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Regulation of Apobec3F and Human Immunodeficiency Virus Type 1 Vif by Vif-Cul5-ElonB/C E3 Ubiquitin Ligase

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The human cytidine deaminase Apobec3F (h-A3F), a protein related to the previously recognized antiviral factor Apobec3G (h-A3G), has antiviral activity against human immunodeficiency virus type 1 (HIV-1) that is suppressed by the viral protein Vif. The mechanism of HIV-1 Vif-mediated suppression of h-A3F is not fully understood. Here, we demonstrate that while h-A3F, like h-A3G, was able to suppress primate lentiviruses other than HIV-1 (simian immunodeficiency virus from African green monkeys [SIVagm] and Rhesus macaques [SIVmac]), the interaction between Vif proteins and h-A3F appeared to differ from that with h-A3G. H-A3F showed no change in its species specificity against HIV-1 or SIVagm Vif when a negatively charged amino acid was replaced with a lysine at position 128, a residue critical for h-A3G recognition by HIV-1 Vif. However, HIV-1 Vif, but not SIVagm Vif, was able to bind h-A3F and induce its polyubiquitination and degradation through the Cul5-containing E3 ubiquitin ligase. Interference with Cul5-E3 ligase function by depletion of Cul5, through RNA interference or overexpression of Cul5 mutants, blocked the ability of HIV-1 Vif to suppress h-A3F. A BC-box mutant of HIV-1 Vif that failed to recruit Cul5-E3 ligase but was still able to interact with h-A3F failed to suppress h-A3F. Interestingly, interference with Cul5-E3 ligase function or overexpression of h-A3F or h-A3G also increased the stability of HIV-1 Vif, suggesting that like the substrate molecules h-A3F and h-A3G, the substrate receptor protein Vif is itself also regulated by Cul5-E3 ligase. Our results indicate that Cul5-E3 ligase appears to be a common pathway hijacked by HIV-1 Vif to defeat both h-A3F and h-A3G. Developing inhibitors to disrupt the interaction between Vif and Cul5-E3 ligase could be therapeutically useful, allowing multiple host antiviral factors to suppress HIV-1.

The human cytidine deaminase Apobec3G (h-A3G) is known to be a broad antiviral factor in humans against human immunodeficiency virus type 1 (HIV-1), simian immunodeficiency virus (SIV), mouse leukemia virus, and hepatitis B virus (13, 18, 21, 23, 30, 35, 43). In the absence of the HIV-1 Vif protein, h-A3G is packaged into viral particles and functions by hypermutating viral DNA in the newly infected cell (13, 18, 21, 23, 30, 43). h-A3G induces C-to-U mutations in the minus DNA strand during reverse transcription, resulting in deleterious G-to-A mutations in the coding strand (13, 18, 21, 23, 33, 40, 43). The HIV-1 Vif protein counteracts this factor in the virus-producing cells by utilizing the Cul5-ElonginB-ElonginC E3 ubiquitin ligase (41) to target h-A3G for degradation through a proteasome-dependent pathway (5, 20, 24, 27, 31, 32, 41).

Cullin-based E3 ligases target substrates for ubiquitin-dependent, proteasome-mediated degradation (6, 28). The Skp1–Cul1–F-box (SCF) and ElonginC–Cul5–SOCS box complexes are well characterized cullin-based ligases. Cullin acts as a scaffold on which other components of the E3 ligase organize in order to bring the substrate into close proximity to the E2 ubiquitin-conjugating enzyme (6, 28). While one E2 may be involved in the ubiquitination of multiple substrates, E3 ligases are substrate specific. Cullin-based E3 ligases display striking similarities: in SCF and ElonginC–Cul5–SOCS box complexes, Skp1 and ElonginC bridge the interaction between the selected cullin and the substrate receptor proteins through specific interactions with the F box and SOCS box, respectively (6, 28). These substrate receptor proteins bind substrates through distinct protein–protein interaction domains (e.g., WD40 in the case of the F-box protein Cdc4 and the β-domain in the case of the SOCS-box protein VHL). HIV-1 Vif is a newly identified substrate receptor protein that selectively assembles with Cul5 but not Cul2 E3 ligase (26, 42) to overcome h-A3G. Exclusion of h-A3G from HIV-1 virions by Vif requires the functional activity of both the Vif-Cul5-ElonginB-ElonginC E3 ubiquitin ligase and proteasomes (20, 41).

Another antiviral factor, Apobec3F (h-A3F), was recently found to exhibit similar suppressive activities against HIV-1 and to be inhibited by HIV-1 Vif (2, 19, 37, 44). The mechanism by which HIV-1 Vif mediates the suppression of h-A3F is not completely understood. H-A3F is closely related to h-A3G within the family of cytidine deaminases located on chromosome 22 (16). h-A3G and h-A3F share a nearly identical 60-amino-acid N-terminal region and are coexpressed in various human tissues, suggesting that their antiviral activities may be coordinated as well (2, 19, 37, 44). However, some functional differences have been noted between these proteins: for example, h-A3G primarily mediates GG-to-GA mutations, whereas h-A3F mostly targets GA-to-AA mutations (2, 19). Some studies have suggested that while h-A3F is clearly able to restrict retroviral infectivity, it may be relatively less potent than h-A3G (2, 19). In addition, mouse leukemia virus is sensitive to

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h-A3G but not to h-A3F (2). This finding has raised the interesting question of whether h-A3F can still act as a broad antiviral factor in humans by providing a cross-species barrier against other viruses. In the present study, we have examined the activity of h-A3F against various primate lentiviruses and characterized its sensitivity to various primate lentivirus Vif proteins. We also investigated the mechanism by which h-A3F is targeted by HIV-1 Vif. Our results indicate that the Cul5-containing E3 ubiquitin ligase is a common pathway utilized by HIV-1 Vif to suppress h-A3F as well as h-A3G.

MATERIALS AND METHODS

Plasmids. Infectious molecular clones of the parental wild-type HIV-1 (HXB2) and Vif mutant virus (HXB2ΔVif) have been described previously (7). Wild-type SIVagm was produced from pSIVagmTan-1 (AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH]). The pSIVagmΔVif mutant, producing the corresponding Vif mutant virus, was constructed as described previously (20). Expression plasmids producing SIVmac, SIVmacΔVif, HIV-1 Vif-Myc, HIV-1 Vif-hemagglutinin (HA), SIVagmVif-Myc, A3G-HA, ubiquitin–c-Myc, and the c-Myc-tagged Cul5 mutant, Cul5ΔNedd8, have been described previously (20, 41). Plasmids producing SIVmacVif with an HA tag were constructed by PCR amplification using the parental wild-type SIVmac (AIDS Research Reagent Program, Division of AIDS, NIAID, NIH; catalog no. 133) as a template and the following primers: forward primer, 5′-GTCGACATGGAGGAGGAAAAGAGGTG-3′, and reverse primer, 5′-GGATCCTCACGCGTAATCTGGGACGTCGTAAGGGTATGCCAGTATTCCTA-3′, containing SalI and BamHI sites, respectively. PCR products were cloned into VR1012 to generate pMacVif-HA. The expression vector for SIVsyk Vif with an HA tag was constructed by PCR amplification using the parental wild-type SIVsyk (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; catalog no. 3287) as a template and the following primers: forward primer, 5′-GTCGACACCATGGAGAAAGAATGGATAG-3′, and reverse primer, 5′-GGATCCTCACGCGTAATCTGGGACGTCGTAAGGGTAGTCTCTGCCTCTTC-3′, containing SalI and BamHI sites, respectively. PCR products were cloned into VR1012 to generate pSykVif-HA.

The V5-tagged human A3F-expressing vector, pcDNA3-A3F-V5, was a kind gift of Zheng and Peterlin at University of California, San Francisco (44). The A3G-HA mutant (D128K) and A3F-V5 mutant (E128K) vectors were made by site-directed mutagenesis PCR of the wild-type plasmids, changing amino acid 128 from D to K in A3G-HA and changing amino acid 128 from E to K in A3F-V5.

Where indicated, pcDNA 3.1-GFP was used as a filler to equalize the total amount of transfected DNA. The VR1012 vector, generously provided by Vical, Inc. (San Diego, CA), and pcDNA3.1 (Invitrogen) were also used as empty vector filler DNA.

Cell lines and antibodies. 293T cells (American Type Culture Collection) and MAGI-CCR5 cells (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum and penicillin-streptomycin (D-10 medium) and passaged upon confluence. The antibodies used for immunoblotting of epitope-tagged proteins were mouse anti-c-Myc monoclonal antibody (MAb) (catalog no. M5546; Sigma), mouse anti-HA MAb (catalog no. MMS-101R-10000; Covance), and mouse anti-V5 MAb (catalog no. 46-0705; Invitrogen). HIV-1 p24 and SIV p27 capsid proteins were detected using anti-p24 MAb (catalog no. 1513; AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) and antiserum from a simian-human immunodeficiency virus-positive rhesus macaque, respectively. Endogenous Cul5 was detected by a monoclonal antibody against Cul5 (41). Antibody against human ribosome P (catalog no. HPO-0100; ImmunoVision, Inc.) was used to detect the protein as a loading control.

Transfections and virus purification. DNA transfection was carried out using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Viruses in cell culture supernatants were cleared of cellular debris by centrifugation at 3,000 rpm for 15 min in a Sorvall RT 6000B centrifuge and filtration through a 0.2-μm-pore size membrane (Millipore). Virus particles were then concentrated by centrifugation through a 20% sucrose cushion at 100,000 × *g* for 1.5 h in a Sorvall Ultra80 ultracentrifuge.

MAGI assay. Virus infectivity was determined with the MAGI assay (36). MAGI-CCR5 cells were plated in six-well plates in D-10 medium 1 day before infection to reach 30 to 40% confluency on the day of infection. Cells were infected by removing medium from each well and adding dilutions of virus in a total volume of 500 μl of D-10 medium with 20 μg/ml of DEAE-dextran. After a 2-h incubation at 37°C in a 5%-CO_2 incubator, 2 ml of D-10 medium was added to each well. After another 46 h of incubation under the same conditions, supernatants were removed, and the cells were fixed for 5 min at room temperature in 800 μl of fixing solution (1% formaldehyde, 0.2% glutaradehyde in phosphate-buffered saline [PBS]). The cells were then washed twice with PBS and incubated with staining solution (20 μl 0.2 M potassium ferrocyanide, 20 μl 0.2 M potassium ferricyanide, 2 μl 1 M $MgCl_2$, 10 μl 40 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for 2 h at 37°C in a non-CO_2 incubator. Staining was stopped by removing the staining solution and thoroughly washing twice with PBS. Positive blue dots were manually counted, and viral infectivity was determined after normalizing the amount of virus input by measurement of p24 antigen by enzyme-linked immunosorbent assay (Perkin-Elmer).

Immunoblot analysis. At 48 h after transfection, cells were collected and washed with PBS, and virus particles were prepared as described above. Cells (1×10^5) and purified virions were lysed in 1× loading buffer (0.08 M Tris, pH 6.8, with 2.0% sodium dodecyl sulfate [SDS], 10% glycerol, 0.1 M dithiothreitol, and 0.2% bromophenol blue). The samples were boiled for 10 min, and proteins were separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto two separate nitrocellulose membranes by passive diffusion for 16 h, producing identical mirror-image blots. Membranes were probed with various primary antibodies against the proteins of interest; secondary antibodies were alkaline phosphatase-conjugated antihuman immunoglobulin G and antimouse immunoglobulin G (Jackson Immunoresearch, Inc.). Staining was carried out with 5-bromo-4-chloro-3-indolyl phosphate (and Nitro Blue Tetrazolium solutions prepared from chemicals obtained from Sigma).

Immunoprecipitation. Transfected 293T cells were harvested and washed twice with cold PBS, then lysed with PBS containing 0.5% Triton X-100 and Complete protease inhibitor cocktail (Roche, Basel, Switzerland) at 4°C for 1 h. Cell lysates were clarified by centrifugation at 10,000 × *g* for 30 min at 4°C. Anti-V5 agarose beads (catalog no. 190-119; Bethyl Laboratory, Inc.) was mixed with the precleared cell lysates and incubated at 4°C for 3 h on an end-over-end rocker. The reaction mixture was then washed three times with cold PBS and eluted with 0.1 M glycine-HCl buffer, pH 2.0. The eluted materials were subsequently analyzed by immunoblotting.

In vivo ubiquitination assay. 293T cells were transfected with expression vectors encoding h-A3F-V5, HIV-1 Vif-Myc, ubiquitin-Myc, and the c-Myc-tagged Cul5ΔNedd8 mutant either alone or in combination, as indicated in the figure panels. Cells were treated with 2.5 μM MG132 (proteasome inhibitor) for 16 h, beginning at 24 h after transfection, and then lysed in lysis buffer (50 mM Tris, pH 7.5, with 150 mM NaCl, 1% Triton X-100, 5 mM iodoacetamide, 10 μM MG132, and Complete protease inhibitor cocktail tablets), followed by centrifugation at 10,000 × *g* for 30 min. Lysates were applied to anti-V5 antibody-conjugated agarose beads (catalog no. 190-119; Bethyl Laboratory, Inc.) and incubated for 3 h at 4°C. After incubation, the beads were washed five times with washing buffer (20 mM Tris, pH 7.5, with 0.1 M NaCl, 0.1 mM EDTA, and 0.05% Tween 20). They were then eluted with elution buffer (0.1 M glycine, pH 2.0), followed by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-c-Myc antibody.

RNAi. RNA interference (RNAi) against Cul5 was carried out using a pool of four duplexed short interfering RNAs (siRNAs) (Dharmacon) with the following sequences: duplex 1, sense, GACACGACGUCUUAUAUUAUU; duplex 1, antisense, 5′-PUAAUAUAAGACGUCGUGUCUU; duplex 2, sense, CGUCUAAUCUGUUAAAGAAUU; duplex 2, antisense, 5′-PUUCUUUAACAGAUUAGACGUU; duplex 3, sense, GAUGAUACGGCUUUGCUAAUU; duplex 3, antisense, 5′-PUUAGCAAAGCCGUAUCAUCUU; duplex 4, sense, GUUCAACUACGAAUACUAAUU; duplex 4, antisense, 5′-PUUAGUAUUCGUAGUUGAACUU.

293T cells were transfected with the Cul5 siRNA pool at a total final concentration of 100 nM using Lipofectamine 2000 (Invitrogen). siCONTROL nontargeting siRNA no. 2 (Dharmacon) was used as a control. The efficiency of Cullin5 mRNA shutdown was determined by quantitative real-time reverse transcription (RT)-PCR, and protein expression was monitored by immunoblotting 2 days after transfection.

qRT-PCR. Quantitative real-time RT-PCR (qRT-PCR) was performed according to standard protocols (15): total RNA from cells was isolated using the RNeasy Mini kit (catalog no. 74104; QIAGEN) according to the manufacturer's instructions, including an on-column DNase digestion step using the RNase-free DNase set (catalog no. 79254; QIAGEN). Up to 10 μg of total RNA was reverse

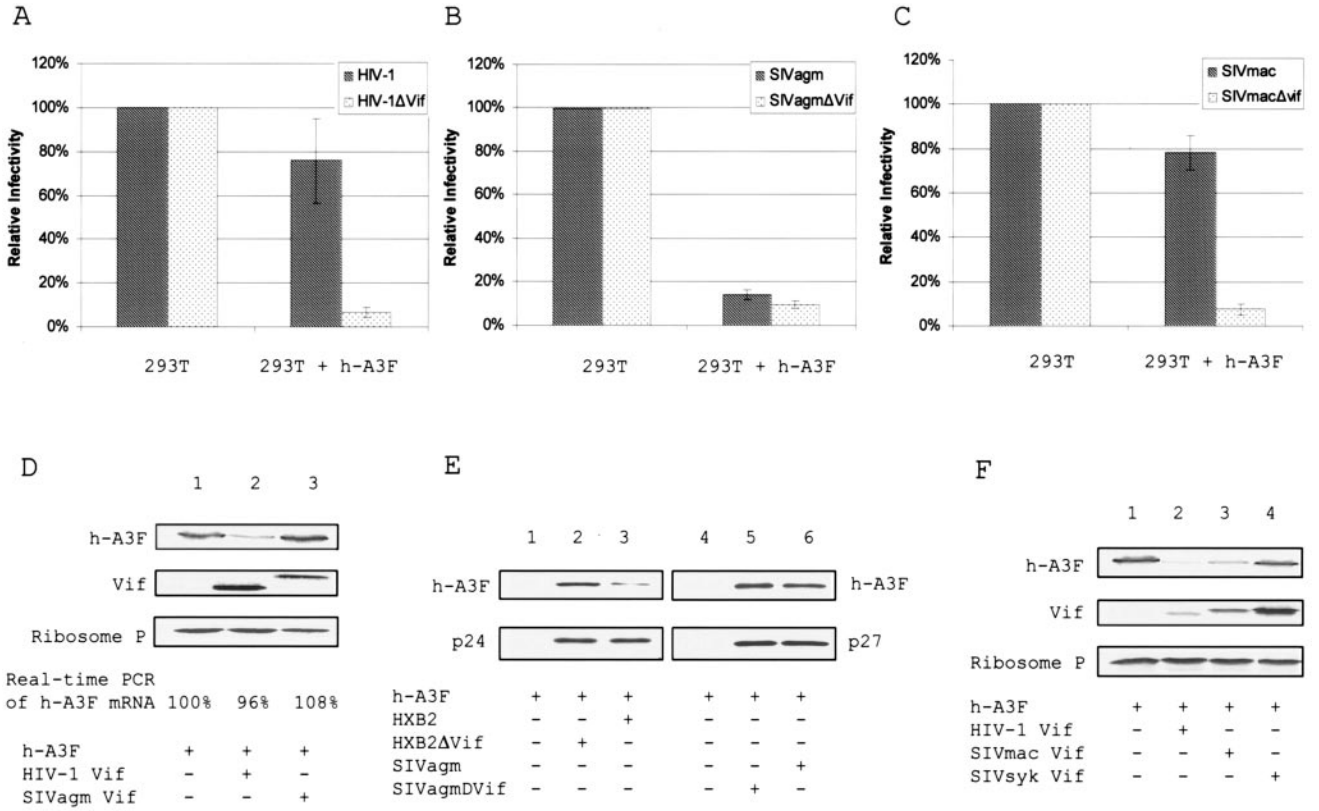

FIG. 1. h-A3F is a broad antiviral factor. (A, B, C) Graphs showing the effect of h-A3F on infectivity of human and nonhuman primate lentiviruses. Wild-type or *vif*-deficient HIV-1, SIVagm, and SIVmac were produced in 293T cells with or without coexpressing h-A3F. Viruses from the supernatants were cultured on Magi indicator cells to test for infectivity. The values indicate percentages of infectivity compared to that of virus produced in the absence of h-A3F. Error bars represent the standard deviation from triplicate wells. (D) Immunoblotting of lysates of 293T cells cotransfected with h-A3F and HIV-1 Vif or SIVagm Vif, showing that h-A3F is degraded by HIV-1 Vif and not by SIVagm Vif. The Ribosome P protein was used as the sample loading control. h-A3F mRNA levels were measured by real-time RT-PCR. (E) Immunoblotting comparing h-A3F content in HIV-1 and SIVagm virions (wild type and *vif* deficient) purified from supernatants of 293T cells cotransfected with plasmids producing virus and h-A3F. h-A3F was packaged efficiently into *vif*-deficient HIV-1, wild-type SIVagm, and *vif*-deficient SIVagm but not wild-type HIV-1. h-A3F was measured after normalizing on the basis of structural Gag protein content (HIV-1 p24 or SIV p27). (F) Immunoblotting of lysates of 293T cells cotransfected with h-A3F and diverse Vif proteins, showing that h-A3F is degraded by HIV-1 Vif and SIVmac Vif but not by SIVsyk Vif. The ribosome P protein was used as the sample loading control.

transcribed using random primers and the Multiscribe reverse transcriptase from the High Capacity cDNA archive kit (catalog no. 4322171; Applied Biosystems) according to the manufacturer's instructions. The cDNA was amplified using Taqman Universal PCR master mix (catalog no. 4304437; Applied Biosystems) and an ABI Prism 7000 sequence detection system (Applied Biosystems). The primer/probe sets were predesigned Taqman gene expression assays specific for Apobec3F and Cul5 (assay ID no. Hs00736570_m1 and Hs00180143_m1, respectively). The probes were designed by the manufacturer to span exon-exon junctions in order to minimize amplification of genomic DNA. qRT-PCR amplification within samples was normalized using the amplification levels of β-actin as an endogenous control (human ACTB endogenous control FAM/MGB probe, part no. 4333762T). The efficiency of the PCR was tested by amplification of the target from serially diluted cDNA generated from reverse transcription of a stock set of human RNAs. Each control or cytokine-treated sample set was assayed in triplicate using each primer/probe set. Data analysis and calculations were performed using the $2^{-\Delta\Delta CT}$ comparative method, as described in reference 1, and converted to percent expression by setting the expression in the control samples to 100%.

RESULTS

Human A3F is a broad cross-species antiviral factor. To examine the antiviral activity of h-A3F against various primate lentiviruses, we compared the viral infectivity of HIV-1 and of SIV from African green monkeys (SIVagm) and Rhesus macaques (SIVmac) produced in the absence or the presence of h-A3F from 293T cells. Wild-type HIV-1 (HXB2), Vif-defective HIV-1 (HXB2ΔVif), wild-type SIV (SIVagm and SIVmac), and Vif-defective SIV (SIVagmΔVif and SIVmacΔVif) were produced in the absence or presence of h-A3F from 293T cells and tested for infectivity in a standard MAGI assay (36). H-A3F dramatically reduced the infectivity of all Vif-defective viruses of HIV-1 (Fig. 1A), SIVagm (Fig. 1B), and SIVmac (Fig. 1C), indicating that it harbors broad antiviral activity.

Consistent with previous reports, HIV-1 with an intact Vif protein retained a relatively high amount of infectivity in the presence of h-A3F (Fig. 1A). However, we found that SIVagm Vif was not effective against h-A3F, since infectivity of the wild-type SIVagm was significantly reduced in the presence of h-A3F (Fig. 1B). In agreement with the infectivity data, we observed that the intracellular level of h-A3F was reduced by the HIV-1 Vif (Fig. 1D, lane 2) but not SIVagm Vif (Fig. 1D,

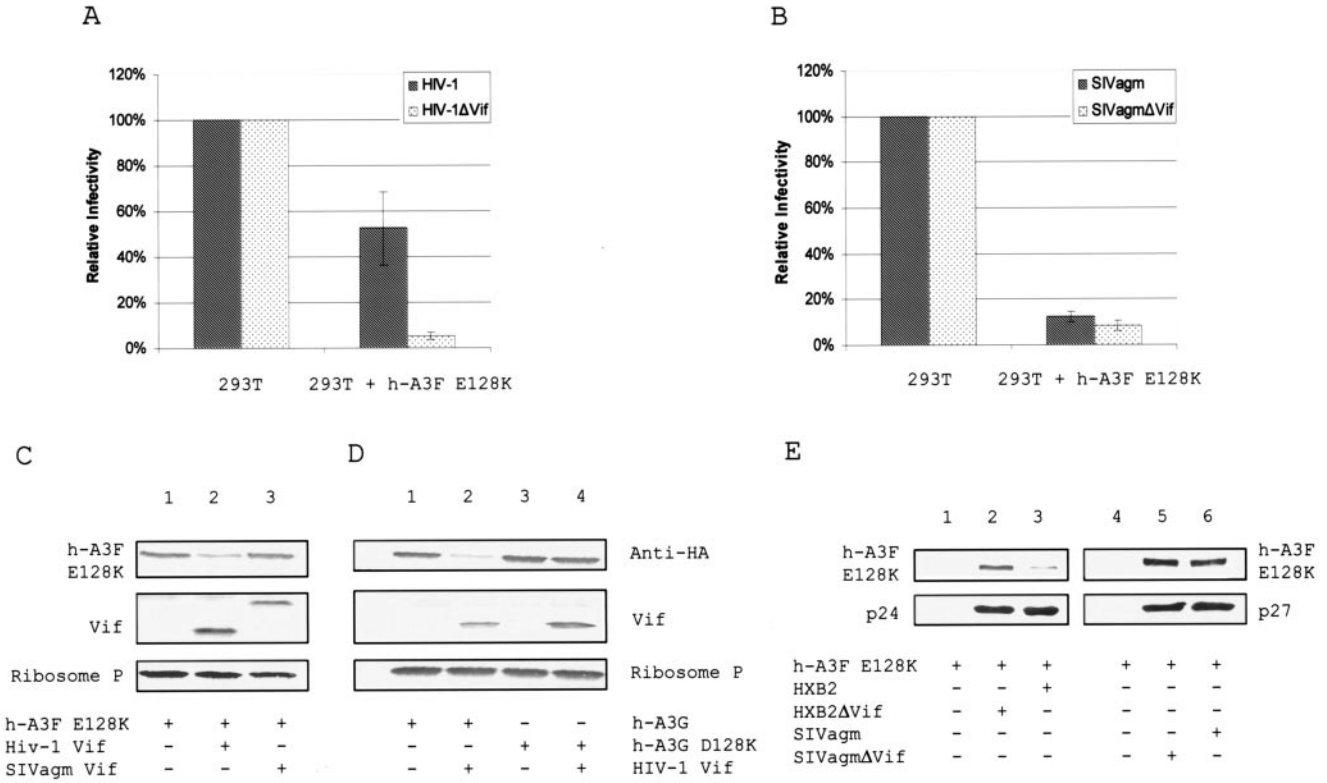

FIG. 2. Altering amino acid 128 in h-A3F does not change species specificity. (A, B) Effect of h-A3F mutant (h-A3F E128K) on HIV-1 and SIVagm infectivity. Wild-type or *vif*-deficient HIV-1 and SIVagm were produced in 293T cells with or without the expression of h-A3F E128K. The virus infectivities were measured by a standard MAGI assay. The values indicate the percentages of infectivity compared to that of virus produced in the absence of h-A3F E128K. Error bars represent standard deviations from triplicate wells. (C) h-A3F E128K is degraded by HIV-1 Vif and not by SIVagm Vif. h-A3F E128K was coexpressed with HIV-1 Vif or SIVagm Vif in 293T cells. The steady-state level of h-A3F E128K was monitored by immunoblotting. The ribosomal P protein was used as the sample loading control. (D) h-A3G but not h-A3G D128K is degraded by HIV-1 Vif. h-A3G or h-A3G D128K was expressed with or without HIV-1 Vif. The steady-state levels of the proteins were measured by immunoblotting. (E) h-A3F E128K is packaged efficiently into *vif*-deficient HIV-1, wild-type SIVagm, and *vif*-deficient SIVagm but not wild-type HIV-1. Wild-type and *vif*-deficient HIV-1 or SIVagm were produced in the presence of h-A3F E128K. After normalizing the virion particles on the basis of virion structural Gag protein content (HIV-1 p24 or SIV p27), the packaged h-A3F E128K was compared by immunoblotting.

lane 3). Expression of HIV-1 Vif had no effect on the mRNA of h-A3F (Fig. 1D), consistent with a previous report that HIV-1 Vif reduces the stability of the h-A3F protein (44). Consequently, wild-type HIV-1 was able to exclude h-A3F from released virions (Fig. 1E, lane 3) compared to HIV-1 ΔVif virions (Fig. 1E, lane 2). In contrast, both wild-type SIVagm and SIVagmΔVif virions contained comparably high levels of h-A3F (Fig. 1E, lanes 5 and 6), consistent with the reduced infectivities of both viruses (Fig. 1B). Unlike SIVagm Vif, SIVmac Vif was able to degrade h-A3F (Fig. 1F, lane 3) and to suppress the antiviral activity of h-A3F (Fig. 1C). It is interesting that SIVmac is closely related to SIVsm and HIV-2 and has been shown to infect human T cells. In addition to being resistant to the SIVagm Vif protein, h-A3F was also relatively resistant to the Vif protein of SIV isolated from Sykes monkeys (SIVsyk) (Fig. 1F, lane 4). These results suggest that h-A3F is more resistant to the Vif proteins from simian lentiviruses more distantly related to HIV-1.

A change in amino acid 128 in h-A3F does not change species specificity. In h-A3G and AGM-A3G, a single amino acid at position 128 mediates their species-specific recognition by the Vif protein of the lentivirus in its natural host (3, 22, 29, 39). By altering the negatively charged aspartic acid at position 128 in h-A3G to a positively charged lysine found in AGM-A3G, the mutated h-A3G (D128K) was made resistant to HIV-1 Vif but became sensitive to SIVagm Vif (3, 22, 29, 39). The position of h-A3F equivalent to 128 of h-A3G also contains a negatively charged amino acid, glutamic acid. (The glutamic acid is, in fact, in position 127 of h-A3F, but will be referred to as amino acid 128, indicating its position when alighed with h-A3G). To determine whether this position in h-A3F also confers a similar specificity to primate lentivirus Vif, we constructed a mutant h-A3F (E128K) by replacing the glutamic acid with a lysine. Unlike the mutant h-A3G D128K, mutant h-A3F E128K was still sensitive to HIV-1 Vif and resistant to SIVagm Vif. That is, h-A3F E128K did not effectively suppress the infectivity of wild-type HIV-1 (Fig. 2A) and was still able to suppress both SIVagm and SIVagmΔVif (Fig. 2B). H-A3F E128K could be degraded by HIV-1 Vif (Fig. 2C, lane 2) but not SIVagm Vif (Fig. 2C, lane 3). On the other hand, HIV-1 Vif could degrade wild-type h-A3G (Fig. 2D, lane 2), but it could not degrade the mutant protein, h-A3G D128K (Fig. 2D, lane 4). Incorporation of h-A3F E128K into wild-type HIV-1 virions was significantly reduced (Fig. 2E, lane 3) from

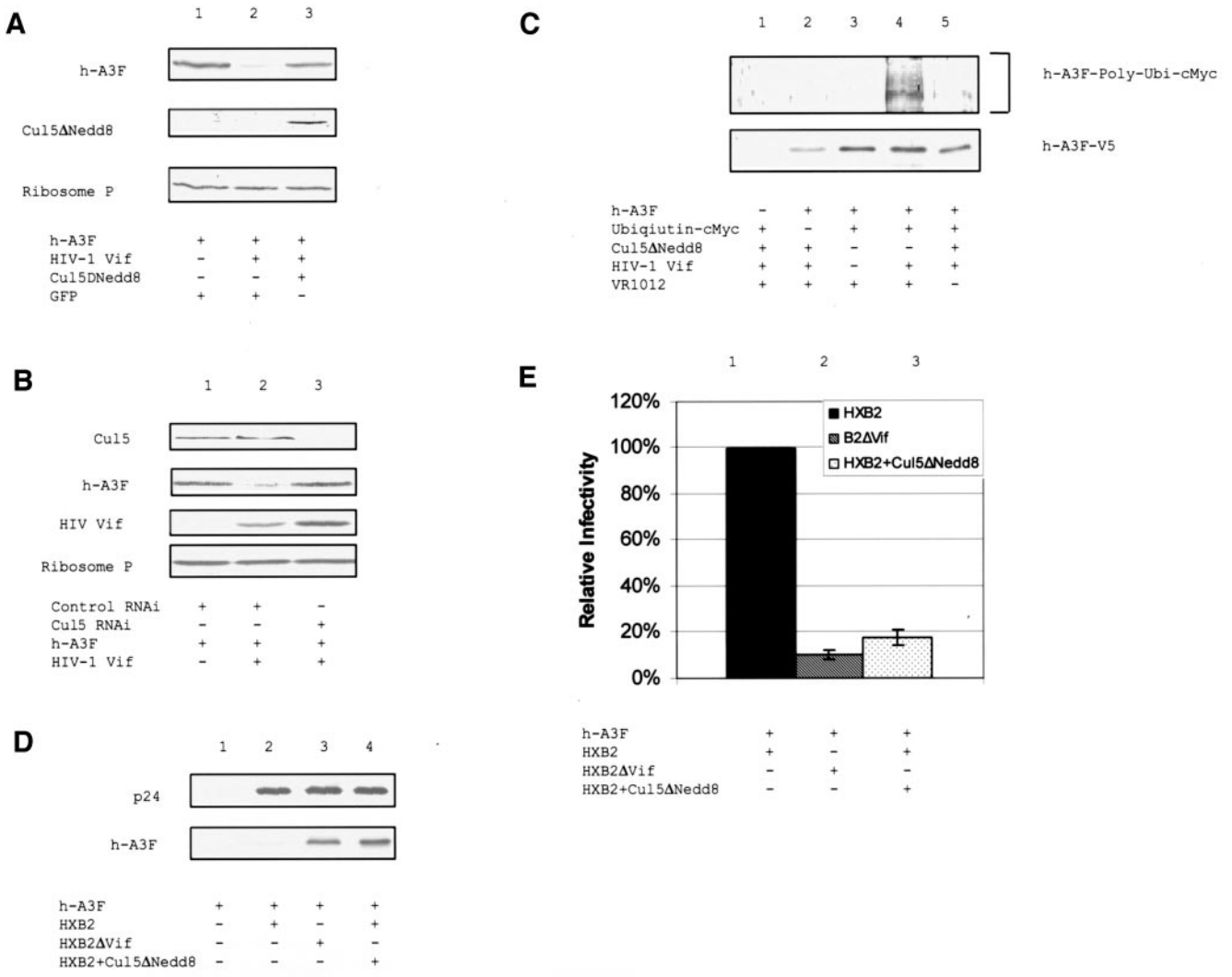

FIG. 3. h-A3F is degraded through the Vif-Cul5 E3 ubiquitin ligase complex pathway. (A) h-A3F was cotransfected with the green fluorescent protein (GFP) expression vector, HIV-1 Vif expression vector, or HIV-1 Vif expression vector plus the Cul5 dominant-negative mutant Cul5ΔNedd8. The steady-state level of h-A3F was measured by immunoblotting. (B) Degradation of h-A3F by HIV-1 Vif is inhibited by Cul5 siRNA. H-A3F was cotransfected with control siRNA (100 nM), HIV-1 Vif, and control siRNA or HIV-1 Vif and Cul5 siRNA (100 nM). Stability of h-A3F and expression of endogenous Cul5 were measured by immunoblotting. (C) h-A3F is polyubiquitinated through the Cul5 E3 ubiquitin ligase complex pathway. V5-tagged h-A3F was cotransfected with HIV-1 Vif and a c-Myc-tagged ubiquitin construct with or without the Cul5 dominant-negative mutant. At 24 h after transfection, the proteasome inhibitor MG132 was added to the cells to block h-A3F degradation. After treatment with MG132 for 16 h, h-A3F was immunoprecipitated from the cells with anti-V5 MAb-conjugated beads. Polyubiquitination of h-A3F was measured by immunoblotting with anti-c-Myc antibody. (D) h-A3F virion incorporation is increased by the Cul5 dominant-negative mutant Cul5ΔNedd8. HIV-1 wild-type or *vif*-deficient virus was produced in 293T cells expressing h-A3F with or without expressing the Cul5 dominant-negative mutant Cul5ΔNedd8. The virus was harvested as indicated in Materials and Methods. The incorporated amount of h-A3F was measured by immunoblotting. (E) HIV-1 wild-type virus infectivity was decreased by the expression of Cul5ΔNedd8 in the presence of h-A3F. The virus-containing supernatants from panel D were subjected to a standard MAGI assay. Values are presented as percent infectivity relative to wild-type HIV-1. Error bars represent standard deviation from triplicate wells.

that with HIV-1ΔVif (Fig. 2E, lane 2), but no significant difference was observed between results with SIVagm (Fig. 2E, lane 6) and SIVagmΔVif (Fig. 2E, lane 5). Therefore, modification of amino acid 128 did not alter recognition of h-A3F by HIV-1 Vif or SIVagm Vif. Although it is likely that amino acids other than 128 within h-A3F confer species specificity, these results indicate that the species-specific interactions between h-A3F and primate lentivirus Vif proteins are different from those involving h-A3G.

Suppression of h-A3F by HIV-1 Vif is inhibited by Cul5 mutants and depletion of Cul5. Next, we wanted to investigate the mechanism by which h-A3F is suppressed by HIV-1 Vif. Previously, we reported that HIV-1 Vif induced h-A3G polyubiquitination and degradation through the activity of Vif-Cul5-ElonginB-ElonginC E3 ligase (41). A dominant-negative Cul5 mutant lacking the Nedd8 binding site, Cul5ΔNedd8, was able to inhibit the degradation of h-A3G by HIV-1 Vif. Similarly, HIV-1 Vif-induced degradation of h-A3F (Fig. 3A, lane 2) was

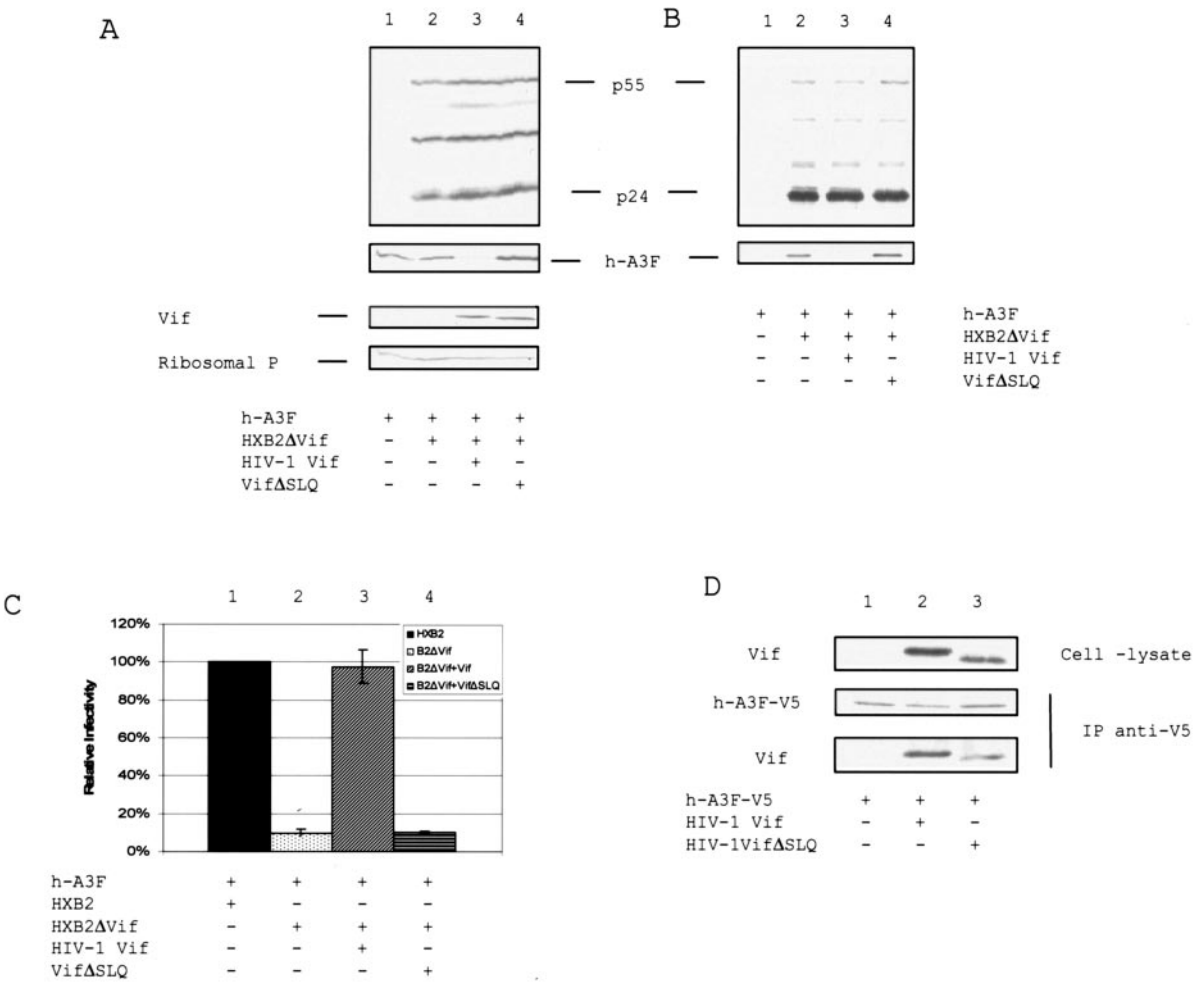

FIG. 4. Vif SLQ motif is required for interaction with the Cul5 complex. (A) The VifΔSLQ mutant fails to induce h-A3F degradation. HXB2ΔVif was cotransfected with h-A3F and HIV-1 Vif or the HIV-1 VifΔSLQ mutant, and h-A3F was detected in cell lysates by immunoblotting. (B) Immunoblotting of viruses purified from supernatants of cells transfected in panel A; h-A3F was detected using anti-V5 MAb. (C) The HIV-1 VifΔSLQ mutant fails to rescue HXB2ΔVif infectivity. Infectivity of HXB2ΔVif viruses produced in the presence of h-A3F and HIV-1 Vif or the VifΔSLQ mutant (lanes 2 to 4) were compared to infectivity of wild-type HXB2 virus produced with h-A3F (lane 1) in a MAGI assay. Values are presented as percent infectivity relative to wild-type virus. Error bars represent standard deviations. (D) HIV-1 Vif and HIV-1 VifΔSLQ interact with h-A3F. V5-tagged h-A3F cotransfected with HIV-1 Vif or the HIV-1 VifΔSLQ construct. At 48 h after transfection, cell lysates were subjected to immunoprecipitation of h-A3F by anti-V5 beads. The interaction between h-A3F and Vif or VifΔSLQ was detected by immunoblotting of immunoprecipitation (IP) samples using antiserum against Vif.

also blocked by the Cul5 mutant, Cul5ΔNedd8 (Fig. 3A, lane 3). We further investigated the involvement of the Cul5 E3 ligase in the activities of HIV-1 Vif by using RNAi with Cul5. Exposure to a pool of four duplexed siRNAs directed against human Cul5 produced a >90% reduction in the Cul5 protein level (Fig. 3B, lane 3). H-A3F was degraded by HIV-1 Vif in the presence of control siRNA (Fig. 3B, lane 2), but this degradation was inhibited when Cul5 protein expression was reduced (Fig. 3B, lane 3). HIV-1 Vif induced polyubiquitination of h-A3F (Fig. 3C, lane 4), which was inhibited in the presence of Cul5ΔNedd8 (Fig. 3C, lane 5). While HIV-1 Vif was able to exclude h-A3F from released virions (Fig. 3D, lane 2), this exclusion did not occur in the presence of Cul5ΔNedd8 (Fig. 3D, lane 4). As a result, Cul5ΔNedd8 also inhibited the infectivity of wild-type HIV-1 in the presence of h-A3F (Fig. 3E, lane 3). Similar results were obtained with a different Cul5 mutant (Cul5ΔRbx1) (data not shown). Therefore, we conclude that HIV-1 Vif induces polyubiquitination and subsequent degradation of h-A3F. These processes could be inhibited by a dominant-negative Cul5ΔNedd8 mutant or depletion of Cul5 by RNAi, suggesting that suppression of h-A3F by HIV-1 is mediated through the Cul5 E3 ligase complex.

Suppression of h-A3F by HIV-1 Vif requires the BC box. HIV-1 Vif is a substrate receptor protein and recruits Cul5-ElonginB-ElonginC through a unique BC-box motif that overlaps the conserved SLQxLA motifs of all lentivirus Vif proteins (26, 42). If recruitment of Cul5-ElonginB-ElonginC is important for HIV-1 Vif function, then the BC-box mutant of HIV-1 Vif should be inactive against h-A3F. Indeed, unlike wild-type HIV-1 Vif (Fig. 4A, lane 3), mutant Vif (VifΔSLQ) could not induce h-A3F degradation (Fig. 4A, lane 4). Whereas HIV-1 Vif could prevent h-A3F incorporation into virions (Fig. 4B,

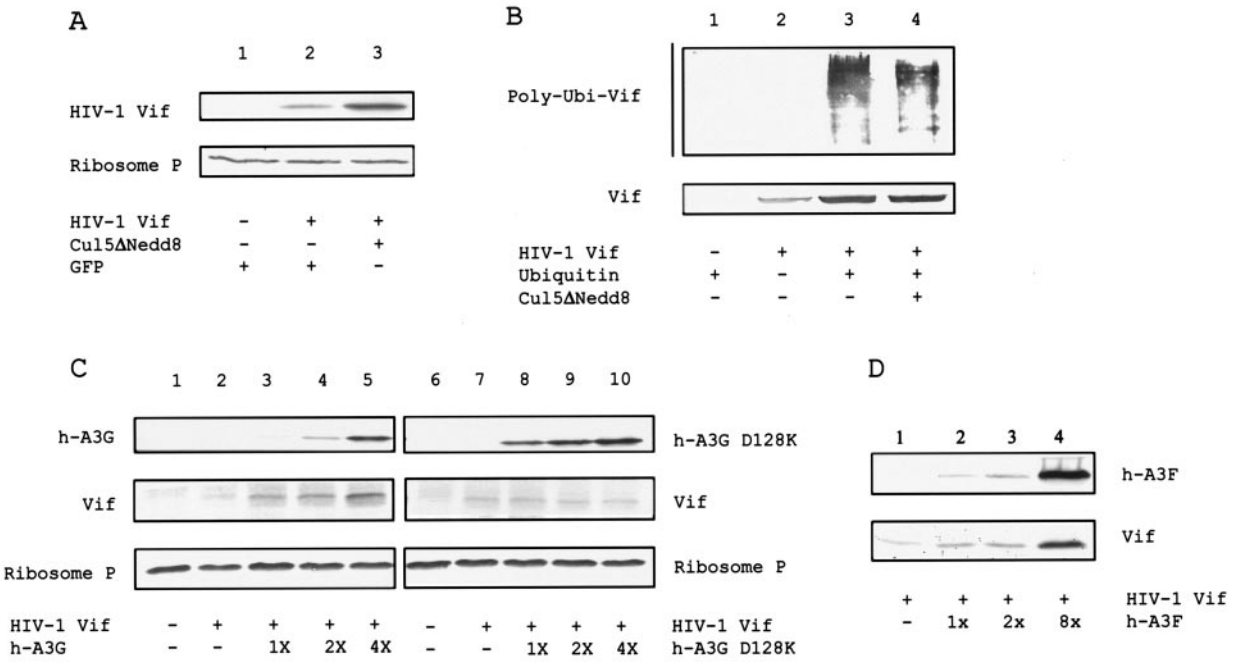

FIG. 5. HIV-1 Vif is regulated by the Cul5-E3 ligase complex. (A) HIV-1 Vif is stabilized by the Cul5ΔNedd8 dominant-negative mutant. HIV-1 Vif was cotransfected with green fluorescent protein (GFP) or the Cul5ΔNedd8 mutant construct. The stability of Vif was detected by immunoblotting using anti-Vif antibody. (B) Vif polyubiquitination is inhibited by Cul5ΔNedd8. HIV-1 Vif was cotransfected with ubiquitin-Myc, with or without Cul5ΔNedd8. At 24 h after transfection, MG132 was added to the cells to block HIV-1 Vif degradation. After treatment with MG132 for 16 h, HIV-1 Vif was immunoprecipitated by anti-HA MAb-conjugated beads. Polyubiquitination of Vif was measured by immunoblotting with anti-c-Myc antibody. (C) HIV-1 Vif is stabilized by the overexpression of target proteins. The HIV-1 Vif plasmid (1 μg) was cotransfected with 0, 1, 2, or 4 μg of h-A3G or h-A3G mutant (D128K). Vif stability was monitored by immunoblotting using anti-c-Myc MAb and h-A3G, and the mutant was detected by anti-HA MAb. Detection of ribosome P was used as a loading control. (D) The HIV-1 Vif plasmid was cotransfected with increasing relative amounts of h-A3F. Vif stability was monitored by immunoblotting using anti-c-Myc MAb, and h-A3F was detected by anti-HA MAb.

lane 3), VifΔSLQ could not (Fig. 4B, lane 4). Consequently, the infectivity of viruses produced in the presence of VifΔSLQ was greatly reduced (Fig. 4C, lane 4) from that of those with wild-type HIV-1 Vif (Fig. 4C, lane 3). Coimmunoprecipitation experiments indicated that the interaction between h-A3F and VifΔSLQ was maintained (Fig. 4D, lane 3), suggesting that binding of HIV-1 Vif to h-A3F alone is not sufficient to block h-A3F activity. Rather, recruitment of the Cul5 E3 ligase through the BC-box (SLQ) motif is apparently critical for Vif-mediated suppression of h-A3F.

Degradation of Vif by Cul5 E3 ligase. We and others have previously shown that HIV-1 Vif itself is also a target of proteasome-mediated degradation (10, 20, 26). While Vif has been shown to be both monoubiquitinated and polyubiquitinated, the mechanism of its regulation is still unclear (8, 27). We observed that the stability of HIV-1 Vif was higher in the presence of Cul5ΔNedd8 (Fig. 5A, lane 3) than in its absence (Fig. 5A, lane 2). The level of polyubiquitination of HIV-1 Vif was also lower when the Cul5 mutant was present (Fig. 5B, lane 4) than when it was absent (Fig. 5B, lane 3). Depletion of Cul5 by siRNA also increased the stability of HIV-1 Vif (Fig. 3B). These results suggest that the substrate receptor HIV-1 Vif could also be a target of the Vif-Cul5 E3 ubiquitin ligase complex. Autoregulation of the SOCS-box- or F-box-containing substrate receptor molecules in cullin-based E3 ligase complexes have been described previously (4, 12, 17, 25, 38, 45). In these situations, an increase in the abundance of the target proteins stabilizes the substrate receptor proteins (12). To determine whether the stability of HIV-1 Vif is affected by its target proteins, we coexpressed a constant amount of the Vif expression vector with increasing amounts of the h-A3G expression vector. As cotransfection of the h-A3G plasmid was increased from one to four times the amount of the HIV-1 Vif plasmid, we observed that Vif became increasingly more stable (Fig. 5C, lanes 3 to 5) than was the case for the situation in which Vif was expressed alone (Fig. 5C, lane 2). Such an increase in the stability of Vif was not observed in the corresponding control experiment, in which Vif was coexpressed with increasing amounts of h-A3GD128K (Fig. 5C, lanes 6 to 10), a mutant A3G that does not interact efficiently with HIV-1 Vif (3, 22, 29). An increase in the stability of Vif was also observed when h-A3F was overexpressed (Fig. 5D).

DISCUSSION

The APOBEC-related family of cytidine deaminases includes 11 members (14). Two of these, h-A3G and h-A3F, are highly expressed in lymphoid cells, the natural targets of HIV-1. Original studies of h-A3G revealed that expression of this antiviral protein is capable of converting so-called permissive cell lines to nonpermissive cell lines that can support replication of HIV-1 only when the virus produces a functional

Vif protein (30). The enzymatic activity of h-A3G, acting on the viral genome, appeared to be the major mechanism of antiviral function against HIV-1, and the viral Vif protein was able to overcome h-A3G through proteasome-mediated degradation involving the Cul5 E3 ligase (13, 18, 21, 23, 41, 43). Subsequently, other members of the cytidine deaminase family were described, including h-A3F, which has a high degree of homology to h-A3G. While the role(s) of other APOBEC-related proteins in antiviral defenses are being investigated, it is becoming clear that HIV-1 Vif must overcome at least h-A3G and h-A3F if it is to help the virus survive. We have determined that HIV-1 Vif is able to utilize the same cellular machinery, the Cul5 E3 ligase, to degrade h-A3F as well as h-A3G, despite the apparent difference between the critical interactions of Vif with these two proteins. Unlike the situation in h-A3G, modification of amino acid 128 in h-A3F does not change its recognition by HIV-1 Vif or SIVagm Vif (Fig. 2). Although the specific amino acid(s) other than position 128 that may confer species specificity on h-A3F remain to be defined, our data suggest that Vif may have evolved distinct protein interfaces in order to interact with both h-A3G and h-A3F. Because measurement of h-A3F mRNA by quantitative real-time RT-PCR showed comparable expression levels when cotransfected with or without HIV-1 Vif (Fig. 1D), the reduced h-A3F protein levels we observed in the presence of HIV-1 Vif are consistent with a proteosome-mediated degradation mechanism (42). We have now shown that HIV-1 Vif induces polyubiquination and degradation of h-A3F, processes that are inhibited by a dominant-negative Cul5ΔNedd8 mutant or RNA interference with Cul5 (Fig. 3). The BC-box (SLQ) motif in HIV-1 Vif is necessary for the interaction with the Cul5 E3 ligase (41). A Vif BC-box mutant that can bind h-A3F but prevents recruitment of the Cul5 E3 ligase failed to suppress h-A3F (Fig. 4). Taken together, these data indicate that the Cul5 E3 ligase complex is critically utilized by HIV-1 Vif to suppress h-A3F as well as h-A3G.

Interestingly, our observation that HIV-1 Vif was also stabilized in the case of Cul5 depletion (Fig. 3B) or in the presence of the dominant-negative Cul5ΔNedd8 mutant (Fig. 5A) led us to believe that the Cul5 E3 ligase could serve the dual purpose of regulating both Vif and its targets. The increased stability of HIV-1 Vif in the presence of excess h-A3G (Fig. 5C) and h-A3F (Fig. 5D) suggests that the interaction of Vif with its target proteins and the Cul5 E3 ligase creates an environment in which Vif itself is protected from polyubiquitination by the same E3 ligase. According to this model, Vif becomes unstable and marked for degradation unless it is functioning as a substrate receptor protein within the E3 ligase complex. Our data are consistent with a recent study showing that polyubiquitination of HIV-1 Vif is reduced in a BC-box mutant of HIV-1 Vif that does not associate with Cul5-ElonginB-ElonginC (26). In that study, polyubiquitination of HIV-1 Vif was also reduced in the presence of Cul5 mutants (26). Regulating Vif protein turnover may serve at least two purposes. First, removal of substrate receptor proteins may allow the other components of the E3 ligase to be recycled. We have observed that other components of the Cul5 E3 ligase complex, namely ElonginB, ElonginC, and Cul5, are not reduced by Vif expression (data not shown). Also, turnover of Vif proteins would limit the amount available to be packaged into the virions (20), which could otherwise be detrimental to virus infectivity (26). We observed that the Cul5 mutant reduced but did not eliminate HIV-1 Vif polyubiquitination (Fig. 5B). This observation raises the question of whether Vif is also the target of other E3 ubiquitin ligases. Interaction of HIV-1 Vif with other E3 ligases, such as Nedd4, AIP4 (8), and Zin (9) has been reported, but whether these proteins contribute to HIV-1 Vif degradation remains an open question.

Studies with nonhuman primate lentiviruses are useful for gaining knowledge about the mechanisms through which human host proteins interact with and defend against HIV-1 and related lentiviruses. It is an intriguing question whether human cytidine deaminases represent a natural barrier in humans to infection by nonhuman primate lentiviruses. Primate lentiviruses such as HIV-1, HIV-2, and SIVmac/sm, which are known to infect human cells in vitro and in vivo, all encode Vif proteins that can efficiently suppress h-A3G and h-A3F. Vif proteins from more distantly related primate lentiviruses, such as SIVagm and SIVsyk, are inefficient at suppressing h-A3G and h-A3F. However, replication of SIVagm in human cells that express h-A3G and h-A3F has been observed (11, 34), suggesting that cytidine deaminase activities and species-specific restriction of SIV Vif function may not be the sole determinants of the lack of human infection by certain SIVs.

In summary, we have demonstrated that h-A3F can act as a broad antiviral factor to suppress various primate lentiviruses. The interactions between Vif proteins and h-A3F or h-A3G seem to be distinct, since the amino acid at position 128 confers species specificity on h-A3G but not h-A3F. It is intriguing that HIV-1 Vif has evolved to mediate the degradation of both antiviral proteins, h-A3G and h-A3F, through a common Cul5-ElonginB-ElonginC E3 ubiquitin ligase. This finding reinforces the idea that the Cul5-ElonginB-ElonginC E3 ubiquitin ligase is a particularly appealing therapeutic target, because by disrupting unique interactions between Vif and Cul5-ElonginB-ElonginC, multiple antiviral factors are released from Vif-mediated suppression and able to overcome the invading viruses.

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