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Multiple lysines combined in HIV-1 Vif determines the responsiveness to CBF-β

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ABSTRACT

The Vif (viral infectivity factor) protein of human immunodeficiency virus type-1 (HIV-1) is critical for HIV-1 infectivity. CBF-β is required for HIV-1 Vif function, as it increases the steady-state level of the HIV-1 Vif protein to promote host restriction factor APOBEC3 degradation. However, the precise mechanism by which CBF-β promotes HIV-1 Vif levels remains unclear. In the present study, we provided evidences that CBF-β promoted steady-state levels of HIV-1 Vif by inhibiting the degradation of HIV-1 Vif through the proteasome pathway. Our results reveal a new mechanism by which a cellular protein supports viral infectivity by inhibiting viral protein degradation.

1. Introduction

Human immunodeficiency virus type-1 (HIV-1) contributes to lifelong infection and can be successfully transmitted between humans. HIV-1 targets important immune cells, such as CD4-positive T cells, destroying the immune system and causing acquired immunodeficiency syndrome (AIDS). AIDS is not a disease, but rather the final stage of HIV-1 infection, and causes millions of deaths each year. There is currently no safe and effective cure for HIV/AIDS. To exploit new antiviral targets, it is important to precisely understand the mechanisms of interaction between viral proteins and the host [1].

HIV and SIV (simian immunodeficiency virus) can be distinguished from other retroviruses by their wide variety of accessory proteins. The compact genome of HIV-1 consists of 9 genes, and four of these genes encode the proteins Vif, Nef, Vpr and Vpu. These proteins interact with cellular factors and pathways, and allow the viruses to evade cell-mediated innate immunity, thereby enabling viral replication [2–7].

Vif is an approximately 23 kDa protein that is encoded by all known lentiviruses with the exception of equine infectious anemia virus (EIAV) [8]. Vif allows viruses to counteract cellular defense factors (APOBEC3), thereby promoting viral infectivity. The APOBEC3 family members are cytosine deaminases. The primate APOBEC3 family consists of seven members. Among these proteins, A3G and A3F exhibit potent antiviral ability. A3G and A3F are packaged into virus particles and induce a C to U transition in the viral minus single-stranded genome during reverse transcription, thereby causing the viral genome to be degraded by uracil DNA glycosylase and introducing a G to A hypermutation in the plus strand genome [9–11]. In addition, A3G and A3F decrease the affinity of $tRNA^{lys3}$ to HIV-1 RNA during priming, thereby blocking reverse transcription [1]. Moreover, these proteins block the integration of the viral genome into the host chromosome [1].

Although several antiviral mechanisms exist, Vif is sufficient for HIV-1 to counteract the effects of A3G and A3F. HIV-1 Vif has been proposed to decrease A3G protein translation and prevent A3G from encapsulating itself into HIV-1 virions [12]. Moreover, HIV-1 Vif recruits the CUL5-ELOG B/C-based E3 ligase complex to induce human and chimpanzee A3G/A3F degradation through the proteasome pathway [13]. Recent studies have highlighted the important role of the cellular transcription cofactor CBF-β in HIV-1 Vif function [14–24]. CBF-β promotes Vif function by reinforcing the interaction between Vif and CUL5 [15,25–27] and enhancing the steady-state levels of Vif, thus promoting its ability to block the antiviral activities of A3G [14,21,28,29]. However, the precise mechanisms through which CBF-β increases the level of HIV-1 Vif remain unclear.

In this study, we found that CBF-β overexpression or treatment with the proteasome pathway inhibitor MG132 increased the levels of HIV-1 Vif and that co-treatment of CBF-β with MG132 did not increase the level of HIV-1 Vif beyond that observed using CBF-β and MG132 in isolation. Moreover, we found that proteasomal

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degradation of HIV-1 Vif requires the lysine residues in Vif; when all lysines were mutated to arginines, the resulting HIV-1 Vif lysine-free mutant could not be degraded through the proteasome pathway and lost its responsiveness to CBF-β. However, the HIV-1 Vif lysine-free mutant retained its ability to immunoprecipitate with CBF-β. Our results demonstrate for the first time that CBF-β increases the steady-state level of HIV-1 Vif by inhibiting its proteasomal degradation. Therefore, altering the HIV-1 Vif-CBF-β interaction and facilitating Vif degradation presents a promising target for anti-HIV-1 drug design.

2. Materials and methods

2.1. Cells and plasmids

293T cells were maintained in DMEM high glucose (HyClone) containing 10% fetal bovine serum (FBS, GIBCO) and 100 U of penicillin and streptomycin (GIBCO). The *CBF-β* gene (Database ID: KM250107) was cloned into the vector VR1012 using specific primers (CBF-F: 5′-ATGGGATCCATGCCGCGCGTCGTGCCTGACCA-GAGAAGCAAGTTC-3′; CBF-R: 5′-TAAAGCGGCCGCG-TAGGGTCTTGTTGTCTTCTTGCCAGTTAC-3′) fused to a C-terminal myc-Flag tag; the obtained construct will be referred to as VR-CBF-mf. The human A3G (A3G) and chimpanzee A3G (A3Gcpz) expression vectors have been described previously [30]. FIV *vif* (Database ID: NC001482), HIV-1 *Vif* (Database ID: AF324493) and HIV-1 Vif-16K/R (all 16 lysines substituted with arginines) cDNAs were codon-optimized and cloned into double HA-tagged pCDNA 3.1(+); these constructs were named FIV-Vif, Vif-WT and Vif 16K/R, respectively. HIV-1 Vif-WT K mutants in which K22, K26, K34, K36, K50, K63, K91 and 92, K141, K155, K157, K160, K168, K176, K179 and K181 were altered to R were named K22R, K26R, K34R, K36R, K50R, K63R, K91-92R, K141R, K155R, K157R, K160R, K168R, K176R, K179R and K181R, respectively. The HIV-1 Vif-16 K/R R mutants in which R22, R26, R34, R36, R50, R63, R91 and 92, R141, R155, R157, R160, R168, R176, R179 and R181 were reverted to K and named R22K, R26K, R34K, R36K, R50K, R63K, R91-92K, R141K, R155K, R157K, R160K, R168K, R176K, R179K and R181K, respectively. These mutants were generated using a site-directed point mutation method and cloned into the double HA-tagged pCDNA 3.1(+).

2.2. Co-immunoprecipitation assay and western blotting

All transfections were performed using the standard calcium phosphate method with 293T cells. Cells were collected 24 h after transfection and lysed in a buffer that contained 150 mM Tris–HCl (pH 7.6), 50 mM NaCl, 5 mM Na_2EDTA, 1% Triton-X100 and 10% glycerol. The cell debris was removed by centrifugation at 10,000 g for 10 min. For immunoprecipitation experiments, a sample of each cell lysate was analyzed using western blotting to determine

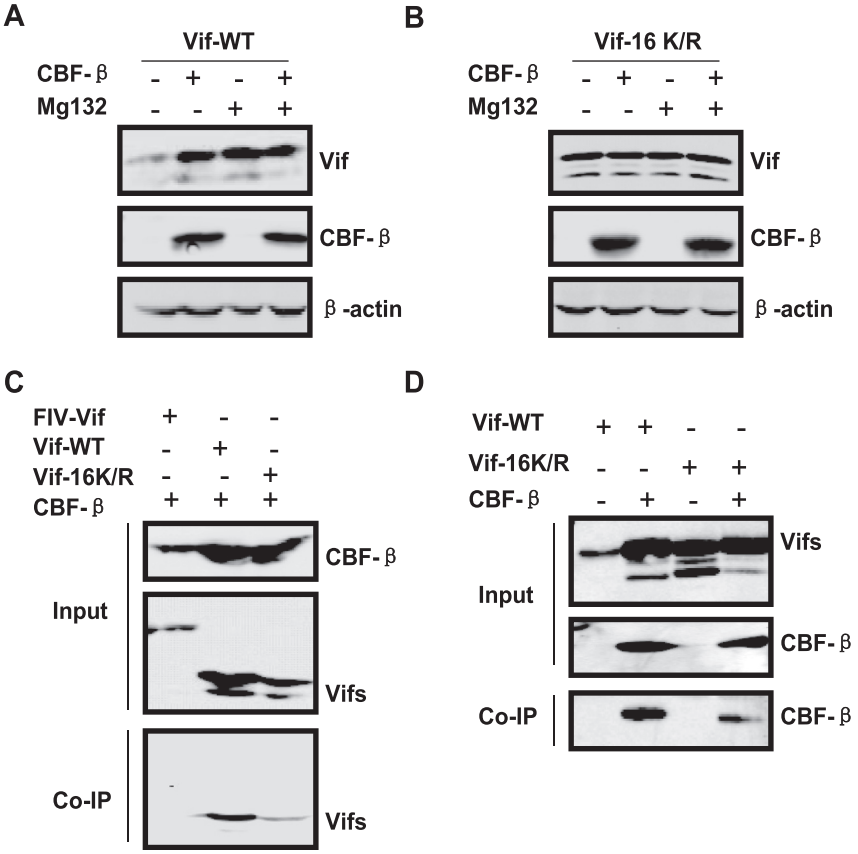

Fig. 1. CBF-β inhibits proteasomal HIV-1 Vif degradation. Cells were co-transfected with 1.5 μg of HIV-1 Vif-WT (A) or a HIV-1 Vif-16K/R (B) expression vector with 0.2 μg of VR1012 or VR-CBF-mf. Twelve hours after transfection, 7.5 μM MG132 or DMSO was added to the medium, and the cells were collected 24 h after transfection. Protein expression was analyzed using western blotting. Vif-WT and Vif 16K/R were detected using an anti-HA antibody, CBF-β was detected using an anti-Flag antibody, and β-actin was detected using an anti-actin antibody. The western blotting results represent the outcomes of three independent experiments. (C) 293T cells were co-transfected with 0.5 μg of VR-CBF-mf and 1.5 μg of the FIV Vif, HIV-1 Vif-WT or HIV-1 Vif 16K/R expression vectors. Twenty-four hours post transfection, the cells were lysed. One portion of each cell lysate was analyzed using western blotting, and the remaining part was mixed with anti-Flag-agarose for 4 h. The cellular (input) and bound proteins (co-IP) were analyzed using an anti-Flag antibody (CBF-β) or an anti-HA antibody (Vifs). (D) HIV-1 Vif-WT vectors (3 μg) or HIV-1 Vif 16K/R vectors (3 μg) were co-transfected with 0.3 μg of VR-CBF-mf or VR1012 into 293T cells. One day after transfection, the cells were harvested and lysed. One portion of each cell lysate was analyzed using western blotting, and the remaining part was mixed with anti-HA-agarose for 4 h. The proteins were analyzed using an anti-Flag antibody (CBF-β) or an anti-HA antibody (Vifs).

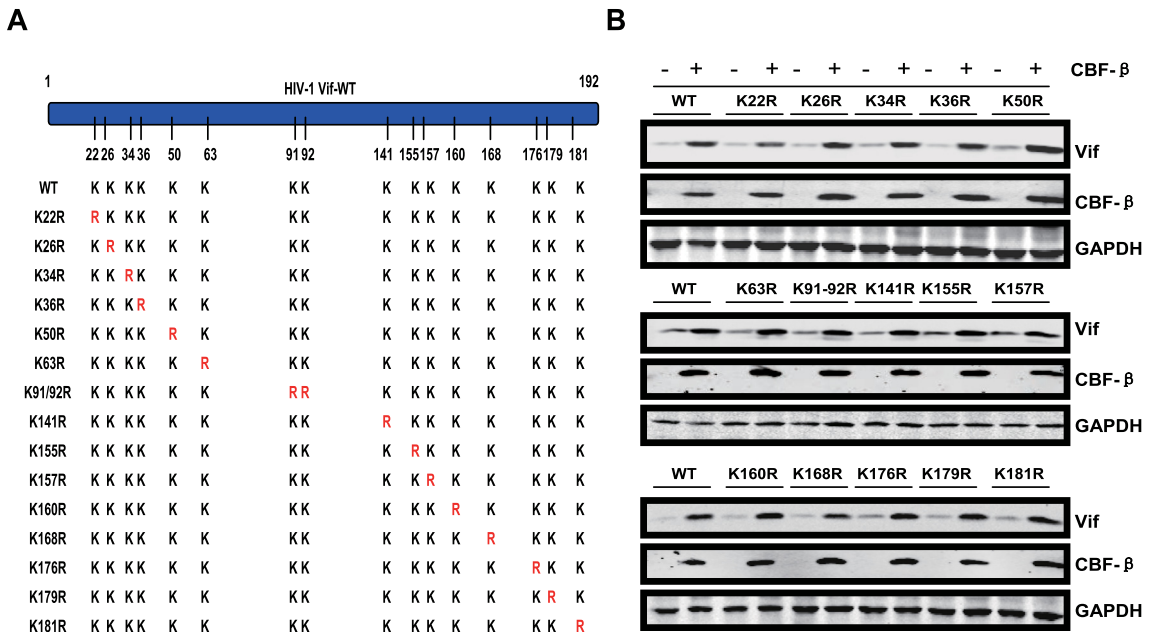

Fig. 2. Single K mutations in HIV-1 Vif have little effect on the responsiveness to CBF-β. (A) HIV-1 Vif-WT and its K to R mutations. HIV-1 Vif comprises 192 amino acid residues, including 16 lysine (K) residues. The numbers indicate the positions of K in the sequence of Vif. The sequence of each K to R mutant is shown below that of wild-type Vif (WT). K22R indicates that K22 of HIV-1 Vif was mutated to R, and similar terminology was used to describe the other Vif mutants. (B) HIV-1 Vif-WT or its mutants (1 μg) were co-transfected with 0.2 μg VR-CBF-mf or VR1012 into 293T cells. Twenty-four hours later, the cells were harvested and lysed, and the contents were analyzed using western blotting. Vif proteins were detected using an anti-HA antibody, and CBF-β was detected using an anti-Flag antibody; GAPDH was detected using an anti-GAPDH antibody as a loading control.

protein expression, and the remaining lysate was mixed with anti-HA or Flag-agarose antibodies at 4 °C for 4 h. The proteins were separated using 15% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Millipore). The membrane was blocked with 3% BSA (Sigma–Aldrich) dissolved in TBS buffer for 1 h and then probed, initially with primary antibodies and then with Alexa Fluor 700-labeled anti-rabbit IgG secondary antibody (KPL) or Alexa Fluor 800-labeled goat anti-mouse IgG secondary antibody (KPL). Other antibodies used in this study included rabbit anti-HA polyclonal antibody (Invitrogen), mouse anti-HA antibody (Sigma–Aldrich), anti-Flag antibody (Sigma–Aldrich), anti-V5 antibody (Invitrogen), anti-β actin antibody (Sigma–Aldrich), anti-GAPDH antibody (Proteintech), anti-HA-agarose antibody (Sigma–Aldrich) and anti-Flag-agarose antibody (Sigma–Aldrich).

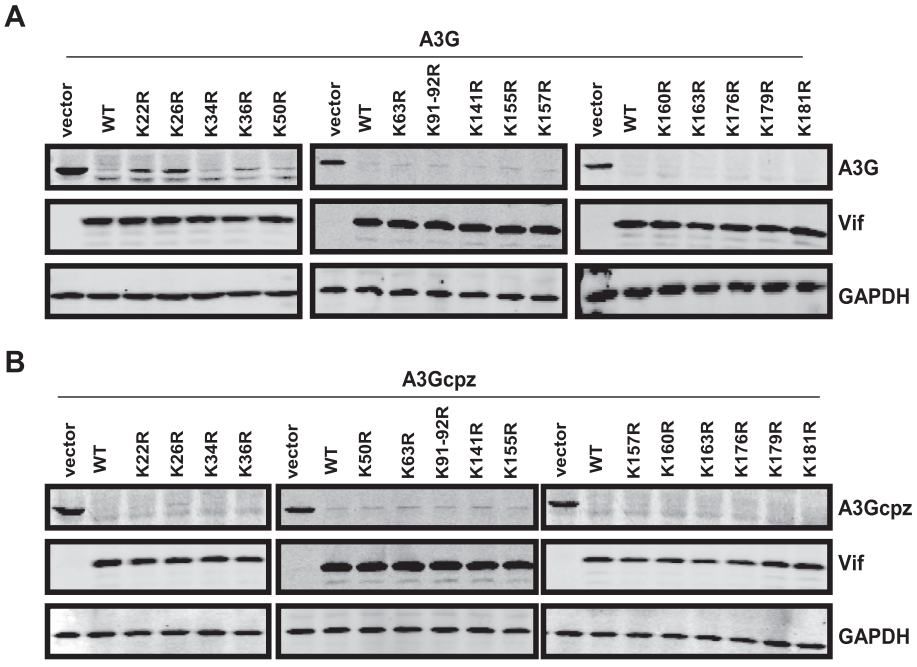

Fig. 3. Single K mutations in HIV-1 Vif have little effect on the responsiveness to A3G and A3Gcpz. 293T cells were co-transfected with 2 μg of human A3G (A3G) (A) or chimpanzee A3G (A3Gcpz) (B) and with Vif or Vif mutant expression vectors. Cells were collected 24 h after transfection, and the constituent proteins were analyzed using western blotting. A3G or A3Gcpz was detected using an anti-V5 antibody.

3. Results and discussion

3.1. CBF-β inhibits the proteasomal degradation of HIV-1 Vif

HIV-1 Vif is unstable and rapidly degraded through the proteasome pathway in cells [31]. Increasing evidence has indicated that knocking down endogenous CBF-β decreases the stability of HIV-1 Vif and that CBF-β overexpression enhances the steady-state level of Vif, thus promoting its function [31]. Some researchers have hypothesized that CBF-β might interact with HIV-1 Vif to decrease its proteasome-dependent degradation, thereby increasing its steady-state level [15,25]. To evaluate this hypothesis, we transfected HIV-1 Vif expression vectors in the presence or absence of CBF-β overexpression or the proteasome inhibitor MG132 in 293T cells. CBF-β overexpression increased the steady-state level of HIV-1 Vif (Fig. 1A) as we previously observed [32], and MG132 treatment increased the steady-state level of HIV-1 Vif to nearly the same level (Fig. 1A). We then treated cells with MG132 with simultaneous co-transfection of CBF-β and HIV-1 Vif. If CBF-β were to increase HIV-1 Vif translation or inhibit HIV-1 Vif degradation through pathways other than the proteasome pathway, CBF-β overexpression in the presence of MG132 would be expected to increase the steady-state level of HIV-1 Vif to a much higher level than that obtained using either CBF-β or MG132 alone. Conversely, if CBF-β were to inhibit proteasomal HIV-1 Vif degradation, then the combined effect of CBF-β and MG132 would be similar to that achieved using either CBF-β or MG132 alone. Over repeated experiments, we found that CBF-β overexpression in the presence of MG132 yielded results that were very similar to those achieved using either CBF-β or MG132 alone, indicating that CBF-β inhibits HIV-1 Vif degradation through the proteasome pathway.

To further confirm our hypothesis, we substituted all of the lysine (K) residues in HIV-1 Vif with arginine (R) to generate HIV-1 Vif-16K/R. Lysine residues are subject to ubiquitination [31]. Vif-16K/R could not be polyubiquitinylated or degraded through the proteasome pathway; thus, the cellular levels remained stable [31]. We found that neither MG132 nor CBF-β overexpression was able to upregulate HIV-1 Vif-16K/R (Fig. 1B). Moreover, treatment with both CBF-β and MG132 also had no effect on the Vif-16K/R levels (Fig. 1B), indicating that CBF-β was unable to rescue the cellular level of Vif mutant which was not degraded through the proteasome pathway.

To exclude the possibility that the Vif-16K/R mutant was not able to interact with CBF-β, we performed a co-immunoprecipitation assay. Unlike HIV-1 Vif, FIV Vif did not interact with CBF-β, and the induction of cat APOBEC3 degradation did not require promotion by CBF-β (manuscript in preparation). We found that CBF-β co-immunoprecipitated with HIV-1 Vif-WT and Vif-16K/R but not FIV Vif (Fig. 1C). Moreover, we also found that HIV-1 Vif-WT and Vif-16K/R co-immunoprecipitated with CBF-β (Fig. 1D). Therefore, the insensitivity of Vif-16K/R to CBF-β could not be attributed to a failure of the interaction between Vif-16K/R and CBF-β. Collectively, our results demonstrate that CBF-β inhibits HIV-1 Vif degradation through the proteasome pathway.

3.2. Single K mutations in HIV-1 Vif did not alter responsiveness to CBF-β

As Vif-WT, but not the lysine-free mutant Vif-16K/R, was responsive to CBF-β overexpression; thus we next analyzed which lysine(s) in Vif-16K/R controlled the sensitivity to CBF-β. We mutated each K residue in HIV-1 Vif-WT to R (Fig. 2A) and then assessed the steady-state levels of Vif-WT and Vif K in the resulting R mutants in the presence and absence of CBF-β overexpression. With the exception of Vif K91-92R, these mutants each contained only one K to R mutation. We found that all Vif mutants were expressed at levels comparable to that of Vif-WT (Fig. 2B). Moreover, CBF-β overexpression increased the cellular levels of all Vif mutants to the same extent as that observed for Vif-WT (Fig. 2B). Our results indicate that single K mutations in HIV-1 Vif do not affect its response to CBF-β.

Previous studies have indicated that K22 and K26 are involved in the interaction with A3G, as the K22D and K26D mutants lost the ability of the Vif-WT to induce A3G degradation [30]. We assessed the ability of all Vif K mutants to induce human A3G (A3G) and chimpanzee A3G (A3Gcpz) degradation. Similar to the results found

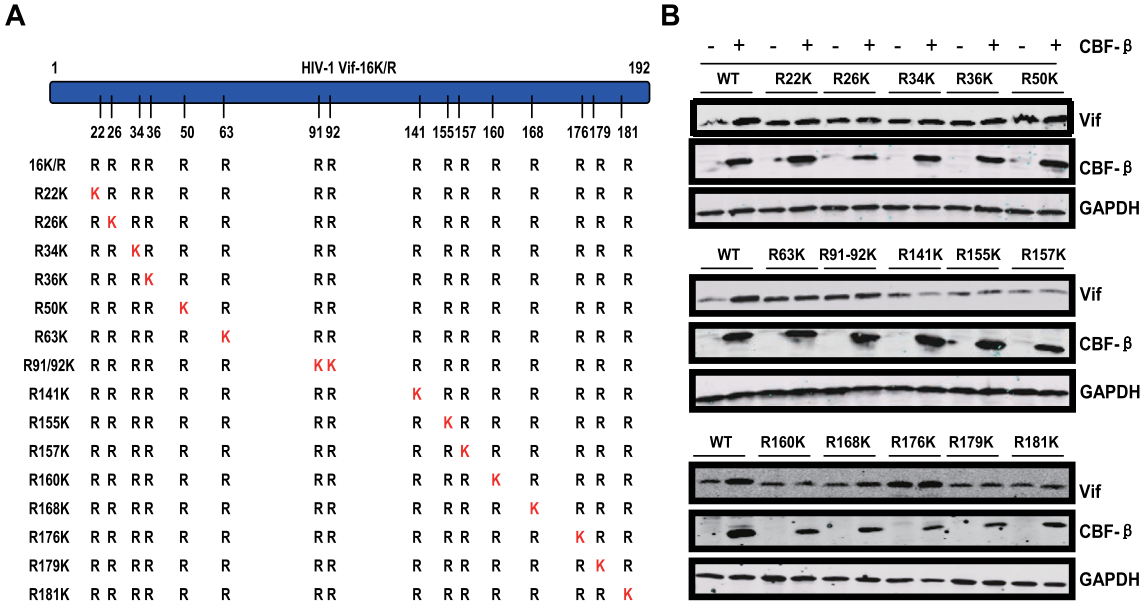

Fig. 4. Multiple K mutations to R in HIV-1 Vif impaired CBF-β responsiveness. (A) HIV-1 Vif-16K/R and its R to K mutants. The HIV-1 Vif-16K/R protein comprises 192 amino acid residues, and 16 of the R residues were mutated to K as indicated. The sequences of the R to K mutants are shown below the Vif-16K/R sequence. R22K indicates that R22 of HIV-1 Vif-16K/R was mutated to K, and similar terminology was used to describe the other Vif mutants. (B) HIV-1 Vif-WT or the HIV-1 Vif-16K/R mutant (1 μg) was co-transfected with 0.2 μg VR-CBF-mf or VR1012 into 293T cells. Twenty-four hours later, the cells were collected and lysed, and the lysate was analyzed using western blotting.

in previous studies [31], the Vif K22R and K26R mutants exhibited an impaired ability to degrade A3G (Fig. 3A); however, other K to R mutations in Vif had no effect on Vif-induced A3G degradation (Fig. 3A). In contrast, all K to R mutants retained the ability to induce A3Gcpz degradation (Fig. 3B), indicating that Vif interacted with A3G and A3Gcpz through distinct mechanisms. Although the K22R and K26R mutants exhibited a reduced ability to induce A3G degradation compared with that of Vif-WT (Fig. 3A), K22R and K26R both showed a sensitivity to CBF-β similar to that of Vif-WT. Our results imply that Vif interacts with CBF-β and A3G through distinct molecular mechanisms.

Because we were unable to determine the exact lysine residue(s) in the primary experiment by mutating K to R in the wild-type Vif protein, we next mutated the R residues in Vif-16K/R back to K (Fig. 4A) to assess the responsiveness of single K residues in Vif to CBF-β. We found that the signals of most of the K mutations of Vif-16K/R were stronger than that of Vif-WT (Fig. 4B) when plasmids were transfected at the same dose. Our results suggested that most of the K to R Vif mutant proteins were more stable [31]. Moreover, CBF-β overexpression increased the steady-state levels of Vif-WT but not those of the R to K mutants of Vif-16K/R (Fig. 4B). We therefore conclude that the responsiveness of Vif to CBF-β is affected by more than one lysine residue. However, we were unable to determine which combination of lysines is essential for CBF-β responsiveness.

Collectively, our results indicate that CBF-β inhibits the proteasomal degradation of HIV-1 Vif, thereby enhancing its steady-state level. Moreover, we found that multiple lysine mutants, but not single K to R mutants, lacked responsiveness to CBF-β, indicating that more than one lysine in HIV-1 Vif mediates the ability of CBF-β to inhibit HIV-1 Vif degradation. Lysine is the target of polyubiquitination, and HIV-1 Vif is first polyubiquitinated and then degraded through the proteasome pathway [31]. Therefore, we hypothesize that CBF-β might decrease the ubiquitination of several lysines in HIV-1 Vif, thereby inhibiting proteasomal HIV-1 Vif degradation.

4. Conclusions

In the present study, we demonstrate that CBF-β inhibits the proteasomal degradation of HIV-1 Vif, thereby increasing the steady-state level of Vif. Our study suggests that perturbing the interaction between Vif and CBF might facilitate Vif degradation, suggesting a potential target for anti-HIV drug design.

Conflict of interest

The authors have declared that no competing interests exist.

Acknowledgments

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