High Level Expression of Human Immunodeficiency Virus Type-1 Vif Inhibits Viral Infectivity by Modulating Proteolytic Processing of the Gag Precursor at the p2/Nucleocapsid Processing Site*

Hirofumi Akari‡, Mikako Fujita‡, Sandra Kao‡, Mohammad A. Khan‡, Miranda Shehu-Xhilaga‡, Akio Adachi‡, and Klaus Strebel‡

From the ‡Laboratory of Molecular Microbiology, NIAID, National Institutes of Health, Bethesda, Maryland 20892-0460, §Tsukuba Primate Center for Medical Science, The National Institute of Infectious Diseases, Ibaraki 305-0843, Japan, and the ¶Department of Virology, The University of Tokushima Graduate School of Medicine, Tokushima 770-8503, Japan

The human immunodeficiency virus type-1 Vif protein has a crucial role in regulating viral infectivity. However, we found that newly synthesized Vif is rapidly degraded by cellular proteases. We tested the dose dependence of Vif in non-permissive H9 cells and found that Vif, when expressed at low levels, increased viral infectivity in a dose-dependent manner. Surprisingly, however, the range of Vif required for optimal viral infectivity was narrow, and further increases in Vif severely reduced viral infectivity. Inhibition of viral infectivity at higher levels of Vif was cell type-independent and was associated with an accumulation of Gag-processing intermediates. Vif did not act as a general protease inhibitor but selectively inhibited Gag processing at the capsid and nucleocapsid (NC) boundary. Identification of Vif variants that were efficiently packaged but were unable to modulate Gag processing suggests that Vif packaging was necessary but insufficient for the production of 33- and 34-kDa processing intermediates. Interestingly, these processing intermediates, like Vif, associated with viral nucleoprotein complexes more rigidly than mature capsid and NC. We conclude that virus-associated Vif inhibits processing of a subset of Gag precursor molecules at the p2/NC primary cleavage site. Modulation of processing of a small subset of Gag molecules by physiological levels of Vif may be important for virus maturation. However, the accumulation of such processing intermediates at high levels of Vif is inhibitory. Thus, rapid intracellular degradation of Vif may have evolved as a mechanism to prevent such inhibitory effects of Vif.

The human immunodeficiency virus type-1 (HIV-1) Vif protein is essential for viral replication in non-permissive cells such as primary CD4+ T lymphocytes and macrophages as well as some T cell lines including H9 and CEM cells (for review, see Refs. 1 and 2). Vif-defective viruses produced from non-permissive cells are defective at an early postentry step of infection and are unable to complete reverse transcription and integration (3–9). In fibroblasts and most T cell lines, however, Vif is not required for the production of infectious HIV-1. This cell type-dependent requirement for Vif implied the involvement of host factor(s). Indeed, the recent identification of CEM15 (APOBEC3G) as a cellular inhibitor of HIV replication (10) confirmed earlier speculations on the existence of an inhibitory factor in non-permissive cell types (11, 12). APOBEC3G was subsequently found to induce hypermutation of the HIV genome by deaminating cytidine residues on the viral minus-strand cDNA resulting in the introduction of guanosine to adenosine mutations in the HIV genome (13–16). Subsequent reports suggested a role of Vif in the inhibition of APOBEC3G packaging into virus particles (17–21). The mechanism of APOBEC3G exclusion from virions, however, remains under investigation. Some reports suggest that Vif induces degradation of APOBEC3G (20, 21), whereas others report an effect on APOBEC3G translation (17, 19) or both (18).

While most current models propose an intracellular interaction of Vif with APOBEC3G, Vif is also packaged into virions. Viruses produced from acutely infected cells incorporate 30–100 copies of Vif (22). In fact, packaging of Vif is specific and requires the interaction of Vif with the viral genomic RNA and the nucleocapsid (NC) domain of the Gag precursor (23). Moreover, virus-associated Vif is partially cleaved by the viral protease (PR) (24). Interestingly, mutations at the processing site that inhibited Vif processing inhibited Vif function, whereas mutations that did not interfere with Vif processing also did not affect Vif function (24). While these findings suggested an important role of virus-associated Vif in virions (24), its specific role remains under investigation.

In the current study we report that newly synthesized Vif is rapidly degraded in transiently transfected HeLa cells with a half-life of less than 30 min. We found that the presence or absence of APOBEC3G had no significant effect on the degradation kinetics of Vif. Based on recent reports demonstrating an interaction of Vif with APOBEC3G (17, 21, 25), we postulated that Vif enhances viral infectivity in a dose-dependent and saturable manner. Accordingly, increasing levels of Vif were expected to result in an increase in viral infectivity reaching a plateau of maximal infectivity once saturating amounts of Vif were reached or exceeded. Furthermore, increasing the amounts of Vif in permissive cell types was not expected to affect viral infectivity, as virus production in such cell types is Vif-independent due to the lack of APOBEC3G in such cells. As expected, physiological expression of Vif increased viral infec-
High Levels of Vif Inhibit Viral Infectivity

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The full-length HIV-1 molecular clone pNL4-3 was used for the production of wild type infectious virus (26). Construction of its variants pNL43-K1 (Env-defective) or pNL4-3vif(--)(Vif-defective) was described previously (27, 28). An Env- and Vif-defective variant, pNL43-K1.vif(--), was constructed by introducing a frameshift mutation in env at a KpnI site in pNL4-3vif(--). Plasmid pHCMV-G contains the vesicular stomatitis virus glycoprotein G (VSV-G) gene expressed from the immediate early gene promoter of human cytomegalovirus (29) and was used for the production of VSV-G pseudotypes. Construction of the APOBEC3G expression vector pHIV-Apo3G is described elsewhere (19). Expression of APOBEC3G from pHIV-Apo3G is under the control of the HIV-1 long terminal repeat and thus requires Tat for expression.

**For expression of Vif in trans**, the subgenomic expression vector pNL-A1 (30) was used. A Vif-defective variant of pNL-A1, pNL-A1vif(--), was used as a control. Vif deletion mutants VifΔD (deletion of residues 23–43), VifΔF (deletion of residues 23–74), and VifΔI (deletion of residues 4–45) were created by two-step PCR amplification. PCR products were column-purified; appropriate pairs were mixed at equimolar ratios and used as templates for a second round of amplification using flanking primers. Final PCR products were cloned into pNL-A1 between the BstEII and EcoRI sites.

**Cells**—H9 and LuSIV cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1-glutamine, and antibiotics. HeLa cells were maintained in complete Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1-glutamine, and antibiotics.

**Transfection and Analysis of Viral Proteins**—H9 cells (4 x 10^4) were transfected by electroporation with 10 μg each of pNL43-K1 or pNL43-K1.vif(--) and 10 μg of pNL-A1 or pNL-A1vif(--). The culture supernatants were harvested 24 h after transfection, filtered through 0.22-μm filters, and concentrated by ultracentrifugation through 20% sucrose for 1 h at 25,000 rpm using an SW41 rotor (Beckman Instruments). Alternatively, HeLa cells (5 x 10^6) were transduced with 2 μg each of variants of pNL4-3 and pNL-A1 using LipofectAMINE PLUS™ (In-vitrogen). Culture supernatants and cells were harvested 24 h later. Cell and viral lysates were analyzed by immunoblotting as described previously (23) using an HIV-1-infected patient serum (APS) and antibodies against Vif (28), NC (kindly provided by Robert Gorelick), reverse transcriptase (Intracell), p24 capsid, and matrix (MA) (provided by S. Zolla-Fazner and P. Spearman, respectively, and obtained through the National Institutes of Health AIDS Research and Reference Reagent Program (31, 32).

**Pulse/Chase Analysis of Vif**—Transfected cells were metabolically labeled for 10 min with [35S]methionine (2 mCi/ml; ICN Biomedical, Inc., Costa Mesa, CA). After the labeling, cells were washed once with phosphate-buffered saline to remove free isotope and suspended in complete RPMI containing all amino acids and 10% fetal bovine serum. Cells were incubated for various times at 37 °C as indicated in the text. Cells were then pelleted and stored at −80 °C. Cell pellets were then lysed using CHAPS buffer (50 mM Tris-hydrochloride, pH 8.0, 5 mM EDTA, 100 mM NaCl, and 0.5% (v/v) CHAPS (3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid)) supplemented with 0.2% deoxycholate, incubated on ice for 5 min, vortexed, and pelleted for 3 min at 15,000 g. Proteins present in the supernatant were used for immunoprecipitation with a Vif-specific polyclonal antibody (Vif93) (28). Immunoprecipitated proteins were solubilized by boiling in sample buffer and separated by SDS-PAGE. Radioactive bands were visualized by fluorography, and quantitation was performed using a Fuji BAS 2000 Bio-Image analyzer.

**Step Gradient Analysis**—Concentrated virus preparations were treated with 0.1% Triton X-100 and harvested with lysis buffer (Promega Corp., Madison, WI). To determine the luciferase activity in the lysates, 50 μl of each lysate were combined with luciferase substrate (Promega Corp.) by automatic injection, and light emission was measured for 10 s at room temperature in a luminometer (Optocomp II, MGM Instruments, Hamden, CT).

**RESULTS**

**Newly Synthesized Vif Is Rapidly Degraded**—Recent work proposed that Vif induces proteasome-dependent degradation of APOBEC3G (20, 21, 25). Although we and others were unable to verify such Vif-dependent degradation of APOBEC3G (17, 19), we nevertheless wanted to assess the possible impact of APOBEC3G on Vif stability. To address this question, we performed pulse/chase analyses in transiently transfected HeLa cells (Fig. 1). To ascertain coexpression of Vif and APOBEC3G in the same cells, Vif was expressed from the subgenomic expression vector pNL-A1 (30), and APOBEC3G was expressed from the Tat-dependent vector pHIV-Apo3G (19). Transfected HeLa cells were labeled for 10 min with [35S]methionine and chased for up to 60 min as described under “Experimental Procedures.” As can be seen in Fig. 1A, Vif was rapidly degraded both in the absence and presence of

![Fig. 1. Newly synthesized Vif is rapidly degraded.](image)
APOBEC3G. Quantitation of the Vif-specific bands (Fig. 1B) revealed no difference in the relative decay rates of Vif. Thus, expression of APOBEC3G had no impact on the intracellular stability of Vif. The intracellular site of Vif degradation is under investigation; however, preliminary data suggest the involvement of the cellular proteasome machinery (35).2

High Level Expression of Vif in Virus-producing Cells Is Detrimental to HIV-1 Infectivity—The rapid intracellular turnover presumably contributes to the low abundance of Vif in virus-infected cells and limits its packaging into virions. This suggests that Vif may function at very low levels. To determine how much Vif is required for maximal viral infectivity, we examined the dose-dependent effect of Vif on viral infectivity in non-permissive H9 cells. Virus stocks were produced in the presence of increasing amounts of Vif and tested for infectivity in a single-round infection assay. To avoid subsequent rounds of infection, an env-defective proviral construct, pNL43-K1.vif(<sup>-</sup>) proviral construct and the VSV-G expression vector, pHCMV-G. In addition, increasing amounts of the Vif expression vector pNL-A1 (0–10 μg) were included as indicated at the bottom. All samples were adjusted to a total of 20 μg of plasmid DNA using the Vif-defective variant pNL-A1vif(<sup>-</sup>). Transfected cells and virus-containing supernatants were harvested 24 h after electroporation, and cell lysates were subjected to immunoblot analysis using a Vif-specific antiserum (α-Vif) or an HIV-positive patient serum (APS). Proteins are identified on the left. Virus-containing supernatants were adjusted for equal reverse transcriptase activity (46) and used for the infection of LuSIV indicator cells. Luciferase activity was determined 24 h after infection and used to calculate the relative infectivity of the individual virus stocks (top). Maximal infectivity was defined as 100%.

APOBEC3G. Quantitation of the Vif-specific bands (Fig. 1B) revealed no difference in the relative decay rates of Vif. Thus, expression of APOBEC3G had no impact on the intracellular stability of Vif. The intracellular site of Vif degradation is under investigation; however, preliminary data suggest the involvement of the cellular proteasome machinery (35).2

High Level Expression of Vif in Virus-producing Cells Is Detrimental to HIV-1 Infectivity—The rapid intracellular turnover presumably contributes to the low abundance of Vif in virus-infected cells and limits its packaging into virions. This suggests that Vif may function at very low levels. To determine how much Vif is required for maximal viral infectivity, we examined the dose-dependent effect of Vif on viral infectivity in non-permissive H9 cells. Virus stocks were produced in the presence of increasing amounts of Vif and tested for infectivity in a single-round infection assay. To avoid subsequent rounds of infection, an env-defective proviral construct, pNL43-K1.vif(<sup>-</sup>) proviral construct and the VSV-G expression vector, pHCMV-G. In addition, increasing amounts of the Vif expression vector pNL-A1 (0–10 μg) were included as indicated at the bottom. All samples were adjusted to a total of 20 μg of plasmid DNA using the Vif-defective variant pNL-A1vif(<sup>-</sup>). Transfected cells and virus-containing supernatants were harvested 24 h after electroporation, and cell lysates were subjected to immunoblot analysis using a Vif-specific antiserum (α-Vif) or an HIV-positive patient serum (APS). Proteins are identified on the left. Virus-containing supernatants were adjusted for equal reverse transcriptase activity (46) and used for the infection of LuSIV indicator cells. Luciferase activity was determined 24 h after infection and used to calculate the relative infectivity of the individual virus stocks (top). Maximal infectivity was defined as 100%.

Fig. 2. Vif is a positive and negative regulator of viral infectivity. A, schematic representation of the experimental procedure. H9 cells are non-permissive and do not support replication of Vif-defective viruses. LuSIV cells are CD4+ indicator cells carrying a luciferase gene under the control of the SIV<sub>HoX</sub> long terminal repeat (33). B, H9 cells were electroporated with constant amounts (5 μg) each of the Vif- and Env-defective pNL43-K1.vif(<sup>-</sup>) proviral construct and the VSV-G expression vector, pHCMV-G. In addition, increasing amounts of the Vif expression vector pNL-A1 (0–10 μg) were included as indicated at the bottom. All samples were adjusted to a total of 20 μg of plasmid DNA using the Vif-defective variant pNL-A1vif(<sup>-</sup>). Transfected cells and virus-containing supernatants were harvested 24 h after electroporation, and cell lysates were subjected to immunoblot analysis using a Vif-specific antiserum (α-Vif) or an HIV-positive patient serum (APS). Proteins are identified on the left. Virus-containing supernatants were adjusted for equal reverse transcriptase activity (46) and used for the infection of LuSIV indicator cells. Luciferase activity was determined 24 h after infection and used to calculate the relative infectivity of the individual virus stocks (top). Maximal infectivity was defined as 100%.

High Level Expression of Vif in Virus-producing Cells Is Detrimental to HIV-1 Infectivity—The rapid intracellular turnover presumably contributes to the low abundance of Vif in virus-infected cells and limits its packaging into virions. This suggests that Vif may function at very low levels. To determine how much Vif is required for maximal viral infectivity, we examined the dose-dependent effect of Vif on viral infectivity in non-permissive H9 cells. Virus stocks were produced in the presence of increasing amounts of Vif and tested for infectivity in a single-round infection assay. To avoid subsequent rounds of infection, an env-defective proviral construct, pNL43-K1.vif(<sup>-</sup>) proviral construct and the VSV-G expression vector, pHCMV-G. In addition, increasing amounts of the Vif expression vector pNL-A1 (0–10 μg) were included as indicated at the bottom. All samples were adjusted to a total of 20 μg of plasmid DNA using the Vif-defective variant pNL-A1vif(<sup>-</sup>). Transfected cells and virus-containing supernatants were harvested 24 h after electroporation, and cell lysates were subjected to immunoblot analysis using a Vif-specific antiserum (α-Vif) or an HIV-positive patient serum (APS). Proteins are identified on the left. Virus-containing supernatants were adjusted for equal reverse transcriptase activity (46) and used for the infection of LuSIV indicator cells. Luciferase activity was determined 24 h after infection and used to calculate the relative infectivity of the individual virus stocks (top). Maximal infectivity was defined as 100%.

High Level Expression of Vif in Virus-producing Cells Is Detrimental to HIV-1 Infectivity—The rapid intracellular turnover presumably contributes to the low abundance of Vif in virus-infected cells and limits its packaging into virions. This suggests that Vif may function at very low levels. To determine how much Vif is required for maximal viral infectivity, we examined the dose-dependent effect of Vif on viral infectivity in non-permissive H9 cells. Virus stocks were produced in the presence of increasing amounts of Vif and tested for infectivity in a single-round infection assay. To avoid subsequent rounds of infection, an env-defective proviral construct, pNL43-K1.vif(<sup>-</sup>) proviral construct and the VSV-G expression vector, pHCMV-G. In addition, increasing amounts of the Vif expression vector pNL-A1 (0–10 μg) were included as indicated at the bottom. All samples were adjusted to a total of 20 μg of plasmid DNA using the Vif-defective variant pNL-A1vif(<sup>-</sup>). Transfected cells and virus-containing supernatants were harvested 24 h after electroporation, and cell lysates were subjected to immunoblot analysis using a Vif-specific antiserum (α-Vif) or an HIV-positive patient serum (APS). Proteins are identified on the left. Virus-containing supernatants were adjusted for equal reverse transcriptase activity (46) and used for the infection of LuSIV indicator cells. Luciferase activity was determined 24 h after infection and used to calculate the relative infectivity of the individual virus stocks (top). Maximal infectivity was defined as 100%.

High Levels of Vif Inhibit Viral Infectivity

2 M. Fujita, manuscript in preparation.
lower and higher levels of Vif are detrimental to virus infectivity.

**Inhibition of Virus Infectivity at High Levels of Vif Is Cell Type-independent**—The results from Fig. 2 are unexpected inasmuch as they are inconsistent with a model that simply envisions a role of Vif in the inactivation of cellular inhibitor(s) such as APOBEC3G. To directly examine the requirement of cellular inhibitory factors for the dose-dependent effect of Vif on viral infectivity, we compared the infectivity of virus stocks produced from non-permissive H9 cells and permissive HeLa cells in the presence of increasing amounts of Vif. In addition, to rule out the possibility that the inhibitory effect of Vif seen following overexpression in *trans* is due to the fact that the proviral vector employed in this study carried a defective *vif* gene, we analyzed a Vif-expressing variant (pNL43-K1) in direct comparison to the Vif-defective pNL43-K1.*vif(−)*. H9 cells (Fig. 3, A and B) and HeLa cells (C and D) were transfected with increasing amounts (1–10 μg) of pNL-A1 together with fixed amounts (5 μg) of pNL43-K1 or pNL43-K1.*vif(−)* as indicated in Fig. 3. In addition, a fixed amount (5 μg) of pHCMV-G was included to produce VSV-G-pseudotyped virus stocks. As before, DNA quantities were adjusted to 20 μg in all samples using appropriate amounts of pNL-A1.vif(−) plasmid DNA. Consistent with the results from Fig. 2, physiological levels of Vif supplied in *trans* or in *cis* produced fully infectious virus from both H9 and HeLa cells, and further increase in the amounts of Vif progressively reduced viral infectivity in H9-derived virus (Fig. 3, A and B). Interestingly, the inhibitory effect of Vif expressed in *cis* and in *trans* appeared to be additive because in the presence of endogenous Vif, smaller amounts of exogenous Vif were necessary for a comparable degree of inhibition (compare A and B of Fig. 3). These results indicate that the inhibitory effect of high levels of Vif on virus infectivity in Fig. 2 was not due to the lack of a functional *vif* gene.

Surprisingly, very similar results were observed when virus was produced from permissive HeLa cells (Fig. 3, C and D);
overexpression of Vif in trans reduced viral infectivity irrespective of the presence or absence of endogenous Vif. Again, endogenous Vif appeared to have an additive effect suggesting a similar molecular mechanism in both cell types. The fact that high level expression of Vif affected viral infectivity in the absence of cellular inhibitory factors suggests that this effect of Vif is unrelated to the noted inhibition of APOBEC3G and thus reflects a separate activity of Vif.

Vif Modulates Gag Processing during Virus Maturation—To investigate the molecular basis of the Vif-induced inhibition of viral infectivity, we performed a biochemical characterization of virions produced in the presence of varying amounts of Vif. The goal was to identify possible effects of Vif on Gag precursor processing or other effects on the viral protein composition that might explain the altered infectivity. In H9 cells, comparable expression and processing of viral proteins including Pr55gar and CA were detected intracellularly using an HIV-positive patient serum (Fig. 4A, APS) irrespective of the level of Vif expression (Fig. 4A, α-Vif). Interestingly, analysis of virus-associated proteins revealed two protein bands of ~33 and 34 kDa, respectively, that were apparent only in viruses produced in the presence of high levels of Vif (Fig. 4B, APS). Both proteins were also recognized by capsid- (Fig. 4B, α-CA) and nucleocapsid-specific (Fig. 4B, α-NC) antisera but not by a matrix-specific antibody (Fig. 4B, α-MA). The reactivity of p33/34 with CA- and NC-specific antibodies identified these proteins as Gag-processing intermediates composed of CA-p2-NC (p33) or CA-p2-NC-p1 (p34). The p33/34 intermediates were significantly less abundant than mature CA or NC suggesting that they represent a minor component of the virions. Importantly, processing of neither p66/p51 reverse transcriptase nor p17 MA was affected by the high levels of Vif (Fig. 4B), indicating that Vif does not act as a general PR inhibitor. In addition, similar Gag-processing intermediates were observed in virus derived from permissive HeLa cells in the presence of high levels of Vif (see Fig. 5). Together, the results from this experiment demonstrate that Vif modulates Gag processing to produce CA-p2-NC intermediates.

Vif and Gag Intermediates Associate with the Nucleoprotein Complex—Our observation that Vif modulates the maturation of Gag precursor molecules without acting as a general PR inhibitor suggests that Vif might inhibit Gag processing through steric interference. It is likely that such interference is caused by a direct interaction of Vif with the Gag precursor at or near the p2/NC cleavage site. In fact, several previous stud-
ies have demonstrated direct interaction of Vif with the viral Gag precursor molecules in vitro (36–38). In addition, our recent observation that mutations in the NC zinc finger domains abolish Vif packaging further points to an interaction of Vif and NC (23). Also, virus-associated Vif is stably associated with the viral core, whereas major portions of the mature CA and NC are sensitive to detergent extraction and thus are either not or only loosely associated with the core (23). If maturation of Gag is blocked through steric interference by Vif, we would assume that the resulting intermediates colocalize with Vif in the viral core. To address this issue, viruses obtained from HeLa cells expressing high levels of Vif (Fig. 5, Vif(+)) or lacking Vif expression (Fig. 5, Vif(−)) were subjected to step gradient centrifugation in the presence of Triton X-100 as reported previously (23). Western blot analysis of individual fractions of the step gradient confirmed the stable association of Vif with the viral core in the insoluble fraction (Fig. 5, lane 3, α-Vif) as well as the presence of MA and major portions of CA and NC in the soluble fraction (Fig. 5, lanes 1). Interestingly, the p33/34 Gag intermediates were resistant to detergent extraction and colocalized with Vif in the viral core fraction (Fig. 5, lane 3, asterisks) in the presence of Vif. These findings indicate that unlike mature CA and NC products, the Gag p33/34 intermediates are tightly associated with the viral nucleoprotein complex, which also contains the viral genomic RNA, reverse transcriptase, integrase, residual Pr55Gag, and Vif (23).

**Modulation of Gag Processing Requires Packaging of Vif and Involves N-terminal Regions in Vif—** In an attempt to determine the domain(s) in Vif contributing to the modulation of proteolytic processing of the Gag precursor molecules, a series of Vif deletion mutants (schematically diagrammed in Fig. 6A) was evaluated. As can be seen in Fig. 6B, comparable amounts of the wild type and mutant Vif proteins were expressed intracellularly (Fig. 6B, Cell), indicating that the mutations did not affect steady-state levels of the resulting proteins. All Vif variants were packaged into virions at levels comparable with those of wild type virus (Fig. 6B, Virus, α-Vif). No aberrant Gag intermediates were apparent in the absence of Vif (Fig. 6B, lane 1, APS) or in the presence of physiological levels of Vif (lane 2, APS and α-NC). In contrast, high level expression of wild type Vif resulted in the appearance of the p33/34 intermediates (Fig. 6B, lane 3, APS and α-NC; marked by asterisks). Deletion of residues 23–43 in Vif did not impair the ability of Vif to modulate Gag processing as shown by the presence of p33/34 at levels comparable with those observed in the presence of wild type Vif (Fig. 6B, compare lanes 3 and 5, Virus, APS and α-NC). However, deletion of residues 4–45 (Fig. 6B, lane 4) completely abolished the ability of Vif to modulate Gag processing despite the presence of high levels of this Vif variant in the virus preparation. Finally, deletion of residues 23–74 (Fig. 6B, lane 6) partially inhibited the ability to modulate Gag maturation. We confirmed that none of these deletions affected the ability of Vif to associate with the nucleoprotein complex.

**Fig. 6. Effect of in-frame deletions on the ability of Vif to modulate maturation of Gag precursor molecules.** A, schematic diagram of Vif deletion mutants employed in this experiment. The amino acid regions deleted are shown on the right. wt, wild type. B, HeLa cells were cotransfected with equal amounts of pNL43-K1.vif(–) and pNL-A1.vif(–) (lane 1), pNL43-K1 and pNL-A1.vif(–) (lane 2), pNL43-K1 and pNL-A1 (lane 3), or pNL43-K1 and various pNL-A1-based Vif deletion mutants as indicated (lanes 4–6). Cell lysates (Cell) and concentrated virus preparations (Virus) were harvested 24 h after transfection and analyzed for viral proteins by immunoblotting using APS, anti-Vif, or anti-NC antibodies as indicated on the right. Viral proteins are identified on the left. Asterisks indicate the positions of p33/34. C, virus-containing supernatants from B were adjusted for equal reverse transcriptase activities and used for the infection of LuSIV indicator cells. Luciferase activity was determined as described under “Experimental Procedures,” and the relative infectivity of the virus stocks was calculated.
(data not shown). Analysis of viral infectivity revealed an inverse correlation with the amounts of p33/34 detectable in the virus preparations (Fig. 6C). In contrast, there was no correlation between inhibition of viral infectivity and packaging of Vif. All Vif variants were expressed at similar levels and were packaged and associated with the viral nucleoprotein complex with similar efficiency. Taken together, these results support the notion that inhibition of virus infectivity is correlated with the presence of p33/34 Gag intermediates rather than with the amounts of Vif packaged. Our data further demonstrate that sequences in the N-terminal domain of Vif, in particular residues 4–22, are critical for the ability of Vif to inhibit Gag processing at the p2/NC site.

**DISCUSSION**

Despite the recent identification of a cellular factor whose inhibitory activity must be overcome by Vif to allow for the production of infectious viruses from non-permissive cell types (10), the molecular mechanism of Vif function and its site(s) of action have remained unclear. We now report for the first time on an activity of Vif that requires its presence in virus particles. Our data suggest that Vif modulates the maturation of a small number of Gag and/or Gag-Pol precursor molecules by physically interacting with the Gag or Gag-Pol precursor at or near the primary cleavage site. Such interaction causes the inhibition of or the delay in processing of Gag at the p2/NC cleavage site resulting in the accumulation of small amounts of p33/34 Gag-processing intermediates. Based on their reactivity with both CA- and NC-specific antibodies the p33/34 intermediates were identified as proteins consisting of CA, NC, and one or both of the spacer peptides p1 and p2. Vif did not affect processing at other proteolytic cleavage sites, suggesting that it selectively blocks processing at the CA-NC boundary through steric interference. It is interesting to note that Vif itself is a substrate for proteolytic processing (24), and it is possible that Vif blocks processing of Gag by acting as a decoy for the viral protease.

Processing of the Gag precursor occurs at five different sites to produce at least six mature processing products (39). This process is highly ordered and starts with an initial cleavage at the p2/NC boundary (40). The resulting processing intermediates are then further processed but at a much reduced rate (41). Inhibition of processing at any of the cleavage sites results in the production of non-infectious virions (41, 42). On the other hand, immunoblot analyses of normal infectious HIV-1 virions invariably identify a series of processing intermediates (see Figs. 2 and 6, Virus, APS; and 4, α-CA) that suggest that Gag processing is incomplete. Notably, most virus preparations contain residual uncleaved Pr55gag precursor. In fact, we noted previously that unlike most mature Gag products, Pr55gag is stably associated with the viral nucleoprotein complex (23). Thus, it is apparent that mature viral particles are composed not only of fully processed Gag and Gag-Pol products but contain residual amounts of Gag precursors and processing intermediates. Whether these processing intermediates have functional significance or are merely signs of an inefficient maturation process remains to be established. However, the noted affinity of Pr55gag as well as the p33/34 intermediates with the viral nucleoprotein complex could imply a role in its formation and/or stabilization.

Vif is known to enhance viral infectivity in a cell type-dependent manner. This phenomenon was recently attributed to the activity of the cellular cytidine deaminase APOBEC3G (13–17). APOBEC3G is packaged into HIV particles where it induces hypermutation of the minus-strand cDNA (10, 14–17). This inhibitory effect of APOBEC3G is counteracted by Vif, which interferes with the packaging of APOBEC3G. How Vif inhibits packaging of APOBEC3G is still under investigation; however, it appears to involve in part a reduction of the intracellular protein levels through proteasome-dependent degradation (18, 20, 21, 25, 35, 43) and requires a physical interaction between the two proteins (17, 18, 21, 35). Thus, like all other viral accessory proteins, Vif appears to be a multifunctional adapter molecule with seemingly opposing effects; binding of Vif to APOBEC3G appears to accelerate its intracellular turnover, whereas the interaction of Vif with the Gag precursor in virions inhibits its proteolytic processing. It is currently unclear whether the domains in Vif required for interaction with APOBEC3G and Gag are the same or map to different regions in the protein. The results from our current study (Fig. 6) suggest that inhibition of Gag processing involves an N-terminal domain in Vif. Although these same mutants were found to also be inactive with respect to APOBEC3G (19), other Vif mutants such as VifΔ23–43 were inactive against APOBEC3G (19) but still inhibited Gag maturation. These results combined with the fact that the effect of Vif on the proteolytic processing of Gag precursors is cell type-independent suggest that the effect of Vif on Gag maturation is mechanistically independent of its neutralization of APOBEC3G. It remains to be investigated whether the interaction of Vif with Gag precursor molecules during or following virus assembly relates to its ability to inhibit packaging of APOBEC3G. This seems possible as the reported reduction of intracellular expression levels of APOBEC3G by Vif does not fully account for the noted exclusion of APOBEC3G from virus particles (17, 19).

The fact that high level expression of Vif is detrimental to viral infectivity confirms that Gag maturation is a carefully balanced process. Thus, rapid degradation of Vif in virus-producing cells may have evolved as a mechanism to preclude the detrimental effects on Gag maturation. Our observation that the accumulation of the CA-p2-NC intermediates is directly correlated with the levels of Vif expression (data not shown) leads us to conclude that similar processing intermediates are produced under physiological conditions but are below the level of detection in our assay system. The fact that packaging of large quantities of Vif N-terminal deletion mutants did not affect Gag maturation (Fig. 6) suggests that the effect of Vif is specific albeit only detectable by currently available immunoblotting techniques at high levels of Vif. Thus, it is possible that minute quantities of CA-p2-NC- and CA-p2-NC-p1-processing intermediates play an important role in viral infectivity. The fact that such intermediates, unlike their mature products, are stably associated with the nucleoprotein complex raises the possibility that the association of these intermediates with Vif and viral genomic RNA promotes the stability or proper conformation of the viral nucleoprotein complex. Such a function would be consistent with previous reports correlating the lack of Vif with a reduced stability of nucleoprotein complexes (8, 44, 45).

**Acknowledgments**—We thank Stephan Bour and Eri Miyagi for helpful discussions and Eric Freed for critical comments on the manuscript. We thank Alicia Buckler-White and Ron Plishka for oligonucleotide synthesis and sequence analysis. We acknowledge Mary Karczewski and Claudia Aberham for construction of the Vif mutants and Robert Gorelick, Susan Zolla-Pazner, and Paul Spearman for antibodies. Antibodies to CA and MA were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program.

**REFERENCES**

1. Bour, S., and Strebel, K. (2000) *Adv. Pharmacol.* 48, 75–120
2. Cullen, B. R. (1998) *Cell* 93, 685–692
3. Akari, H., Uchiyama, T., Fukumori, T., Iida, S., Koyama, A. H., and Adachi, A. (1999) *J. Gen. Virol.* 80, 2945–2949
4. Chowdhury, I. H., Chao, W., Potash, M. J., Sova, P., Gendelman, H. E., and Vaksy, D. J. (1996) *J. Virol.* 70, 5336–5345
5. Courcoux, M., Patience, C., Rey, F., Blanc, D., Harmache, A., Sire, J., Vigne, R., and Spire, B. (1995) *J. Virol.* 69, 2068–2074
6. Goncalves, J., Korin, Y., Zack, J., and Gabuzda, D. (1996) *J. Virol.* 70,
High Levels of Vif Inhibit Viral Infectivity

8701–8709
7. Reddy, T. R., Kraus, G., Yamada, O., Looney, D. J., Suhasini, M., and Wong-Staal, F. (1995) J. Virol. 69, 3549–3553
8. Simon, J. H., and Malim, M. H. (1998) J. Virol. 70, 5297–5305
9. von Schwedler, U., Seng, J., Aiken, C., and Trono, D. (1999) J. Virol. 67, 4945–4955
10. Sheehy, A. M., Gaddis, N. C., Choi, J. D., and Malim, M. H. (2002) Nature 418, 646–650
11. Madani, N., and Kabat, D. (1998) J. Virol. 72, 10251–10255
12. Simon, J. H., Gaddis, N. C., Fouchier, R. A., and Malim, M. H. (1998) Nat. Med. 4, 1397–1400
13. Harris, R. S., Bishop, K. N., Sheehy, A. M., Craig, H. M., Petersen-Mahrt, S. K., Watt, I. N., Neuberger, M. S., and Malim, M. H. (2003) Cell 113, 803–809
14. Zhang, H., Yang, B., Pomerantz, R. J., Zhang, C., Arunachalam, S. C., and Gao, L. (2003) Nature 424, 94–98
15. Mangeat, B., Turelli, P., Caron, G., Friedli, M., Perrin, L., and Trono, D. (2003) Nature 424, 99–103
16. Lecossier, D., Bouchonnet, F., Clavel, P., and Hance, A. J. (2003) Science 300, 1112
17. Marian, R., Chen, D., Schrofelbauer, B., Navarro, F., Konig, R., Bollman, B., Munk, C., Nymark-McMahon, H., and Landau, N. R. (2003) Cell 114, 21–31
18. Stopak, K., de Noronha, C., Yenemoto, W., and Greene, W. C. (2003) Mol. Cell 12, 591–601
19. Kao, S., Khan, M. A., Miyagi, E., Pishka, R., Buckler-White, A., and Strebel, K. (2003) J. Virol. 77, 11398–11407
20. Sheehy, A. M., Gaddis, N. C., and Malim, M. H. (2003) Nat. Med. 9, 1404–1407
21. Mann, M., Rose, K. M., Kozak, S. L., and Kabat, D. (2003) Nat. Med. 9, 1398–1403
22. Kao, S., Akari, H., Khan, M. A., Dettenhofer, M., Yu, X. F., and Strebel, K. (2003) J. Virol. 77, 1131–1140
23. Khan, M. A., Aberham, C., Kao, S., Akari, H., Gorelick, R., Bour, S., and Strebel, K. (2001) J. Virol. 75, 7252–7265
24. Khan, M. A., Akari, H., Kao, S., Aberham, C., Davis, D., Buckler-White, A., and Strebel, K. (2002) J. Virol. 76, 9112–9123
25. Yu, X., Xu, Y., Liu, B., Luo, K., Kong, W., Mao, P., and Yu, X. F. (2003) Science 302, 1056–1060
26. Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rahson, A., and Martin, M. A. (1986) J. Virol. 59, 284–291
27. Bour, S., and Strebel, K. (1996) J. Virol. 70, 8285–8300
28. Karzwezi, M. K., and Strebel, K. (1996) J. Virol. 70, 494–507
29. Yee, J. K., Miyanojara, A., LaPorte, P., Bouie, K., Burns, J. C., and Friedmann, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8564–8568
30. Strebel, K., Daugherty, D., Clouse, K., Cohen, D., Folks, T., and Martin, M. A. (1987) Nature 328, 728–730
31. Geryn, M. K., Gianakakos, V., Sharpe, S., and Zolla-Pazner, S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1624–1628
32. Varthakavi, V., Browning, P. J., and Spearman, P. (1999) J. Virol. 73, 10298–10338
33. Ross, J. W., Maughan, M. F., Liao, Z., Hildreth, J. E., and Clements, J. E. (2000) Virology 273, 307–315
34. Aiken, C. (1997) J. Virol. 71, 5871–5877
35. Mehle, A., Strack, E., Ansuta, P., Zhang, C., McPike, M., and Gabuzda, D. (2004) J. Biol. Chem. 279, 7792–7798
36. Bardy, M., Gay, B., Bernbard, S., Chazal, N., Courouci, M., Vigne, R., Decroly, E., and Boulanger, P. (2001) J. Gen. Virol. 82, 2719–2733
37. Bousquet, M., Courouci, M., Bertoin, G., Baudat, Y., Gabuzda, D., Blanc, D., Chazal, N., Boulanger, P., Sire, J., Vigne, R., and Spire, B. (1997) J. Virol. 71, 9358–9365
38. Have, I., Hono, S. S., Fournier, C., Gay, B., Tournier, J., Carriere, C., Courouci, M., Vigne, R., Spire, B., and Boulanger, P. (1998) J. Gen. Virol. 79, 1069–1081
39. Henderson, L. E., Swdner, R. C., Copeland, T. D., Oroszlan, S., and Beneveniste, R. E. (1990) J. Med. Primatol. 19, 411–419
40. Erickson-Vitanen, S., Manfredi, J., Vitanen, P., Tribe, D. E., Tritch, R., Hutchinson, C. A., III, Leeb, D. D., and Swanstrom, R. (1989) AIDS Res. Hum. Retroviruses 5, 577–591
41. Pettit, S. C., Moody, M. D., Wehbie, R. S., Kaplan, A. H., Nantteret, P. V., Klein, C. A., and Swanstrom, R. (1994) J. Virol. 68, 8017–8027
42. Wiegars, K., Rutter, G., Kottler, H., Teusner, U., Hohenberg, H., and Kraushlich, H. G. (1998) J. Virol. 72, 2846–2854
43. Conticello, S. G., Harris, R. S., and Neuberger, M. S. (2003) Curr. Biol. 13, 2009–2013
44. Ohagen, A., and Gabuzda, D. (2000) J. Virol. 74, 11055–11066
45. Hoflund, S., Ohagen, A., Lawrence, K., and Gabuzda, D. (1994) Virology 201, 349–355
46. Willey, R. L., Smith, D. H., Lasky, L. A., Theodore, T. S., Earl, P. L., Moss, B., Capon, D. J., and Martin, M. A. (1988) J. Virol. 62, 139–147