Human immunodeficiency virus type 1 (HIV-1) encodes the viral infectivity factor (Vif) to induce proteasomal degradation of APOBEC3G (A3G) (4, 17, 19, 21, 23, 27), a potent host restriction factor of HIV-1 (20). A functional Cul5-Vif-APOBEC3 ubiquitin ligase complex is required for Vif to induce APOBEC3 degradation (15, 22, 28, 29). A3G polyubiquitination has been shown in vivo and in vitro (4, 5, 12, 17, 19, 21, 27). HIV-1 Vif is also ubiquitinated and degraded by the proteasomal pathway (1, 7, 14, 18, 19). Dang et al. mutated all 20 lysines in A3G to arginine and found that lysine-free A3G (A3G20K/R) was still degraded in a Vif-dependent manner; however, they could not detect the polyubiquitination of A3G20K/R (5). The authors argued that polyubiquitination and degradation of APOBEC3 are independent of the degradation of Vif. Furthermore, an in vivo polyubiquitination assay showed that lysine-free APOBEC3 was also polyubiquitinated. These data suggest that polyubiquitination of APOBEC3G, not that of HIV-1 Vif, is crucial for APOBEC3G degradation.

Proteasomal degradation of APOBEC3G is a critical step for human immunodeficiency virus type 1 (HIV-1) replication. However, the necessity for polyubiquitination of APOBEC3G in this process is still controversial. In this study, we showed that although macaque simian immunodeficiency virus (SIVmac) Vif is more stable than HIV-1 Vif in human cells, SIVmac Vif induces degradation of APOBEC3G as efficiently as HIV-1 Vif. Overexpression of APOBEC3G or lysine-free APOBEC3G stabilized HIV-1 Vif, indicating that APOBEC3G degradation is independent of the degradation of Vif. Furthermore, an in vivo polyubiquitination assay showed that lysine-free APOBEC3G was also polyubiquitinated. These data suggest that polyubiquitination of APOBEC3G, not that of HIV-1 Vif, is crucial for APOBEC3G degradation.

Human immunodeficiency virus type 1 (HIV-1) encodes the viral infectivity factor (Vif) to induce proteasomal degradation of APOBEC3G (A3G) (4, 17, 19, 21, 23, 27), a potent host restriction factor of HIV-1 (20). A functional Cul5-Vif-APOBEC3 ubiquitin ligase complex is required for Vif to induce APOBEC3 degradation (15, 22, 28, 29). A3G polyubiquitination has been shown in vivo and in vitro (4, 5, 12, 17, 19, 21, 27). HIV-1 Vif is also ubiquitinated and degraded by the proteasomal pathway (1, 7, 14, 18, 19). Dang et al. mutated all 20 lysines in A3G to arginine and found that lysine-free A3G (A3G20K/R) was still degraded in a Vif-dependent manner; however, they could not detect the polyubiquitination of A3G20K/R (5). The authors argued that polyubiquitination and degradation of HIV-1 Vif are essential for A3G degradation. Here we show evidence that polyubiquitination of A3G, and not that of HIV-1 Vif, is essential for the degradation of A3G.

It has been reported that Vif from other lentiviruses, such as rhesus macaque simian immunodeficiency virus 251 (SIVmac), could also subvert the antiviral function of human A3G through the Cullin5 E3 complex (8, 15, 16, 26). To determine whether the Cullin5 E3 complex mediates degradation of different Vif proteins, HIV-1 Vif, SIVmac Vif, and SIVTan Vif were cotransfected with either empty vector or Cul5ΔNedd8 (27), into 293T cells. Because HIV-1 Vif is regulated by Cullin5 E3 ligase, HIV-1 Vif expression levels increased in the presence of Cul5ΔNedd8, as expected (Fig. 1D, lane 2 versus lane 1). By contrast, SIVmac Vif and SIVtan Vif expression levels did not dramatically increase when the function of the Cullin5 E3 complex was blocked by Cul5ΔNedd8 coexpression (Fig. 1D, lanes 4 and 6), indicating that SIVmac Vif and SIVtan Vif are more stable than HIV-1 Vif in 293T cells.

HIV-1 Vif has been shown to induce degradation of lysine-free A3G (A3G20K/R) and to overcome its anti-HIV function (5). We wanted to test if other lentiviral Vif proteins, such as those of SIV, can induce A3G20K/R degradation and overcome its antiviral function. An HIV-1 Vif-deficient proviral construct (HXB2ΔVif) was cotransfected with A3G or A3G20K/R (from Y. H. Zheng, Michigan State University) and either HIV-1 Vif or SIVmac Vif into 293T cells. Forty-eight hours later, supernatants were harvested for determining infectivity by a multinuclear activation of a galactosidase indicator (MAGI) assay. The viral particles were normalized by standard HIV-1 p24 enzyme-linked immunosorbent assay (ELISA). Both A3G and A3G20K/R dramatically decreased the infectivity of Vif-deficient HIV-1 (Fig. 2A, lanes 2 and 5). However, the infectivity of Vif-deficient HIV-1 was restored in the presence of HIV-1 Vif and SIVmac Vif provided in trans (Fig. 2A, lanes 3, 4, 6, and 7). These data indicate that SIVmac Vif is able to overcome the antiviral function of both A3G and A3G20K/R as efficiently as HIV-1 Vif.

We then wanted to determine if SIVmac Vif could overcome A3G20K/R by a degradation mechanism. We transfected A3G, A3G20K/R, SIVmac Vif, HIV-1 Vif, and Cul5ΔNedd8 into 293T cells. Both HIV-1 Vif and SIVmac Vif induced degradation of A3G and A3G20K/R (Fig. 2B, lanes 2, 4, 7, and.
The degradation of A3G and A3G20K/R was blocked by Cul5/H9004Nedd8 coexpression (Fig. 2B, lanes 3, 5, 8, and 10). Since HIV-1 Vif is also degraded by the Cul5 complex (14, 18), we observed an increase in HIV-1 Vif expression of 2.5- to 3-fold in the presence of Cul5/H9004Nedd8 (Fig. 2B, lane 3 versus lane 2 and lane 8 versus lane 7, Western blot; Fig. 2C, densitometry calculation). Surprisingly, when Cul5/H9004Nedd8 was cotransfected with SIVmac Vif, although A3G and A3G20K/R expression significantly increased, there was almost no change in the expression of SIVmac Vif (Fig. 2B, lane 5 versus lane 2 and lane 10 versus lane 9, Western blot; Fig. 2C, densitometry calculation). These data suggest that SIVmac Vif degrades A3G and A3G20K/R through the Cul5 proteasomal degradation pathway without being degraded itself and argues against the Vif and A3G codegradation model proposed by Dang et al. (5).

We previously showed that A3G and APOBEC3F expression increases HIV-1 Vif stability (14) and wanted to test if expression of A3G20K/R also increases the stability of HIV-1 Vif. Different amounts of A3G or A3G20K/R DNA were co-transfected with HIV-1 Vif in 293T cells, and Vif expression was analyzed by Western blotting. A3G and A3G20K/R expression increased HIV-1 Vif expression in a dose-dependent manner (Fig. 3A and B). CHX was also used to study the half-life of HIV-1 Vif when A3G or A3G20K/R was coexpressed. Twenty-four hours posttransfection, CHX was used to treat the transfected cells to stop translation. HIV-1 Vif was rapidly degraded by 80% in 120 min without A3G expression, while approximately 20 to 30% of Vif was degraded in 120 min when A3G or A3G20K/R was coexpressed (Fig. 3C and D). With these findings taken together, we concluded that HIV-1 Vif is more stable when A3G or A3G20K/R is coexpressed. According to this model, expression of A3G or A3G20K/R would have no effect on the half-life of Vif or even shorten its half-life by engaging more Vif molecules in the degradation pathway. As shown in Fig. 3, our data indicate that expression of A3G or A3G20K/R stabilizes HIV-1 Vif functions as a vehicle to transport A3G to proteasomes for degradation. According to this model, expression of A3G or A3G20K/R would have no effect on the half-life of Vif or even shorten its half-life by engaging more Vif molecules in the degradation pathway. As shown in Fig. 3, our data indicate that expression of A3G or A3G20K/R stabilizes HIV-1 Vif functions as a vehicle to transport A3G to proteasomes for degradation. According to this model, expression of A3G or A3G20K/R would have no effect on the half-life of Vif or even shorten its half-life by engaging more Vif molecules in the degradation pathway. As shown in Fig. 3, our data indicate that expression of A3G or A3G20K/R stabilizes HIV-1 Vif functions as a vehicle to transport A3G to proteasomes for degradation.
induce A3G degradation without being degraded. It has also been reported recently that MDM2, a human E3 ligase, induces polyubiquitination and degradation of HIV-1 Vif and reversely increases A3G levels (11). This might be another unique feature for the rapid degradation of HIV-1 Vif in human cells.

To determine if HIV-1 Vif could induce the polyubiquitination of A3G20K/R, we transfected A3G, A3G20K/R, hemag-

FIG. 2. SIVmac Vif degrades A3G and A3G20K/R without being degraded by the Cullin5 E3 complex. (A) HIV-1 Vif-deficient proviral construct B2NΔVif was cotransfected with A3G, A3G20K/R, HIV-1 Vif, or SIVmac Vif as indicated. Viral supernatants were collected and tested by MAGI assay for infectivity. HIV-1 p24 ELISAs were used to normalize viral loading. Error bars represent the standard deviations of the results of three independent experiments. (B) A3G or A3G20K/R was cotransfected with SIVmac Vif, HIV-1 Vif, and Cul5ΔNedd8 as indicated. c-Myc (Cul5ΔNedd8 and Vif), V5 (A3G and A3G20K/R), and actin antibodies were used to analyze protein expression by Western blotting. (C) Relative expression levels of Vif were calculated by analyzing the results shown in panel B. The expression of untreated Vif was set to 1. The results are representative of at least three independent experiments.

FIG. 3. HIV-1 Vif is more stable when A3G or A3G20K/R is coexpressed. (A) Different doses (1 μg, 2 μg, and 4 μg) of A3G and A3G20K/R (indicated by black triangles) were cotransfected with HIV-1 Vif. pcDNA3.1 empty vector was transfected as a control lacking A3G. V5 (A3G and A3G20K/R) and c-Myc (HIV-1 Vif) antibodies were used to visualize protein expression levels in Western blots. (B) Relative expression levels of Vif were analyzed by quantifying the intensity of the Western blot. Expression for a control lacking A3G was set to 1. (C) HIV-1 Vif was cotransfected with pcDNA3.1, A3G, and A3G20K/R. Twenty-four hours after transfection, CHX (100 μg/ml) was used to monitor the half-life of HIV-1 Vif. (D) Relative expression levels of Vif were calculated by analyzing the intensity of the Western blot. Vif expression at minute zero before treatment was set to 1. The results are representative of at least three independent experiments.
lysine residues by deleting the lysine-containing region (to residues in the C-terminal tag region. We removed the two suggested by Iwatani et al. (10), A3G20K/R has two lysine Cullin5 mutant expression vectors as indicated in Fig. 4. As glutinin (HA)-tagged ubiquitin, HIV-1 Vif, and c-Myc-tagged Nedd8 as indicated. Twenty-four hours posttransfection, MG132 (2.5 μM) was used to treat cells for 16 h. A3G, A3G20K/R, and A3G20K/RΔ2K were immunoprecipitated by Ni-NTA agarose affinity gel (Qiagen) under denaturing and native hybrid conditions. (A) Ubiquitinated forms of A3G, A3G20K/R, and A3G20K/RΔ2K were visualized by anti-HA antibody. (B) A3G, A3G20K/R, and A3G20K/RΔ2K were stained by anti-V5 antibody. The results are representative of at least three independent experiments. WB, Western blotting.

FIG. 4. HIV-1 Vif induces A3G20K/R polyubiquitination. A3G, A3G20K/R, and A3G20K/RΔ2K were transfected with HA-tagged ubiquitin (Ub-HA), HIV-1 Vif, and Cul5Nedd8 as indicated. Twenty-four hours posttransfection, MG132 (2.5 μM) was used to treat cells for 16 h. A3G, A3G20K/R, and A3G20K/RΔ2K were immunoprecipitated by Ni-NTA agarose affinity gel (Qiagen) under denaturing and native hybrid conditions. (A) Ubiquitinated forms of A3G, A3G20K/R, and A3G20K/RΔ2K were visualized by anti-HA antibody. (B) A3G, A3G20K/R, and A3G20K/RΔ2K were stained by anti-V5 antibody. The results are representative of at least three independent experiments. WB, Western blotting.

Our data show that SIVmac Vif degrades A3G and A3G20K/R through the Cullin5 proteasomal degradation pathway without the degradation of SIVmac Vif itself (Fig. 1 and 2). This result argues against the model of codegradation of HIV-1 Vif and A3G. When the A3G substrate was expressed, HIV-1 Vif became more stable, supporting the model that HIV-1 Vif functions as an adaptor protein. In the absence of A3G, HIV-1 Vif undergoes ubiquitination within the Cul5Nedd8 complex in an autocatalytic manner, as proposed by Zhou and others (9, 13, 25, 30) for F-box proteins. In the presence of A3G, HIV-1 Vif was shielded from degradation and stabilized (Fig. 3). Most importantly, we demonstrated that HIV-1 Vif induced the polyubiquitination of A3G20K/R and A3G20K/RΔ2K, supporting the idea that HIV-1 Vif induces degradation of A3G, not that of HIV-1 Vif, is crucial for the degradation of A3G. With increasing attention being paid to the mechanisms of interactions between HIV-1 Vif and A3G, fully understanding how HIV-1 Vif induces the polyubiquitination and degradation of A3G will have a significant impact on novel anti-HIV drug design.

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