-A3F, 5 μg of either WT or mutant pcDNA-HVif, and 1.2 μg pGL. To maintain equal amounts of DNA, pcDNA3.1-noMCS was used when needed. For analysis of A3F from other primates, the transfections were performed similarly, except that the expression plasmids for myc-tagged agmA3F and rhA3F (66) were used. For the analysis of hA3C and hA3DE, the pFLAG-A3C and pFLAG-A3DE expression plasmids were used. For hA3C, cells were transfected with 0.8 μg of WT or mutant pFLAG-A3C, 5 μg of either WT or mutant pcDNA-HVif, and 1.2 μg pGL. For hA3DE, cells were transfected with 6 μg of WT or mutant pFLAG-A3DE, 6 μg of either WT or mutant pcDNA-HVif, and 1.2 μg pGL. Transfected cell lysates were harvested as previously described (44), and FLAG-APOBEC3, Vif, and α-tubulin were analyzed by Western blotting. For the detection of myc-tagged proteins, rabbit anti-c-Myc monoclonal antibody (Sigma) at a 1:5,000 dilution was used, followed by a horseradish peroxidase-labeled goat anti-rabbit secondary antibody (Sigma) at a 1:10,000 dilution.

RESULTS

Identification of specific residues in hA3F involved in inhibition by HIV-1 Vif. Previously, amino acids 283 to 300 in the C-terminal half of A3F were shown to be important for its interaction with Vif, while amino acids within the region of positions 126 to 132 in the N-terminal half of hA3G are important for its interactions with Vif (21, 44). To determine if any of the residues in hA3F's region comprising positions 283 to 300 were particularly important for Vif interactions, a series of mutants were made in pFLAG-A3F by changing hA3F’s residues to the corresponding residues in hA3G (Fig. 1A). The functionality of these mutants was tested by examining their ability to inhibit the infectivity of vif-deleted HIV-1 in single-cycle assays (Fig. 1B). In addition, the susceptibility of the hA3F mutants to neutralization by WT Vif and an hA3F-specific mutant, called the F binder (43), was tested (Fig. 1B). The F binder Vif mutant has five alanine substitutions in a region of Vif (46YRHH44) that is critical for interactions with hA3G but not hA3F (43). Figure 1B shows that all mutants tested had antiviral activity in the absence of Vif, although the FG5 mutant had about 4-fold-reduced inhibitory activity and about 2.5-fold-reduced viral infectivity compared to the levels for the infection without A3F. The amino acid changes in FG5 (G285Q-V287M) did not affect this mutant’s ability to inhibit HIV-1 in the absence of Vif, nor did the mutations affect its susceptibility to WT Vif or the F binder. When two additional mutations were added to FG5 to make FG2, the ability to rescue infectivity in the presence of this mutant was completely abolished for WT Vif and nearly completely abolished for the F binder. Three additional changes to the FG2 construct yielded the FG3 construct. The ability of both WT Vif and the F binder to rescue infectivity in the presence of this mutant was completely diminished. The results from these experiments indicate that residues 289 to 294 of hA3F are important for neutralization by Vif. Constructs FG4 and FG5 had all of the mutations of the previous constructs as well as some additional mutations; rescue of infectivity by WT Vif or the F binder was not observed in the presence of these mutants.

To examine the Vif-induced degradation of the FGF constructs, the steady-state levels of these proteins in the presence
of excess Vif were examined by Western blotting (Fig. 1C). These results showed that WT hA3F and the FGF1 construct were degraded by Vif but that the other FGF constructs were not. These results are consistent with the infectivity data in Fig. 1B.

To determine whether the mutant constructs retained their ability to bind to Vif, co-IP experiments were performed. For these experiments, WT Vif, the F binder, and an hA3G-specific Vif mutant called the G binder were used. The G binder Vif mutant has four alanine substitutions in a region of Vif (14DRMR17) which is critical for interactions with hA3F but not hA3G (43). The FLAG-tagged WT and hA3F mutants were immunoprecipitated, and Vif’s ability to coimmunoprecipitate with these proteins was tested (Fig. 1D). As observed previously, WT Vif and the F binder, but not the G binder, were able to coimmunoprecipitate with WT hA3F (43, 44). Binding of the different Vif proteins to FGF1 was unaltered, while binding to FGF2 was reduced, and binding to FGF3, FGF4, and FGF5 was greatly reduced. The mutations in FGF2 significantly reduced Vif sensitivity in addition to reducing Vif binding, suggesting that the residues mutated in FGF2 contribute to both Vif binding and Vif sensitivity.

Amino acid E289 in the EFLARH sequence of hA3F is critical for HIV-1 Vif sensitivity. The results from the FGF mutants (Fig. 1) indicate that when mutations were introduced in the region comprising amino acids 289 to 294, Vif’s ability to rescue infectivity in the presence of hA3F and Vif-hA3F binding was decreased. To further investigate the roles of these amino acids in the interactions with Vif, one or only a few residues within this region were mutated and tested. In the absence of Vif, each of the mutants inhibited HIV-1 infectivity (Fig. 2A). Interestingly, for a single hA3F(E289K) mutant or a double hA3F(E289K-L291I) mutant (called EL/H11022KI), the abilities of WT Vif and the F binder to neutralize this mutant were completely abolished; however, a single hA3F(L291I) mutant (Fig. 2A) or a hA3F(L291A) mutant (data not shown) were completely neutralized by WT Vif and the F binder. While the single mutants hA3F(A292S), hA3F(R293K), and hA3F(H294N) were neutralized by WT Vif and the F binder, the triple mutant hA3F(A292S-R293K-H294N) (called ARH/SKN) in this region could not be neutralized.

FIG. 1. Localization of the Vif interaction domain in hA3F. (A) Schematic representation of hA3F mutants used to determine the residues in hA3F which are important for interactions with HIV-1 Vif. The top line depicts amino acids 283 to 300 of WT hA3F, and the second line shows the sequence within the same region in hA3G. The third line through the seventh line show the amino acid substitutions in hA3F for five FGF chimeras. Dots indicate identical amino acids. (B) Effect of hA3F and the FGF chimeras on HIV-1 infectivity in the presence and absence of HIV-1 Vif. Single-cycle viruses were produced from 293T cells that were transfected with pHIV-eGFP, pHCMV-G, an hA3F expression plasmid, and either no Vif, WT Vif, or the F binder. The p24 CA levels were determined and used to normalize the levels for virus samples prior to infection of TZM-bl indicator cells. The infectivity of the viruses was determined by quantitation of luciferase activity. Data are plotted as relative infectivity levels, with the level for the hA3F-free virus (not shown) set to 100%. Error bars indicate standard errors of the means (SEM) of results from six infectivity experiments performed with three independent virus stocks. (C) Effects of WT HIV-1 Vif on the degradation of WT hA3F or the FGF chimeras. Cotransfections of 293T cells were carried out with an hA3F expression plasmid and either no Vif or WT Vif expression plasmid. Cell lysates were analyzed by Western blotting using anti-FLAG, anti-Vif, or anti-α-tubulin antibodies. The results are representative of two independent experiments. (D) Co-IP assays to determine binding of Vif to FGF chimeras. 293T cells were cotransfected with expression plasmids for FLAG-A3F or FLAG-FGF chimeras along with expression plasmids for WT Vif, the F binder, or the G binder. Cell lysates and immunoprecipitated proteins were analyzed by Western blotting using anti-FLAG and anti-Vif antibodies. The cell lysates were also analyzed for α-tubulin as a loading control. The results are representative of three independent experiments. WT, wild type; +, with; −, without.
These results were further investigated by examining the steady-state levels of WT hA3F or its mutants in the presence of WT Vif (Fig. 2B). These experiments showed that the protein levels of all of the mutants are similar to that of WT hA3F in the absence of Vif but that hA3F(E289K) and the double mutant (EL>KI) were resistant to Vif-induced degradation, while hA3F(L291I) was not. Only the triple mutant (ARH>SKN) with amino acids 292 to 294 mutated was completely resistant to Vif-induced degradation.

Co-IP assays were also performed with the mutants in which...
infectivity assays were performed with the Vif(SEMQ) mutant coimmunoprecipitated smaller amounts of WT Vif and even less F binder Vif, indicating that this mutation decreased binding to Vif. The hA3F(ARH→SKN) mutant also coimmunoprecipitated smaller amounts of WT Vif or F binder Vif. All other hA3F single mutants with amino acids 292 to 294 mutated did not have an impaired ability to bind WT Vif or the F binder.

Residue F290 is conserved between hA3F and hA3G and it is located next to the critical E289 residue in A3F. Therefore, we tested the effects of mutating this amino acid to tyrosine or tryptophan but saw no effect on the antiviral activity of these mutations or their interactions with Vif (data not shown).

Because a single change in the E289 residue of A3F can critically affect interactions with Vif, further analyses were focused on this residue. To examine the effects of different amino acids at the E289 position, various mutations were made at this site and the mutants were tested in the single-cycle infectivity assay for their ability to be neutralized by WT Vif or the F binder. The results in Fig. 2D showed that, like lysine, the positively charged arginine residue at the E289 position is resistant to Vif neutralization, since the hA3F(E289R) mutant retained its ability to inhibit viral infectivity in the presence of WT Vif or the F binder. Although the hA3F(E289D) mutant retains the negative charge at this position, it has a shorter side chain; neutralization of this mutant by Vif and the F binder was incomplete. The hA3F(E289Q) mutant does not retain the negative charge, but its side chain is similar in length to that of glutamate; despite retaining a similar side chain length, this mutant was only partially neutralized by WT Vif and the F binder. Finally, the hA3F(E289A) mutant does not retain the charge or the length of the side chain; this mutant was insensitive to Vif and was only partially neutralized by either WT Vif or the F binder. Together, these results suggest that both a negative charge and the length of the side chain at amino acid 289 in hA3F are important for Vif’s ability to rescue viral infectivity in the presence of hA3F. The lysozymes of the viral producer cells were also examined by Western blot analysis (Fig. 2E). In the presence of WT Vif and F binder Vif, the levels of WT A3F were substantially reduced, but the levels of the E289K mutant were not noticeably altered, confirming that this mutant was resistant to Vif-mediated degradation. The E289R, E289D, E289Q, and E289A mutants were partially sensitive to degradation induced by the Vif proteins.

To determine if a Vif mutant that has an overall negative charge in the 14DRMR\(^{17}\) region could compensate for the switch in charge and neutralize the hA3F(E289K) mutant, infectivity assays were performed with the Vif(SEMQ) mutant (43, 45), which has 14DRMR\(^{17}\) mutated to 14SEMQ\(^{17}\). This mutant is able to rescue infectivity in the presence of WT hA3G and a Vif-resistant hA3G(D128K) mutant but not in the presence of WT hA3F (43, 45). The results in Fig. 2F show that the HIV-1 Vif(SEMQ) mutant partially rescues infectivity in the presence of WT hA3G and hA3G(D128K) as expected but that this mutant cannot rescue the infectivity in the presence of WT hA3F or hA3F(E289K).

The Vif interaction sites of hA3F and hA3G are not functionally transferable. Two distinct Vif interaction domains have been identified in hA3F and hA3G (44). To determine whether these two domains could be functionally transferred between these two proteins, mutants of hA3F and hA3G in which the hA3G-Vif binding sequence was added to A3F (called A3F-G\(_{125-131}\)-F) and the hA3F-Vif binding sequence was added to A3G (called A3G-F\(_{297-302}\)-F) were made (Fig. 3A). The abilities of the G binder Vif mutant to degrade A3F-G\(_{125-131}\)-F and the F binder Vif mutant to degrade A3G-F\(_{297-302}\)-G were tested. In each case, the mutant proteins were stably expressed in the absence of Vif, but both were susceptible to degradation induced by WT Vif (Fig. 3B). Like WT hA3F, the A3F-G\(_{125-131}\)-F protein was susceptible to the F binder but not the G binder. Furthermore, like WT hA3G, the A3G-F\(_{297-302}\)-G protein was susceptible to the G binder but not the F binder. These results indicated that the Vif interaction sites in hA3F and hA3G could not be easily transferred to the other A3 protein.

Glutamates in the EFLARH sequences of hA3C and hA3DE are critical for HIV-1 Vif-mediated degradation. Sequence alignment results revealed that the EFLARH sequence that is important for hA3F-Vif interactions is present in other A3 proteins, including hA3C and hA3DE (Fig. 4A). To determine if the glutamates in these sequences are important for HIV-1 Vif-induced degradation, site-directed mutagenesis was performed on hA3C and hA3DE. The antiviral activity of these proteins against HIV-1 is not as potent as that of hA3G and hA3F (9, 62, 71), but hA3C and hA3DE are susceptible to HIV-1 Vif-induced degradation (6, 9, 42, 62, 68, 69). Therefore, to assess the importance of the glutamate residue of
the EFLARH region in hA3C-Vif and hA3DE-Vif interactions, the steady-state levels of the WT and mutant A3 proteins in the absence and presence of HIV-1 Vif were examined (Fig. 4B).

In agreement with previous findings (6, 9, 42, 62, 68, 69), we observed that hA3C and hA3DE were susceptible to degradation induced by WT Vif (Fig. 4B). In addition, we observed that hA3C and hA3DE were susceptible to degradation induced by F binder Vif but not G binder Vif. These results are in agreement with the previous observation that Vif interacts with hA3F, hA3C, and hA3DE through distinct domains that do not interact with A3G (6, 42). When the glutamic acid in the EFLARH sequence was mutated to lysine (Fig. 4A), the hA3C(E106K) and the hA3DE(E302K) mutants were resistant to Vif-induced degradation (Fig. 4B), indicating that this residue plays an important role in the Vif sensitivity of these human A3 proteins. To ensure that the hA3C(E106K) and hA3DE(E302K) mutants did not have overall protein-folding defects, the antiviral activities of these proteins were tested against HIV-1 (http://home.ncifcrf.gov/hivdrp/FigureS1_Smith_and_Pathak_J_Virol_100810.pdf). Although hA3C is not a potent inhibitor of HIV-1, the hA3C(E106K) mutant did not lose any antiviral activity; the hA3DE(E302K) mutant also had antiviral activity that was similar to that of hA3DE. These results suggested that the glutamic acid mutations in these proteins did not result in large protein-folding defects that were responsible for the Vif resistance phenotype.

The EFLARH sequence in agmA3F but not rhA3F is important for HIV-1 Vif-induced degradation. The EFLARH sequence is also present in agmA3F and rhA3F. The expression levels of these nonhuman primate A3F proteins were analyzed in the absence and presence of WT Vif, the F binder, and the G binder (Fig. 5A). Previously, it was demonstrated that agmA3F, but not rhA3F, is sensitive to Vif-induced degradation when equivalent amounts of A3F and Vif expression plasmids were used in cotransfections (66). To test Vif susceptibility, we used a 1:10 ratio of A3F/Vif expression plasmid and observed that agmA3F was sensitive to Vif-induced degradation but that rhA3F was not; these observations were in agreement with the previously reported results (66). To examine whether Vif-induced degradation of agmA3F was dependent on the glutamate residue in the EFLARH sequence, this amino acid was changed to lysine to generate agmA3F(E289K); the expression levels of this mutant in the absence and presence of WT Vif, the F binder, and the G binder were determined (Fig. 5B). As with hA3F, the E289K mutant of agmA3F rendered the A3F protein resistant to Vif-induced degradation by either the WT Vif or the F binder. To
ensure that the agmA3F(E289K) mutant retained functionality, its antiviral activity was tested against HIV-1 (http://home.ncifcrf.gov/hivdp/FigureS1_Smith_and_Pathak_J_Virol_100810.pdf). The agmA3F and agmA3F(E289K) proteins had similar effects on inhibition of HIV-1.

**DISCUSSION**

The results of these studies show that the 289EFLARH294 region of hA3F is critical for interactions with HIV-1 Vif that induce its proteasomal degradation. These studies further narrow down the region of hA3F that is important for HIV-1 Vif interaction from our previously identified region of amino acids 283 to 300 (44) to six amino acids, of which only four appear to be important for binding to Vif. Furthermore, the amino acids equivalent to E289 in hA3C, hA3DE, and agmA3F are critical for the HIV-1 Vif-mediated degradation of these proteins, suggesting that the HIV-1 Vif interaction domain is conserved in three human and at least some nonhuman primate A3 proteins. Although the structure of hA3F is not yet determined, we used the available structure of the C-terminal domain of hA3G to gain insights into this Vif-interacting region (Fig. 6A). Interestingly, the A3G residue K297, which is equivalent to E289 in hA3F, is exposed on the surface, consistent with its importance in interaction with HIV-1 Vif; similarly, the A3G residue I299, which is equivalent to L291 in hA3F, is buried within the protein, consistent with the observation that the L291I mutation had little or no effect on HIV-1 Vif-mediated degradation. The A3G residue K301, which is equivalent to the hA3F residue R293, is also surface exposed; thus, if hA3F and hA3G are highly similar in structure as expected, the E289 and R293 residues should be adjacent to each other on the exposed side of helix 3. We hypothesize that these charged residues bind to HIV-1 Vif through electrostatic interactions with the Vif DRMR region. Consistent with this hypothesis, the DRMR->SEMQ mutant of HIV-1 Vif failed to induce degradation of hA3F. However, extensive mutational analyses of both the EFLARH region of hA3F and the DRMR region of HIV-1 Vif would be required to fully test this hypothesis.

Although the E289 residue and the EFLARH region appear to play a critical role in Vif interactions, other regions of hA3F and HIV-1 Vif may interact and contribute to the stability of binding. In addition to the DRMR region (43), other amino acids in HIV-1 Vif have been shown to be important for degradation of hA3F, including W11 (43, 52), Q12 (43), V25 (6, 11), Y69 and L72 (19, 42, 58), E76 and W79 (19, 52, 58), and 81LGxG64 and 17EDRW174 (8) (Fig. 6B). Thus, it is certainly possible that A3F has multiple determinants that contribute to its interaction with Vif. In agreement with this, Lassen et al. recently reported that the N terminus of A3F has domains that are involved in regulating Vif sensitivity (23).

Single amino acid changes in A3 proteins have previously been shown to significantly affect interactions with HIV-1 Vif. The D128 residue of A3G was previously shown to render hA3G resistant to degradation mediated by HIV-1 Vif (4, 32, 45, 57). Subsequent studies have shown that A3G residues 126 to 132 are involved in interaction with Vif (44). Mutations of the analogous amino acids in hA3F(E127) (28), hA3DE(D140) (9), and hA3C(Y140) (69) did not confer Vif resistance, suggesting that the Vif interaction mediated by this region of A3G is not conserved. Recently, a single amino acid change in A3H haplotype I (K121E), which is different from the A3G region comprising amino acids 126 to 132, was shown to render this protein partially sensitive to HIV-1 Vif (25, 70).

The hA3F Vif-interacting region appears to be conserved among several human and nonhuman A3 proteins (Fig. 6C). The overall sequence similarities between the C-terminal halves of hA3F and hA3C and between the whole hA3F and hA3DE proteins are ~77% (9, 68), and each of these proteins has the EFLARH sequence. It was previously reported that hA3C and hA3DE are susceptible to HIV-1 Vif (6, 9, 42, 62, 68, 69); however, the specific amino acids in these proteins that are important for Vif interactions have not previously been identified. Our results showed that mutation of the amino acid equivalent to E289 rendered these proteins resistant to Vif-mediated degradation, suggesting that the EFLARH region in these proteins is involved in interactions with HIV-1 Vif that mediate proteasomal degradation.

It was previously shown that a deletion mutation in the N-terminal region (residues 2 to 47) of hA3C before the active site was not susceptible to HIV-1 or African green monkey simian immunodeficiency virus (SIVagm) Vif, suggesting that an important determinant for Vif-induced degradation is located in this region of A3C (69). Interestingly, we found that the E106 residue, located just after the active site (Fig. 4A), is important for HIV-1 Vif susceptibility. The results of these two studies suggest that more than one region of hA3C may be involved in the Vif-mediated degradation of this protein.

The hA3F Vif-interacting region is also present in the agmA3F (Chlorocebus aethiops) and rhA3F (Macaca mulatta) proteins, which have high overall sequence similarity to hA3F (88% and 87% identity, respectively) (66). Our results showed that agmA3F, but not rhA3F, is susceptible to HIV-1 Vif-mediated degradation, in agreement with previous reports (54, 66). Mutation of E289 in agmA3F rendered this protein resistant to Vif-mediated degradation. It is not clear why rhA3F remains resistant to HIV-1 Vif, but presumably, other structural features of rhA3F interfere with HIV-1 Vif binding or a subsequent step that is essential for Vif-induced degradation.

The gorilla A3C (Gorilla gorilla) and the chimpanzee A3D (Pan troglodytes) proteins also contain the EFLARH region, but whether these proteins are sensitive to HIV-1 Vif has not been determined. Interestingly, rhA3D was previously shown to be sensitive to HIV-1 Vif (54). While A3D isoform 1 contains an EFLATH sequence, the rhA3D sequence that was used to determine Vif sensitivity contained the EFLARH region. In addition, rhA3C, which has a KFLARH sequence, was found to be resistant to HIV-1 Vif-induced degradation (54). These results are consistent with the prediction of our studies and suggest that HIV-1 Vif interacts with rhA3D by use of the EF LARH region but cannot interact with rhA3C. The EFLARH region is not present in hA3G, hA3B, hA3A, and hA3H.

We also determined whether EFLARH-like sequences were present in the N-terminal domains of A3 proteins that contain two zinc-binding motifs; however, none of the human or nonhuman primate A3 proteins analyzed contained an exact match to this sequence. The EFLA/EH sequences that are present in hA3F and hA3DE are most likely not involved in neutralization by Vif, since the E289K mutation in hA3F and the analogous mutation in hA3DE conferred Vif resistance to these
proteins. In addition, the EFLRH region in rhA3F is also not likely to be involved in neutralization by Vif, since rhA3F is naturally resistant to Vif-mediated degradation.

In summary, we have demonstrated that residues 289EFLARH294 in hA3F are important for HIV-1 Vif interactions. In particular, the glutamate residue is critical for HIV-1 Vif-mediated degradation of hA3F as well as other A3 proteins, such as hA3C, hA3DE, and agmA3F. The identification of the HIV-1 Vif interaction region in hA3F may facilitate the development of novel antiviral drugs that target this region.

ACKNOWLEDGMENTS

We especially thank Wei-Shau Hu for intellectual input throughout the project. We also thank Gisela Heidecker, Chawaree Chaipan, FIG. 6. Model structure of the HIV-1 Vif binding domain of hA3F. (A) Model structure of the C-terminal domain of hA3G (Protein Data Bank accession number 2KEM (17) is shown. hA3G amino acids 297 to 302 of α-helix 3, which are equivalent to hA3F amino acids 289 to 294, are shown in space-filling form, with color coding as indicated. The remaining hA3G protein is shown in ribbon form; the α-helices are shown in red, and the β-sheets are shown in cyan. The figure was generated using the Accelrys Discovery Studio Visualizer (version 2.5) software program. (B) Schematic representation of hA3F-Vif and hA3G-Vif interactions. The major determinants of hA3F-Vif interactions and hA3G-Vif are shown. The determinants that were used in this study are shown extending out from each protein. The residues shown are those that have been shown to decrease interactions with A3F (top) or A3G (bottom) upon mutagenesis but to retain binding to the other APOBEC3 protein or cullin 5. (C) Sequence alignments of seven human A3 proteins and the A3F, A3C, and A3DE proteins from several nonhuman primates. The sequences are compared to the hA3F C-terminal EFLARH sequence and surrounding amino acids (left) and the N-terminal EFLAEH sequence and surrounding amino acids (right). Protein alignments were done using the BioEdit Sequence Alignment Editor, version 7.0.5.3 (16). The GenBank accession numbers of the sequences used in these comparisons were NP_660341.2 (A3F isoform α; Homo sapiens), AAH17371.1 (A3C; H. sapiens), NP_689639.2 (A3D; H. sapiens), NP_068594.1 (A3G; H. sapiens), EAW0281.1 (A3B; H. sapiens), NP_663745.1 (A3A; H. sapiens), ACK77727.1 (A3H; H. sapiens), NP_001055832.1 (A3F; M. mulatta), XP_525657.2 (A3D; P. troglodytes), XP_001094452.2 (A3G; M. mulatta), and NP_001009001.1 (P. troglodytes). The sequence for A3F (C. aethiops) was provided in reference 66.

proteins. In addition, the EFLAEH region in rhA3F is also not likely to be involved in neutralization by Vif, since rhA3F is naturally resistant to Vif-mediated degradation.

In summary, we have demonstrated that residues 289EFLARH294 in hA3F are important for HIV-1 Vif interactions. In particular, the glutamate residue is critical for HIV-1 Vif-mediated degradation of hA3F as well as other A3 proteins, such as hA3C, hA3DE, and agmA3F. The identification of the HIV-1 Vif interaction region in hA3F may facilitate the development of novel antiviral drugs that target this region.

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REFERENCES


