

# Identification of Two Distinct Human Immunodeficiency Virus Type 1 Vif Determinants Critical for Interactions with Human APOBEC3G and APOBEC3F<sup>∇</sup>

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**Human cytidine deaminases APOBEC3G (A3G) and APOBEC3F (A3F) inhibit replication of Vif-deficient human immunodeficiency virus type 1 (HIV-1). HIV-1 Vif overcomes these host restriction factors by binding to them and inducing their proteasomal degradation. The Vif-A3G and Vif-A3F interactions are attractive targets for antiviral drug development because inhibiting the interactions could allow the host defense mechanism to control HIV-1 replication. It was recently reported that the Vif amino acids D<sup>14</sup>RMR<sup>17</sup> are important for functional interaction and degradation of the previously identified Vif-resistant mutant of A3G (D128K-A3G). However, the Vif determinants important for functional interaction with A3G and A3F have not been fully characterized. To identify these determinants, we performed an extensive mutational analysis of HIV-1 Vif. Our analysis revealed two distinct Vif determinants, amino acids Y<sup>40</sup>RHHY<sup>44</sup> and D<sup>14</sup>RMR<sup>17</sup>, which are essential for binding to A3G and A3F, respectively. Interestingly, mutation of the A3G-binding region increased Vif's ability to suppress A3F. Vif binding to D128K-A3G was also dependent on the Y<sup>40</sup>RHHY<sup>44</sup> region but not the D<sup>14</sup>RMR<sup>17</sup> region. Consistent with previous observations, subsequent neutralization of the D128K-A3G antiviral activity required substitution of Vif determinant D<sup>14</sup>RMR<sup>17</sup> with SEMQ, similar to the SERQ amino acids in simian immunodeficiency virus SIV<sub>AGM</sub> Vif, which is capable of neutralizing D128K-A3G. These studies are the first to clearly identify two distinct regions of Vif that are critical for independent interactions with A3G and A3F. Pharmacological interference with the Vif-A3G or Vif-A3F interactions could result in potent inhibition of HIV-1 replication by the APOBEC3 proteins.**

The human immunodeficiency virus type 1 (HIV-1) virion infectivity factor (Vif) is a 23-kDa nonstructural protein essential for viral replication in certain nonpermissive cell types (6, 15, 25, 29, 33). Sheehy et al. first showed that the expression of APOBEC3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like protein 3G [A3G]) resulted in the nonpermissive cell phenotype (23). In the absence of Vif, A3G, a cytidine deaminase, is packaged into newly formed virions; during reverse transcription of the viral genome in the target cells, A3G deaminates the minus-strand DNA, leading to G-to-A hypermutation of the viral genome (8, 11, 16, 43). Vif overcomes the effects of A3G by binding to it and targeting it for degradation via the cellular ubiquitin/proteasomal pathway (2, 13, 18, 19, 24, 28, 41). Since the discovery of A3G, other APOBEC family members have been shown to be capable of inhibiting HIV-1. Of these, the most relevant is APOBEC3F (A3F), which is less potent than A3G (12, 27, 35, 42) but is expressed in CD4<sup>+</sup> T cells, a natural target of HIV-1 infection. Furthermore, A3F is susceptible to Vif-induced degradation, albeit with lower sensitivity than A3G (12, 42).

Extensive mutational analyses of Vif have revealed that the S<sup>144</sup>LQXLA<sup>150</sup> motif is essential for targeting A3G for proteasomal degradation (18, 19, 41). Vif interacts with the cellular

Cullin 5/ElonginBC E3 ubiquitin ligase complex via its SLQXLA motif, which leads to the polyubiquitination of A3G and its subsequent degradation. Substitution of the SLQ portion of the SLQXLA motif is sufficient to prevent A3G degradation (10, 24, 41). Recently, a novel zinc-binding motif, H<sup>108</sup>-X<sub>5</sub>-C-X<sub>17-18</sub>-C-X<sub>3-5</sub>-H<sup>139</sup> (HCCH), has also been shown to be important in A3G degradation (14, 36). Stabilization of the HCCH domain by incorporation of a zinc molecule was shown to be necessary for the Vif-Cullin 5 interaction and indeed appeared to be essential for the specificity of Cullin selection. Two additional domains, the central hydrophilic E<sup>88</sup>WRKKR<sup>93</sup> domain and the proline-rich P<sup>161</sup>PLP<sup>164</sup> domain, have been shown to be important in enhancing steady-state levels of Vif and in binding to tyrosine kinases, respectively (4, 5). The E<sup>88</sup>WRKKR<sup>93</sup> domain may be important for maintaining sufficient levels of Vif for APOBEC3 inhibition. However, it remains to be shown what role these domains play, if any, in Vif-targeted degradation of the APOBEC3 proteins.

While the Vif domains important in targeting A3G and, presumably, A3F for degradation have been well characterized, the regions involved in the interaction between the Vif and APOBEC3 proteins have not been clearly defined. Prior to the discovery of the APOBEC3 proteins, an extensive series of alanine substitution and deletion mutations of Vif were characterized (26). However, the intracellular locations of several of the Vif mutants were altered, and the exact role of these amino acids in Vif function could not be determined. The only region identified by this analysis was the SLQXLA motif, which was later shown to play an important role in Vif func-

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tion; however, other regions important for Vif function were not revealed in this analysis. More recently, a number of studies have been carried out that have identified Vif residues important for the inhibition of A3G and A3F that are not located in the HCCH or SLQXLA motifs. Simon et al. reported the presence of single-amino-acid changes in Vif sequences isolated from HIV-1-infected patients that were sufficient to prevent APOBEC3 neutralization (27). Interestingly, a subset of the mutants was functional against A3G but not A3F and vice versa. A similar phenomenon was also observed with specific tryptophan residues (31). Schrofelbauer et al. (22) have also reported that the residues D<sup>14</sup>RMR<sup>17</sup> are important in A3F inhibition and in the degradation of a previously identified Vif-resistant mutant of A3G (D128K-A3G) (1, 17, 21, 38). Amino acid 128 of A3G was shown to be solely responsible for the species-specific inhibition of A3G by Vif (1, 17, 21, 38). Substitution of D128 in human A3G for K128, found in African green monkey (agm) A3G, results in Vif-resistant human A3G. This has been suggested to occur either because D128K-A3G is no longer able to interact with HIV-1 Vif (1, 17, 21) or because, following D128K-A3G-Vif binding, a subsequent downstream step is inhibited (38). Schrofelbauer et al. showed that substitution of D<sup>14</sup>RMR<sup>17</sup> with SERQ, the equivalent residues found in agm Vif, was sufficient to allow functional interaction of HIV-1 Vif with rhesus macaque A3G and agmA3G, as well as human A3G and D128K-A3G. This interaction was also observed with only a three-amino-acid change of DRMR to SEMQ. This removal of species restriction was concluded to be due to the negative charge of residues 14 and 17 of Vif and their interaction with the positively charged residue at position 128 in A3G (22). However, with the exception of the W11A substitution that was shown by Tian et al. to no longer bind to A3F, none of the work described above has demonstrated a direct link to Vif-APOBEC3 binding. Consequently, whether these mutations play a specific role in Vif-APOBEC3 interactions or a subsequent step has not been elucidated.

To identify the determinants of Vif that interact with A3G and A3F, we employed a strategy of sequential double-alanine-substitution mutagenesis starting at the N terminus of Vif. Analysis of these mutants for their ability to rescue HIV-1 from inhibition by the APOBEC3 proteins and for their ability to bind to the APOBEC3 proteins in a coimmunoprecipitation (co-IP) assay led to the discovery of two distinct regions of Vif involved in A3G and A3F binding. Interestingly, mutation of the A3G-binding region resulted in an increase in Vif function against A3F, whereas the reverse observation was not made for the A3F-binding region. Further studies showed that the D128K-A3G mutant bound to the A3G-binding domain but that residues in the A3F-binding domain were critical for its subsequent degradation. These studies are the first to identify the presence of two distinct regions of Vif that are involved in interaction with A3G and A3F. Identification of these determinants will aid in the rational design of inhibitors that interfere with the Vif-APOBEC3 interactions and provide new avenues for anti-HIV therapy.

#### MATERIALS AND METHODS

**Plasmid construction and cell culture.** Alanine substitution mutations were constructed in a codon-optimized Vif expression vector, pcDNA-HVif (20), by

using a QuikChange II site-directed mutagenesis kit (Stratagene). Where possible, additional silent mutations were also inserted, forming new restriction enzyme sites, to allow for easy screening of colonies. The presence of the desired mutations and the absence of undesired mutations in the mutant HIV expression vectors were verified by DNA sequencing. The N-terminal FLAG-tagged A3F expression vector, pFLAG-A3F, was constructed by PCR amplification of A3F from pcDNA3.1-APOBEC3F (12) using the forward primer 5'GATCGCGGCCGCTATGAAGCCTCACTCAGAAAC3', containing an additional NotI restriction enzyme site, and the reverse primer 5'GATCTCTAGAATGAGCAGGAGGCTAGAGGAG3', containing an additional XbaI restriction enzyme site. The resulting PCR amplicon was digested with NotI plus XbaI and cloned into NotI-plus-XbaI-digested pFLAG-A3G, which expresses FLAG-tagged A3G, thereby removing A3G and replacing it with A3F. The absence of undesired mutations in the resulting pFLAG-A3F was verified by DNA sequencing. The empty expression vector pcDNA3.1noMCS was made by digestion of pcDNA-APO3G (9) with PmeI, removal of the A3G fragment, and religation of the vector backbone.

The modified human embryonic kidney cell line 293T (39) and the HeLa-derived HIV-1 reporter cell line TZM-bl (3, 34), which encodes the firefly luciferase gene under the control of HIV-1 Tat-responsive promoter, were maintained in complete medium (CM) which consisted of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, and 1% glutamine.

**Virus production and titration.** For virus production, 293T cells, seeded at  $8 \times 10^5$  cells per well in six-well plates, were transfected with the following plasmids: 3.33  $\mu$ g of the HIV-1 vector genome pHDV-EGFP (32); 0.67  $\mu$ g of the vesicular stomatitis virus glycoprotein expression plasmid; pHCMV-V-G (40); 0.67  $\mu$ g of either pcDNA-APO3G, which expresses A3G, pcDNA3.1-APOBEC3F, which expresses A3F, or pA3G-D128Kmyc (38), which expresses A3G with the D128K substitution (D128K-A3G); and 4.5  $\mu$ g of either wild-type (WT) or mutant pcDNA-HVif. The virus-containing supernatant was harvested 48 h after transfection, filtered through a 0.45- $\mu$ m filter, and diluted in CM. TZM-bl cells were seeded at  $4 \times 10^3$  cells per well in white flat-bottom 96-well plates and infected with virus 24 h later. Another 72 h later, the culture medium was removed and replaced with 100  $\mu$ l of CM without phenol red and 100  $\mu$ l of Britelite luciferase solution (PerkinElmer). After a 1-min incubation, the level of luciferase activity was measured using a LUMIstar Galaxy luminometer.

**Co-IP and protein visualization.** 293T cells were seeded at  $4 \times 10^6$  cells per 100-mm dish and were transfected 24 h later with 6  $\mu$ g of either pFLAG-A3G, pFLAG-A3F, or pFLAGAPOD128K (37); 6  $\mu$ g of either WT or mutant pcDNA-HVif; 1.2  $\mu$ g pGL; and 9  $\mu$ g pcDNA3.1noMCS. A 1:5 molar ratio of APOBEC3 (0.8  $\mu$ g) to Vif (4  $\mu$ g) was also analyzed for the multiple-alanine-substitution mutants. The supernatant was removed 48 h posttransfection, and the cells were washed twice in 10 ml of phosphate buffered saline. The cells were then lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing Protease Inhibitor Cocktail (Sigma), by incubation with gentle agitation for 30 min. The cellular debris was removed by centrifugation at  $10,000 \times g$  for 10 min, and the resulting supernatant was added to an anti-FLAG M2 agarose affinity gel (Sigma). The FLAG-tagged APOBEC3 proteins were allowed to bind to the agarose affinity gel by gentle rotation at 4°C overnight, after which any unbound proteins were removed by extensive washing. The bound protein complexes were then eluted by competition with a 3 $\times$  FLAG peptide (Sigma). The eluted complexes, as well as the input cell lysates, were then analyzed by polyacrylamide gel electrophoresis and Western blotting. The APOBEC3 proteins were detected using a mouse anti-M2 antibody (Sigma) at a 1:5,000 dilution, followed by a horseradish peroxidase (HRP)-labeled goat anti-mouse secondary antibody (Sigma) at a 1:10,000 dilution; the Vif proteins were detected using a rabbit anti-Vif polyclonal antibody (7) at a 1:5,000 dilution, followed by an HRP-labeled goat anti-rabbit secondary antibody (Sigma) at a 1:10,000 dilution. As a control for the amount of total protein,  $\alpha$ -tubulin was detected using mouse anti- $\alpha$ -tubulin antibody (Sigma) at a 1:1,000 dilution, followed by an HRP-labeled goat anti-mouse secondary antibody at a 1:10,000 dilution. The proteins were visualized using a Western Lighting Chemiluminescence Reagent Plus kit from PerkinElmer, and the band intensities were measured using an Alpha Innotech ChemiImager 5500.

#### RESULTS

**Identification of two distinct regions of Vif involved in inhibition of A3G and A3F.** To determine the region(s) of Vif important in APOBEC3 binding, a series of sequential double-alanine-substitution mutants was generated in pcDNA-HVif, a

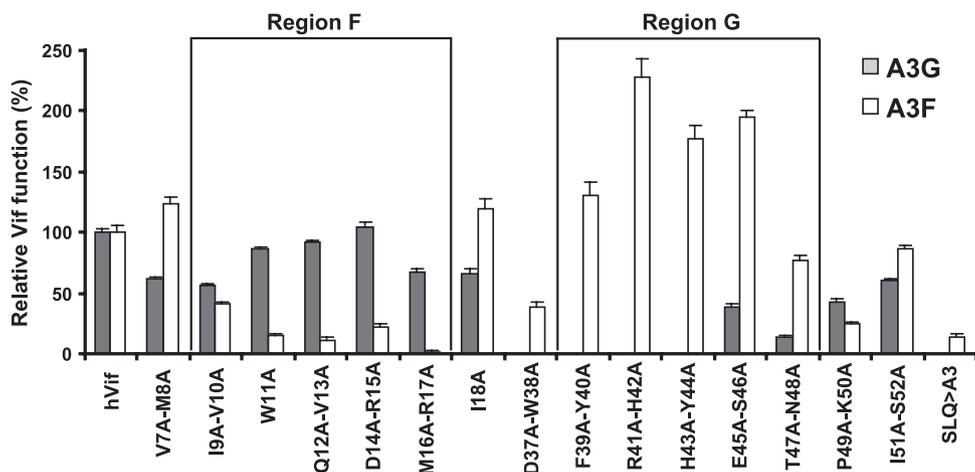


FIG. 1. The effect of double-alanine substitutions within Vif on its function against A3G and A3F. Double-alanine-substitution mutants were generated, and their effect on Vif function against A3G and A3F was determined. For determination of Vif mutant activity against A3G (gray bars) and A3F (white bars), 293T cells were transfected with the WT or mutant Vif expression plasmid, pHDV-EGFP, a vesicular stomatitis virus glycoprotein expression plasmid, and either an A3G-expression plasmid or an A3F-expression plasmid. The infectivity of the virus produced from the transfected cells was determined by infection of TZM-bl indicator cells and quantitation of luciferase enzyme produced in the TZM-bl cells after infection. The average relative light units (RLU) in the presence of A3G with WT Vif were 145,544 and 51,091 in the presence of A3F with WT Vif. Background levels were an average of 480 RLU. The data shown are plotted as the level of mutant Vif function as a percentage, relative to WT Vif activity (set to 100%), with standard errors of the means (SEM) from two independent experiments. Region F designates mutations in Vif that reduce activity against A3F but not A3G; region G designates mutations in Vif that reduce activity against A3G but not A3F.

codon-optimized HIV-1 Vif expression vector. The initial panel of mutants targeted Vif residues 2 to 60. Because some of the single-residue mutants were already constructed (for example, W11A and I18A), the double mutants were designed to target other residues. The effect of these mutations on Vif function was then measured as the ability of the Vif mutants to rescue HIV-1 replication from inhibition by A3G and A3F. The level of Vif function was then calculated as a ratio of the mutant Vif function to the WT Vif function against A3G and A3F (Fig. 1). Among the mutants tested, four different phenotypic classes were observed: those that remained fully functional against both A3G and A3F (e.g., V7A-M8A in Fig. 1), those that reduced function against both A3G and A3F (e.g., P49A-K50A), and those that had lost function against A3F but not A3G (e.g., Q12A-V13A) or against A3G but not A3F (e.g., R41A-H42A). Mutants that had lost function against both of the APOBEC3 proteins were not examined further, as it was unknown if the introduced mutations had resulted in protein misfolding or altered intracellular localization. Of particular interest were the mutants that fell into the last two categories. Since they were still fully functional against either A3G or A3F, it was surmised that they were correctly folded and localized within the cell. Of these mutants, all were expressed at WT levels in the absence of any APOBEC3 proteins, with the exception of E45A-S46A, which showed a reduced expression level (data not shown). Therefore, we have identified two distinct regions of Vif important for its function against the APOBEC3 proteins, termed region F and region G (Fig. 1). Region F, located between amino acids I9 and R17, is important in Vif function against A3F, while region G, located between amino acids F39 and N48, is important for Vif function against A3G. Interestingly, several of the mutants located in region G exhibited reduced Vif function against A3G but enhanced function against A3F. As expected, a control Vif mutant

(SLQ>A3) containing a triple-alanine substitution of the essential SLQ residues within the SLQXLA motif (10, 24, 41) exhibited a severe reduction in Vif function against both A3G and A3F. The mutants were also tested for their ability to inhibit A3G activity by using a previously described scintillation proximity assay for cytidine deaminase activity (30), the results of which closely matched those obtained from the infectivity assay (data not shown).

To establish whether regions G and F were directly involved in Vif-APOBEC3 binding, we performed co-IP assays using A3G and A3F proteins tagged at their N terminus with the FLAG epitope (Fig. 2). The ability of the FLAG-tagged APOBEC3 proteins to coimmunoprecipitate with the mutant Vif proteins was determined by polyacrylamide gel electrophoresis and Western blotting (Fig. 2A and B). In each case, the amounts of APOBEC3 and Vif in both the input cell lysates and the coimmunoprecipitates were measured. To normalize for the amount of total cell lysate protein added to the co-IP assay, the level of  $\alpha$ -tubulin in the input cell lysate was determined. The binding efficiency relative to WT Vif, shown below the co-IP samples in Fig. 2A and B, was calculated as a ratio of the level of the coimmunoprecipitated Vif to the level of the input Vif, normalized to the level of A3G or A3F protein in the co-IP assay.

We first analyzed the effects of the double-alanine-substitution mutations in region G on binding to A3G (Fig. 2A, left panel; binding efficiencies from two independent experiments are shown). The mutants spanning amino acids F39 to Y44 showed reduced binding to A3G. The binding efficiencies of these mutants ranged from  $23\% \pm 7\%$  for F39A-Y40A to  $8\% \pm 4\%$  for H43A-Y44A, and all are statistically significantly different from that of WT Vif ( $t$  test;  $P < 10^{-5}$ ). Although the mutants spanning amino acids E45 to K50 appear to be less abundant than WT Vif in the A3G co-IP samples, they also

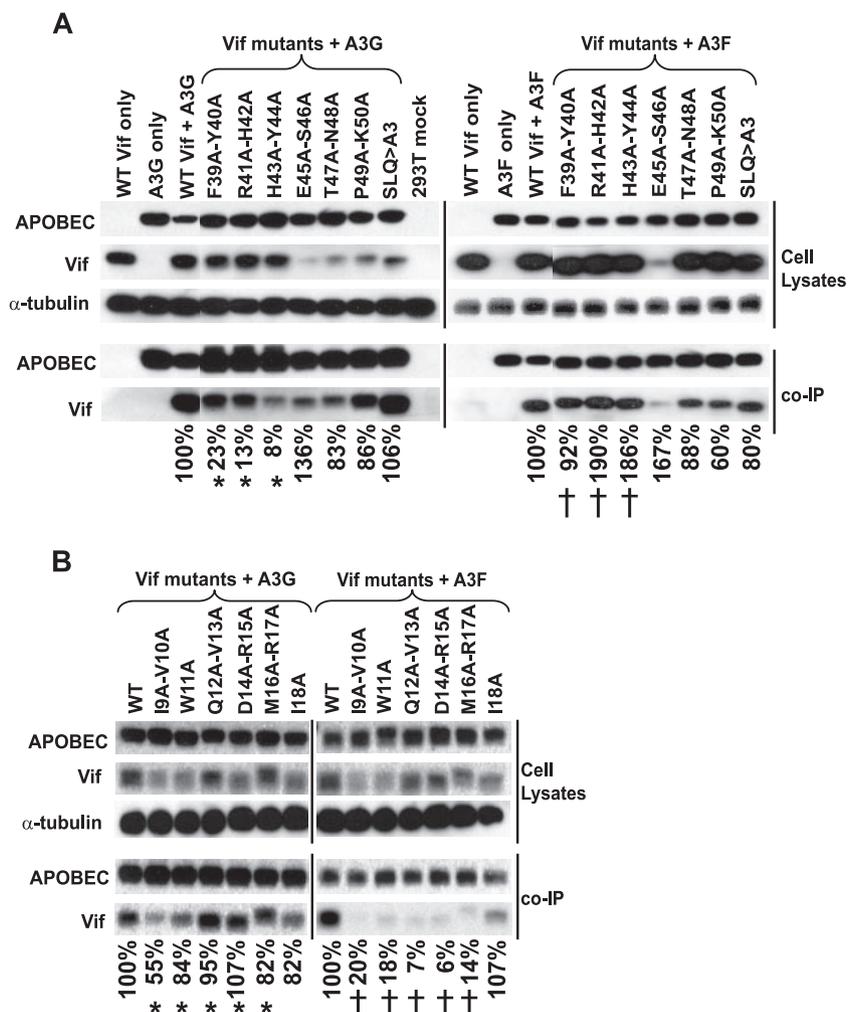


FIG. 2. Effects of single- and double-alanine-substitution mutations on binding to A3G and A3F. (A) Effects of double-alanine substitutions in region G on binding to A3G (left panel) and A3F (right panel). Either FLAG-A3G or FLAG-A3F was cotransfected into 293T cells with WT Vif- or Vif mutant-expressing plasmids. Cells were transfected with WT Vif only, A3G only, A3F only, and A3G and A3F with the SLQ Vif mutant, and mock-transfected cells served as controls. The transfected cell lysates were analyzed by Western blotting for expression of A3G or A3F (APOBEC) or Vif. The cell lysates were also analyzed for  $\alpha$ -tubulin to control for the total amount of cell lysate analyzed. Additionally, the cell lysates were analyzed in a co-IP assay using anti-FLAG antibody to immunoprecipitate FLAG-A3G (left panel) or FLAG-A3F (right panel). The immunoprecipitated proteins were analyzed by Western blotting using anti-FLAG and anti-Vif antibodies. Band intensities were quantified from two independent experiments, and the binding efficiency of each mutant was calculated as the ratio of coimmunoprecipitated Vif to the levels of input Vif in cell lysates, normalized to the amount of immunoprecipitated A3G or A3F. The asterisks (\*) and daggers (†) indicate double-alanine-substitution mutants that significantly reduced A3G binding but either enhanced or did not reduce A3F binding. (B) Effects of single- and double-alanine substitutions in region F on binding to A3G (left panel) and A3F (right panel). Either FLAG-A3G or FLAG-A3F was cotransfected into 293T cells with WT Vif- or Vif mutant-expressing plasmids. Cells transfected with WT Vif only served as a control. The transfected cell lysates were analyzed for expression of FLAG-A3G, FLAG-A3F, Vif, and  $\alpha$ -tubulin as described above. The cell lysates were also analyzed in co-IP assays, the band intensities from two independent experiments were quantified, and the binding efficiency of each mutant was calculated as described above. The asterisks (\*) and daggers (†) indicate double-alanine-substitution mutants that significantly reduced A3F binding (right panel) but did not influence A3G binding (left panel).

had lower steady-state levels than WT Vif in the input cell lysates and therefore do not possess a binding defect. In fact, for the mutant E45A-S46A, the co-IP samples displayed an enrichment of Vif, suggesting enhanced binding to A3G. Interestingly, mutants T47A-N48A and P49A-K50A do not appear to be as abundant as WT Vif in the presence of A3G, suggesting that the reduction in steady-state levels of these particular Vif mutants was related to A3G coexpression. As expected, the SLQ>A3 mutation did not affect the efficiency of the Vif-A3G interaction in the co-IP assays. Furthermore, in

the presence of WT Vif, there was a reduction (3.2-fold) in the level of A3G in the input cell lysates; however, because the A3G amounts are in large excess relative to the anti-FLAG antibody in the co-IP assay, this reduction did not significantly affect the efficiency of the co-IP. Moreover, in calculations of the binding efficiency, any differences in the amount of APOBEC3 bound during co-IP is accounted for by normalizing for the level of bound APOBEC3.

We also analyzed the effects of double-alanine-substitution mutations in region G on binding to A3F (Fig. 2A, right panel).

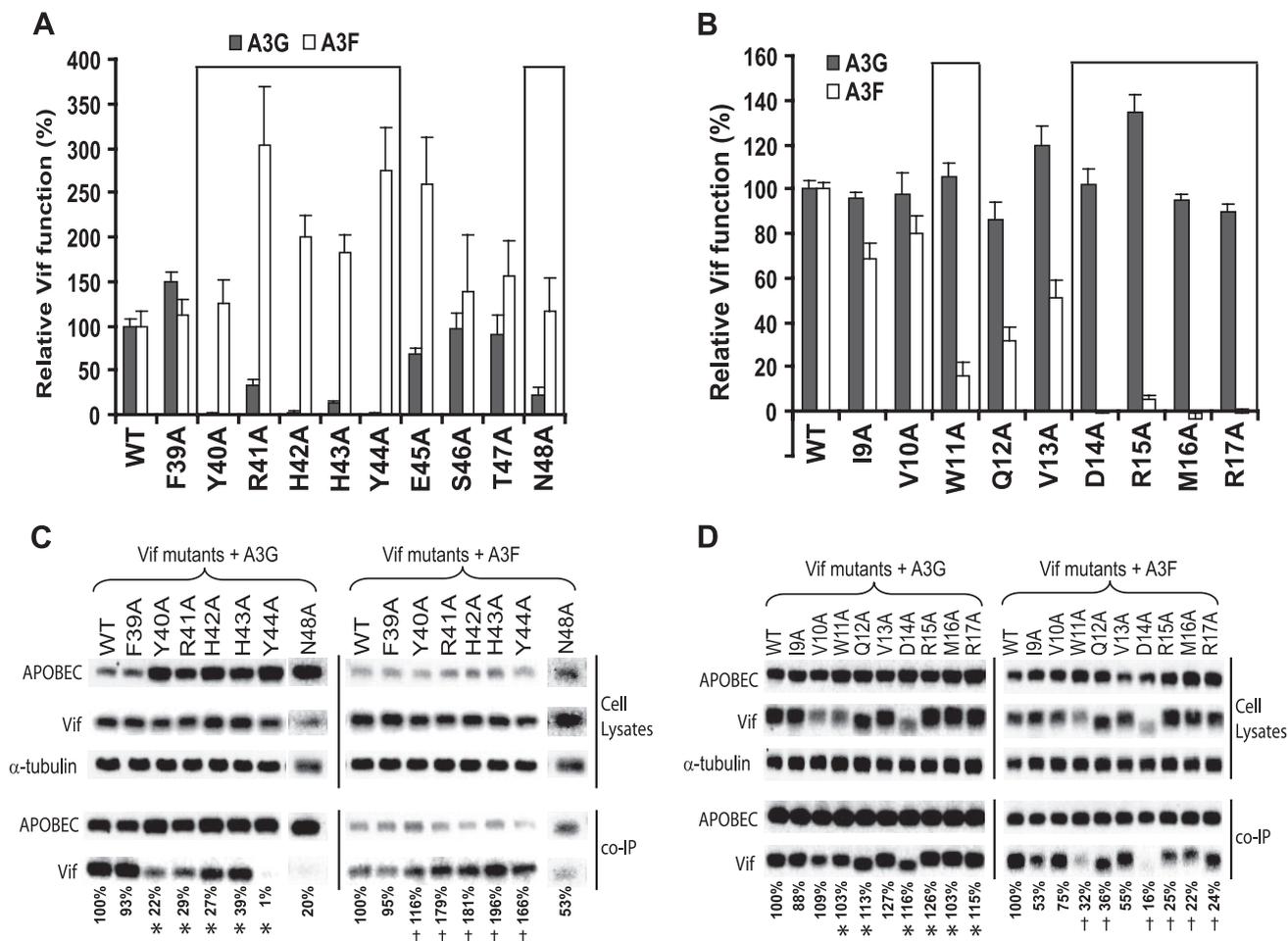


FIG. 3. The effects of single-alanine-substitution mutations on Vif function and binding to APOBEC3 proteins. (A) The effects of single-alanine substitutions in region G on Vif's function against A3G and A3F. The function of Vif against A3G and A3F was determined using the assay described in the Fig. 1 legend. Single-alanine substitutions of Y40 to Y44 and N48 significantly reduced Vif function against A3G but either enhanced or did not influence Vif function against A3F. The average RLU in the presence of A3G with WT Vif were 200,550 and 130,886 in the presence of A3F with WT Vif. Background levels were an average of 350 RLU. The results are presented as a percentage of WT Vif binding with SEM from two independent experiments. (B) The effect of single-alanine substitutions in region F on Vif function against A3G and A3F. Substitutions of W11 or D14 to R17 significantly reduced Vif function against A3F but not A3G. The average RLU in the presence of A3G with WT Vif were 117,796 and 64,457 in the presence of A3F with WT Vif. Background levels were an average of 430 RLU. The results are presented as a percentage of WT Vif binding with SEM from two independent experiments. (C) Effects of single-alanine substitutions in region G on binding to A3G (left panel) and A3F (right panel). Western blotting analysis and co-IP assays were performed as described in Fig. 2 legend. The asterisks (\*) and daggers (†) indicate single-alanine-substitution mutants that significantly reduced A3G binding (left panel) but either enhanced or did not influence A3F binding (right panel). (D) Effects of single-alanine substitutions in region F on binding to A3G (left panel) and A3F (right panel). Western blotting analysis and co-IP assays were performed as described in the Fig. 2 legend. The asterisks (\*) and daggers (†) indicate single-alanine-substitution mutants that significantly reduced A3F binding (right panel) but did not significantly influence A3G binding (left panel). A representative analysis is shown, and the results from two independent experiments are presented as a percentage of WT Vif binding.

The Vif mutants that exhibited reduced binding to A3G did not display reduced binding to A3F (compare the lanes marked with an asterisk to those marked with a dagger). Interestingly, the mutants spanning amino acids R41 to S46 showed enhanced binding to A3F that was consistent with their increased ability to neutralize A3F (Fig. 1). The results also indicated that, in contrast to the results obtained in the presence of A3G, coexpression of A3F did not influence the steady-state levels of the T47A-N48A and P49A-K50A mutants. Again, as expected, the SLQ>A3 mutation did not influence the ability of Vif to coimmunoprecipitate with A3F. In the presence of WT Vif, there was a reduction (1.5-fold) in the

level of A3F in the input cell lysates. Although this level of reduction was less than that seen for A3G (3.2-fold), it supports the previous observations that A3F is partially Vif resistant (12, 42).

Next, we analyzed the effects of the alanine substitution mutations in region F on binding to A3G (Fig. 2B, left panel; binding efficiencies from two independent experiments are shown). None of the mutations tested significantly affected the Vif-A3G binding in the co-IP assays. In contrast, reduced binding to A3F was seen for amino acids spanning I9 to R17 of region F (Fig. 2B, right panel; compare the lanes marked with an asterisk to those marked with a dagger). The binding effi-

ciencies of these mutants to A3F ranged from  $20\% \pm 4\%$  for I9A-V10A to  $6\% \pm 0.24\%$  for D14A-R15A and all are statistically significantly different from WT Vif ( $t$  test;  $P < 10^{-3}$ ). Taken together, these results strongly suggest the presence of two APOBEC3 binding regions in Vif, one spanning amino acids F39 to Y44 that specifically binds to A3G and one spanning amino acids I9 to R17 that specifically binds to A3F.

**Single-amino-acid substitutions in regions G and F suppress Vif function.** To characterize regions G and F further, single-alanine-substitution mutations were introduced at positions F39 to N48 and I9 to R17, respectively. The resulting mutants were tested for their ability to rescue HIV-1 from inhibition by A3G and A3F (Fig. 3). Analysis of the single-alanine substitutions in region G showed that amino acids Y40 to Y44 and N48 were critical for Vif's function against A3G (Fig. 3A). The same single-amino-acid substitutions either did not significantly influence (e.g., Y40A) or enhanced (e.g., R41A) Vif's ability to neutralize A3F.

We also analyzed the effects of the single-alanine-substitution mutations in region F on Vif's activity against A3F (Fig. 3B). The results showed that amino acids D14 to R17 were critical for Vif function against A3F. To a lesser extent, W11 and Q12 were also important for Vif-mediated neutralization of A3F. In contrast, the mutations in region F had no effect on Vif function against A3G.

We then examined the effects of the single-alanine-substitution mutations in region G on Vif-A3G and Vif-A3F binding (Fig. 3C). As expected, the mutations that displayed the most significant reductions in Vif function against A3G (Fig. 3A) also showed the most severe defect in binding to A3G in co-IP assays (Fig. 3C, left panel). The Vif-A3G binding efficiency was most severely reduced by the single Y44A substitution, to 1% of that of the WT Vif control. Other single-alanine substitutions reduced binding to A3G to various degrees, ranging from 20 to 39% (Fig. 3C, left panel, lanes marked with asterisks). The F39A mutant, which did not influence the function of Vif against A3G, also did not influence Vif-A3G binding in co-IP assays. In all cases where a reduction in binding was seen, this reduction was statistically significant ( $t$  test;  $P < 10^{-2}$ ). Additionally, we tested the effects of the single-alanine-substitution mutations in region G on A3F binding in co-IP assays (Fig. 3C, right panel). In agreement with their effects on HIV-1 infectivity (Fig. 3A), the single-alanine substitutions R41A to Y44A, which reduced A3G binding, showed a slight, twofold increase in binding to A3F (Fig. 3C, compare lanes labeled R41A to Y44A and marked with an asterisk to those marked with a dagger).

Finally, we analyzed the effects of single-alanine substitutions in region F on Vif-APOBEC3 binding (Fig. 3D). In agreement with their effect on Vif function, the mutations D14A to R17A reduced the ability of Vif to bind to A3F in co-IP assays (Fig. 3D, right panel). The W11A and Q12A mutations also reduced Vif-A3F binding but to a lesser extent. In contrast, these single-alanine-substitution mutations did not affect Vif-A3G binding (Fig. 3D, compare lanes marked with an asterisk with those marked with a dagger).

**Multiple-alanine substitutions in regions G and F almost completely abolish Vif-APOBEC3 binding.** Most of the single- and double-alanine-substitution mutations in regions G and F

did not completely inhibit APOBEC3 binding. To examine the combined effects of the inhibitory mutations in regions G or F, we constructed two additional mutants in which multiple-alanine substitutions were introduced in each region. In region G, amino acids Y40 to Y44 were replaced by alanines to generate YRH<sub>40-44</sub>>A5, whereas in region F, amino acids D14 to R17 were replaced by alanines to create DRMR>A4. The effects of these multiple-alanine substitutions on Vif's ability to neutralize A3G and A3F were then determined (Fig. 4A). The YRH<sub>40-44</sub>>A5 mutation almost completely abolished Vif neutralization of A3G, but increased its ability to block A3F. In contrast, the DRMR>A4 mutation did not influence the ability of Vif to neutralize A3G but almost completely blocked its ability to inhibit A3F function.

Next, we determined the effects of the multiple alanine substitutions on binding to A3G and A3F (Fig. 4B). The YRH<sub>40-44</sub>>A5 mutation almost completely abrogated Vif-A3G binding, reducing it to 6% of the WT Vif control value, but only slightly reduced Vif-A3F binding, to 63% of the control value. In contrast, the DRMR>A4 mutation almost completely eliminated Vif-A3F binding, reducing it to 7% of that of the WT Vif control; the same mutation only reduced Vif-A3G binding to 67% of that of the control.

To confirm that these observations were not the result of overexpression of the APOBEC3 proteins in relation to Vif, we repeated the experiment using a 1:5 molar ratio of APOBEC3:Vif (Fig. 4C). Under these conditions, a much greater reduction in the levels of both A3G and A3F in the presence of WT Vif was observed, although, as expected, the level of reduction of A3F (15-fold) was less than that of A3G (25-fold). However, despite the reduction in input APOBEC3, there was still sufficient APOBEC3 present for co-IP. In complete agreement with the results obtained using a 1:1 ratio of APOBEC3:Vif, the YRH<sub>40-44</sub>>A5 mutation prevented Vif-A3G binding and the DRMR>A4 mutation prevented Vif-A3F binding. In both cases, there was only minimal impact on the binding of the YRH<sub>40-44</sub>>A5 and DRMR>A4 Vif mutants to A3F and A3G, respectively. In support of the binding data, the level of A3G in the input cell lysates was severely decreased in the presence of DRMR>A4 but not YRH<sub>40-44</sub>>A5, whereas the level of A3F in the input cell lysates was unaffected by DRMR>A4 but severely reduced by YRH<sub>40-44</sub>>A5. These results highlight the importance of the two distinct regions in Vif binding to and degradation of A3G and A3F. Furthermore, since the same mutations had little or no effect on binding to and degradation of the other APOBEC3 protein, they confirm that the mutations do not drastically alter the overall structure of Vif. As the double-alanine mutants I9A-V10A and Q12A-V13A also appeared to have detrimental effects on Vif binding to A3F (Fig. 2B), we constructed the mutant IVWQVDRMR>A9, which had nine sequential alanine substitutions. However, when it was tested for its function against A3G and A3F, we found not only that it was nonfunctional against A3F but also that it had lost function against A3G, suggesting that these mutations altered the overall structure of Vif (data not shown).

**D128K-A3G requires the Y<sup>40</sup>RHHY<sup>44</sup> region to bind to Vif, but its subsequent degradation is dependent on the amino acids located at positions 14 to 17.** It was recently reported that substitution of the D<sup>14</sup>RMR<sup>17</sup> residues with SERQ (22), the equivalent amino acids in agm Vif, or SEMQ, allowed HIV-1

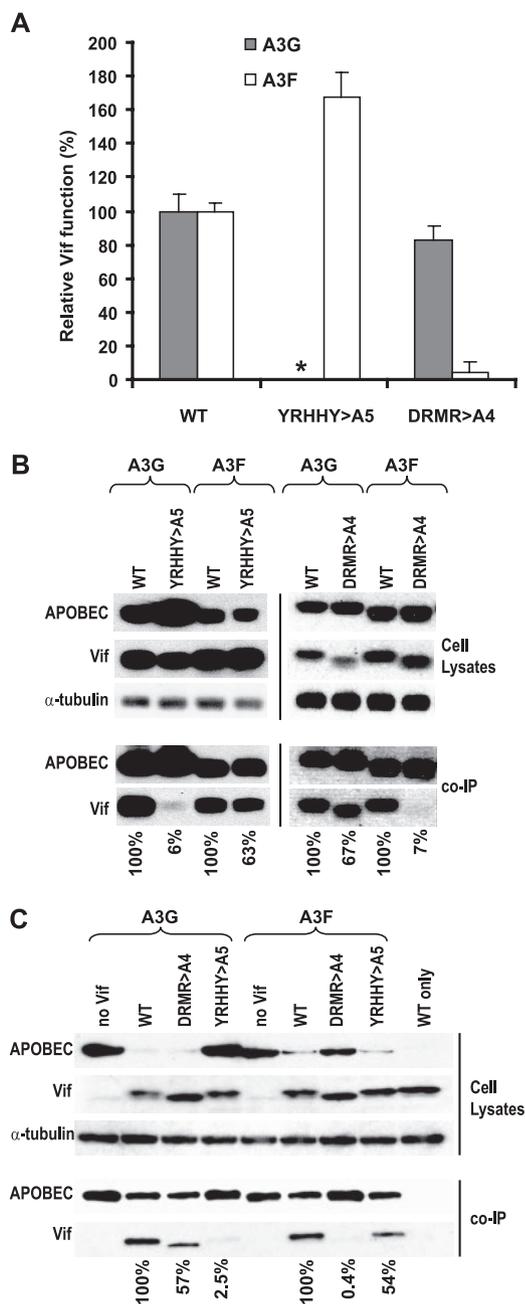


FIG. 4. Effects of multiple-alanine substitutions in regions G and F on Vif function and APOBEC3 binding. (A) Amino acids Y40 to Y44 in region G were replaced by alanines to create YRHHY>A5; amino acids D14 to R17 in region F were replaced by alanines to generate DRMR>A4. The effects of these mutations on Vif function were determined as described in the Fig. 1 legend. The asterisk denotes that the effect of YRHHY>A5 mutation on function against A3G was tested, but the bar graph and the error bars are too small to be seen. The average RLU in the presence of A3G with WT Vif were 78,964 and 45,260 in the presence of A3F with WT Vif. Background levels were an average of 265 RLU. The results are presented as a percentage of WT Vif binding with SEM from two independent experiments. (B) Effects of multiple-alanine substitutions on APOBEC3 binding at a 1:1 molar ratio. The effects of the YRHHY>A5 and the DRMR>A4 mutations on A3G and A3F binding at a 1:1 molar ratio of APOBEC3:Vif are shown in the left and right panels, respectively. Western blotting analysis and co-IP assays were performed as described in the Fig. 2 legend. A representative analysis is shown, and the results from two independent experiments are presented as a percentage of WT Vif

Vif to neutralize the function of a Vif-resistant mutant of A3G (D128K-A3G) (1, 17, 21, 38). To investigate this further, we analyzed the single- and multiple-alanine-substitution mutants in region F for their ability to inhibit D128K-A3G (Fig. 5A). The results showed that, similar to WT HIV-1 Vif, the single- and multiple-alanine-substitution mutants were unable to efficiently block the antiviral activity of D128K-A3G. The R15A mutant exhibited a modest, 5.5-fold increase in function against D128K-A3G; however, the D128K-A3G still inhibited HIV-1 infectivity 40-fold in the presence of the R15A mutant compared to the control infection in the absence of A3G. None of the region G mutants showed any function against D128K-A3G (data not shown).

To examine the role of the D<sup>14</sup>RMR<sup>17</sup> region in blocking the antiviral activity of D128K-A3G further, we replaced the D<sup>14</sup>RMR<sup>17</sup> residues with SEMQ and tested the ability of the mutant to inhibit A3G, A3F, and D128K-A3G (Fig. 5B). In agreement with results reported by Schrofelbauer et al. (22), the SEMQ Vif mutant was able to rescue virion infectivity in the presence of A3G and D128K-A3G but not A3F. We then determined the ability of the SEMQ Vif mutant to bind to A3G, A3F, and D128K-A3G in co-IP assays (Fig. 5C). The results showed that the SEMQ mutant was unaffected in its ability to bind to WT A3G. However, in agreement with the results obtained with the DRMR>A4 mutant (Fig. 4B), the ability of the SEMQ mutant to bind to A3F was reduced to 6% of that of the WT Vif control. Furthermore, the WT Vif and the SEMQ mutant Vif were both able to bind to the D128K-A3G in the co-IP assay. Consistent with its ability to neutralize D128K-A3G, co-expression of the SEMQ mutant Vif resulted in a significant reduction in the amount of the D128K-A3G in the cell lysate (Fig. 5C, compare D128K-A3G in the presence of WT Vif versus SEMQ mutant Vif in the cell lysates).

We also assessed the binding of D128K-A3G to the DRMR>A4 and YRHHY>A5 Vif mutants (Fig. 5D). The results showed that, similar to WT A3G, the D128K-A3G mutant was able to bind to both WT Vif and the DRMR>A4 mutant Vif but not the YRHHY>A5 mutant Vif. Taken together, these data suggest that D128K-A3G binds to HIV-1 Vif via the Y<sup>40</sup>RHHY<sup>44</sup> domain but that a subsequent downstream step is blocked by the presence of DRMR and not SEMQ at amino acids 14 to 17.

## DISCUSSION

The results of these studies show that the Y<sup>40</sup>RHHY<sup>44</sup> residues of Vif are important for binding to A3G, whereas the D<sup>14</sup>RMR<sup>17</sup> residues are important for binding to A3F. Our results that the YRHHY>A5 and DRMR>A4 mutations reduced A3G and A3F binding to 6 and 7%, respectively, indicate that these are the primary determinants that are critical

binding. (C) Effects of multiple-alanine substitutions on APOBEC3 binding at a 1:5 molar ratio of APOBEC3:Vif. The effects of the YRHHY>A5 and the DRMR>A4 mutations on A3G and A3F binding at a 1:5 molar ratio of APOBEC3:Vif are shown. Western blotting analysis and co-IP assays were performed as described in the Fig. 2 legend.

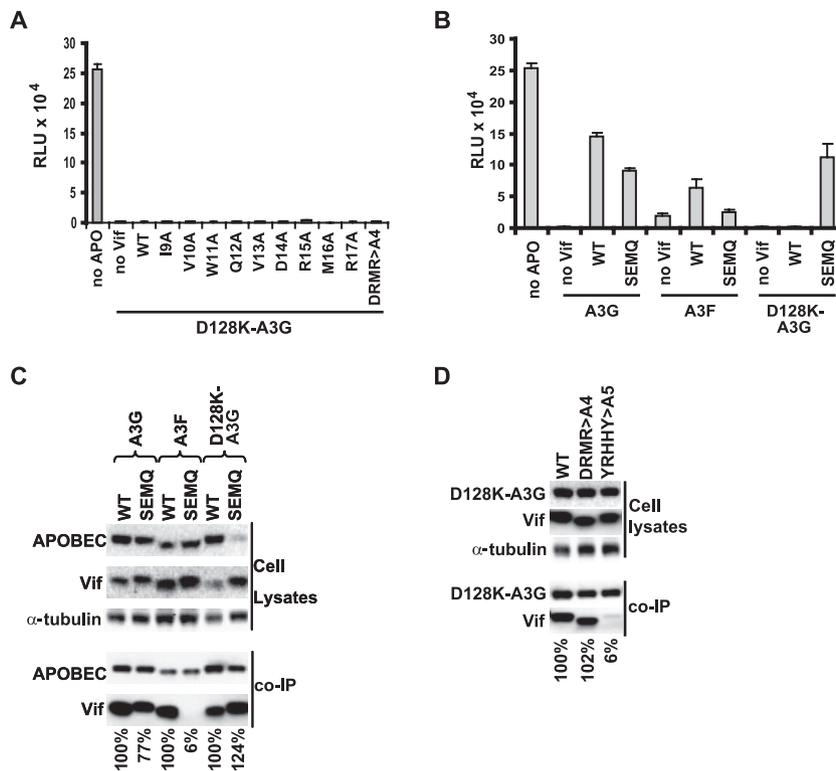


FIG. 5. Effects of single- and multiple-substitution mutations on Vif function and binding to A3G, A3F, and D128K-A3G. (A) Effect of the single-alanine mutants in region F on Vif function against D128K-A3G. The effects of these mutations on Vif function against D128K-A3G were determined as described in the Fig. 1 legend. The results shown are the RLU and SEM from two independent experiments. (B) Effect of the DRMR>SEMQ substitution on Vif function. The effects of the DRMR>SEMQ mutation on Vif function against A3G, A3F, and D128K-A3G were determined as described in the Fig. 1 legend. The results shown are RLU and SEM from three independent experiments. (C) Effect of the DRMR>SEMQ mutation on binding to A3G, A3F, and D128K-A3G. Western blotting analysis of cell lysates, co-IP assays, quantitation of band intensities, and calculation of APOBEC3 binding efficiencies were performed as described in the Fig. 2 legend. A representative blot is shown. The results from two independent experiments are presented as percentages of WT Vif binding. (D) Effect of the DRMR>A4 and YRHHY>A5 mutations on Vif binding to D128K-A3G. Western blotting analysis of cell lysates, co-IP assays, quantitation of band intensities, and calculation of D128K-A3G binding efficiencies were performed as described in the Fig. 2 legend. A representative blot is shown, and the results from two independent experiments are presented as a percentage of WT Vif binding.

for the APOBEC3 interactions. In agreement with our results, it was previously reported that a Y<sup>40</sup> substitution reduced function against A3G, and D<sup>14</sup>RMR<sup>17</sup> substitutions reduced function against A3F (22, 27). In addition to the YRHHY and DRMR residues, other amino acids in Vif are likely to also contribute to APOBEC3 binding. Consistent with previous reports (27, 31), our results showed that substitution of W<sup>11</sup>, and to a lesser extent Q<sup>12</sup>, reduced Vif's ability to neutralize A3F but not A3G. Our results and previous observations by Simon et al. also indicate that the K22E, E45G, and N48A substitutions suppressed Vif's function against A3G but not A3F (27). Nevertheless, the observation that the YRHHY>A5 and DRMR>A4 mutants almost completely abolish A3G and A3F binding, respectively, imply that these are the primary determinants for physical Vif-APOBEC3 interactions. However, it remains possible that the physical interactions between the two proteins involve other determinants that are indirectly affected by the mutations. Additional biochemical and structural studies are needed to ascertain whether the DRMR and YRHHY residues are directly involved in physical interactions with the APOBEC3 proteins.

Sequence comparisons of HIV-1 and SIV<sub>AGM</sub> Vif proteins

show that SIV<sub>AGM</sub> Vif contains the partial YRHH region, yet it is unable to bind to human A3G (1, 17, 21). This could be due to sequence and structural differences between agmA3G and human A3G but may also be due to the involvement of other, as-yet-unidentified regions in Vif. Landau and coworkers previously showed that substitution of D<sup>14</sup>RMR<sup>17</sup> with SERQ or SEMQ led to a loss of species specificity of Vif, allowing it to function against rhesus macaque A3G, agmA3G, human A3G, and D128K-A3G (22). Additionally, replacing DRMR with SEMQ led to a loss of Vif function against A3F; our results presented here are in agreement and further demonstrate that the loss of Vif function against A3F is directly related to a loss of A3F binding.

In agreement with our previously published results (38), we observed that D128K-A3G binds to WT Vif; in addition, we show in this study that it also binds to the SEMQ and DRMR>A4 mutants but not the YRHHY>A5 mutant. The result that only the SEMQ mutant Vif inhibited D128K-A3G suggests that an ability to bind to D128K-A3G does not necessarily lead to an ability to degrade it. Therefore, although the Y<sup>40</sup>RHHY<sup>44</sup> domain is necessary for binding to A3G, we hypothesize that the D<sup>14</sup>RMR<sup>17</sup> domain is involved in a second-

ary step that is necessary for A3G degradation. The residues DRMR, AAAA, and SEMQ at positions 14 to 17 are all able to degrade WT A3G, but only the SEMQ mutant is able to degrade the D128K-A3G. It is possible that the negative charge, provided by the SEMQ mutation, is needed to overcome the positive charge provided by the D128K substitution, thereby allowing the proposed secondary step needed for degradation to occur. On the other hand, A3F binds directly to the DRMR region, and it remains to be seen if any other regions of Vif are necessary for a secondary step after A3F binding.

The observed increase in Vif function against A3F with the YRHYY>A5 mutant suggests that the bulky YRHYY residues at the A3G-binding site partially occlude the A3F-binding DRMR domain. Substitution of the bulky YRHYY residues with the smaller alanine residues may allow more efficient interaction to occur between A3F and Vif. Taken in conjunction with data showing that A3F is less potent than A3G (12, 27, 35, 42), these results lead us to hypothesize that Vif evolved to efficiently target the more potent A3G, to the detriment of its ability to target the less potent A3F. This hypothesis may help explain the partial Vif resistance that has been observed for A3F (12, 42).

The interactions between the APOBEC3 proteins and Vif are attractive targets for development of new antiretroviral agents. These studies have identified the primary human APOBEC3 interaction domains in HIV-1 Vif, which may facilitate the rational design of inhibitors that block the interaction and provide a new approach for controlling HIV-1 infection.

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