HIV-1 Vif Blocks the Antiviral Activity of APOBEC3G by Impairing Both Its Translation and Intracellular Stability

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Summary

The human immunodeficiency virus type 1 (HIV-1) re-
lies on Vif (viral infectivity factor) to overcome the po-
tent antiviral function of APOBEC3G (apolipoprotein B
mRNA-editing enzyme, catalytic polypeptide-like 3G,
also known as CEM15). Using an APOBEC3G-specific
antiserum, we now show that Vif prevents virion incor-
poration of endogenous APOBEC3G by effectively de-
pleting the intracellular levels of this enzyme in HIV-
1-infected T cells. Vif achieves this depletion by both
impairing the translation of APOBEC3G mRNA and
accelerating the posttranslational degradation of the
APOBEC3G protein by the 26S proteasome. Vif physi-
cally interacts with APOBEC3G, and expression of Vif
alone in the absence of other HIV-1 proteins is suffi-
cient to cause depletion of APOBEC3G. These findings
highlight how the bimodal translational and posttrans-
lational inhibitory effects of Vif on APOBEC3G combine
to markedly suppress the expression of this potent
antiviral enzyme in virally infected cells, thereby effec-
tively curtailing the incorporation of APOBEC3G into
newly formed HIV-1 virions.

Introduction

The function of HIV-1 Vif is to suppress a powerful anti-
viral host defense mechanism mediated by the DNA
editing enzyme APOBEC3G. In the absence of Vif, the
replicative capacity of HIV-1 and other primate lentivi-
ruses is severely impaired in most cell culture (Fisher et
al., 1987; Strebel et al., 1987; von Schwedler et al., 1993)
and in vivo systems (DesRosiers et al., 1998).

Early studies demonstrated that HIV-1 requires Vif for
effective viral spread in primary CD4+ T cells, which are
natural targets of HIV-1 infection. Similarly, some cell
lines such as the H9 T cell line are also "nonpermissive"
because Vif is required for the production of fully infec-
tious virus from these cells. However, HIV-1 does not
require Vif for viral spread in "permissive" T cell lines
like SupT1 or Jurkat T cells (Gabuzda et al., 1992; Sova
and Volsky, 1993).

Infectivity studies of virions produced by heterokary-
ons formed between permissive and nonpermissive
T cell lines revealed that nonpermissive cells produce
an inhibitory factor(s) that impairs the infectivity of HIV-1
virions (Madani and Kabat, 1998; Simon et al., 1998).

These studies laid the groundwork for the seminal identi-
fication of this antiviral factor as APOBEC3G (CEM15)
(Sheehy et al., 2002). APOBEC3G is homologous to the
APOBEC1 RNA editing enzyme (Sheehy et al., 2002),
and both APOBEC3G and APOBEC1 are able to mutate
DNA (Harris et al., 2002). When APOBEC3G expression
vector DNA is transfected into permissive cells, these
cells are converted to the nonpermissive phenotype
(Sheehy et al., 2002).

APOBEC3G is incorporated into newly formed virions
making it available to act in the subsequent target cell
(Sheehy et al., 2002). In these targets, APOBEC3G in-
duces extensive dC to dU mutations in the viral minus
(first cDNA) strand DNA during reverse transcription,
resulting in G to A substitutions in the viral plus (geno-
mic) strand (Harris et al., 2003; Lecossier et al., 2003;
Mangeat et al., 2003; Zhang et al., 2003).

The mutations induced by APOBEC3G are so exten-
sive that they effectively terminate the viral life cycle
during or at some point after reverse transcription (Harris
et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003;
Zhang et al., 2003). Antiviral effects of APOBEC3G are
not limited solely to HIV-1 but also extend to other retro-
viruses including murine leukemia virus (MLV), simian
immunodeficiency virus, and equine infectious anemia
virus (EIAV) (Harris et al., 2003; Mangeat et al., 2003).
Of note, MLV and EIAV do not encode Vif or Vif-like
proteins and thus may be particularly susceptible to the
antiviral effects of APOBEC3G.

Although the DNA-hypermutating activity of APO-
BEC3G appears to explain its potent antiviral effects,
little is known about how Vif overcomes the effects of
APOBEC3G. Our studies have focused on this key ques-
tion and demonstrate that Vif reduces the level of APO-
BEC3G protein in virally infected cells through inhibitory
effects on both APOBEC3G translation and intracellu-
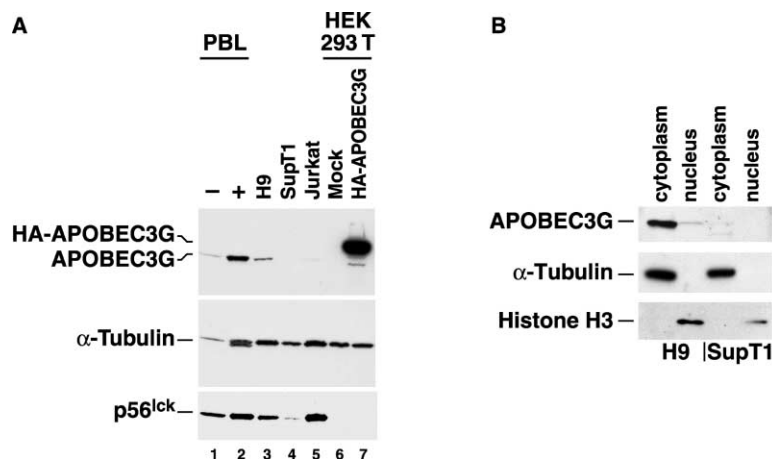lar survival.

Results

Native APOBEC3G Is Selectively Expressed in Nonpermissive T Cells and Is Principally Localized in the Cytoplasm

In order to analyze the endogenous APOBEC3G protein,
we developed a rabbit polyclonal antibody that specifi-
cally reacts with the C-terminal 16 amino acids of this
~46 kDa enzyme. Although APOBEC3G mRNA is ex-
pressed in nonpermissive but not in permissive cells
(Sheehy et al., 2002), it is not known whether the corre-
sponding APOBEC3G protein is stably expressed in
nonpermissive cells. We assessed the expression pat-
tern of APOBEC3G protein in nonpermissive and permis-
sive cell lines by immunoblotting whole-cell lysates with
the anti-APOBEC3G antibody (Figure 1A). In nonpermis-
sive T cells including PHA-stimulated primary peripheral
blood lymphocytes (PBLs) and H9 cells, the antibody
reacted with a ~46 kDa protein (Figure 1A, lanes 2 and
3) that was not recognized by preimmune serum isolated
from the same rabbit prior to immunization with C-ter-

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A

PBL

HEK 293 T

HA-APOBEC3G
APOBEC3G

α-Tubulin

p56^lck

1 2 3 4 5 6 7

B

APOBEC3G

α-Tubulin

Histone H3

H9 | SupT1

Figure 1. Expression and Localization of Endogenous APOBEC3G Protein

(A) Immunoblot analysis of APOBEC3G expression in nonpermissive and permissive cells. Cells were lysed and analyzed by SDS-PAGE and immunoblotting with anti-APO-BEC3G antiserum, anti-tubulin, and anti-p56^lck. To confirm specificity of the antiserum, 293T cells were transfected with a HA-APO-BEC3G expression vector. PBL, peripheral blood lymphocytes; +, PHA-stimulated cells; −, unstimulated cells.

(B) Subcellular localization of APOBEC3G. Nuclear and cytoplasmic fractions were prepared from H9 (nonpermissive) or Supt-1 (permissive) cells and analyzed by immunoblotting with antiserum reacting with the native APOBEC3G protein. Note that APOBEC3G is predominantly localized in the cytoplasm although small amounts of this protein are also present in the nucleus.

minal APOBEC3G peptide (data not shown). The ∼46 kDa protein band was absent or barely detecable in lysates prepared from the permissive cell lines Supt-1, Jurkat, or 293T cells (Figure 1A, lanes 4–6). α-Tubulin was expressed at similar levels in both the nonpermissive and permissive cell lysates confirming equal loading of cellular proteins. Interestingly, PHA stimulation of PBLs increased the expression of endogenous APOBEC3G (compare lanes 1 and 2) as well as of α-tubulin. Since α-tubulin was upregulated by PHA stimulation, we used p56^lck as a loading control. Although p56^lck was expressed at nearly equivalent levels in stimulated and unstimulated PBLs, APOBEC3G expression appeared to be moderately induced following mitogen activation.

To further confirm the specificity of the anti-APO-BEC3G antibody, cellular lysates from 293T cells transfected with either control vector DNA (mock) or HA-APOBEC3G expression vector were immunoblotted with the anti-APOBEC3G antiserum. An ∼50 kDa protein was detected in the lysate from the HA-APOBEC3G-transfected cells but was not detected in the lysate from mock-transfected 293T cells (Figure 1A, lanes 6 and 7). The decreased mobility of this protein band was consistent with the inclusion of a triple-HA epitope tag in the APOBEC3G cDNA. Together, these findings demonstrate that the rabbit anti-APOBEC3G antibody specifically reacts with the endogenous human APOBEC3G enzyme, confirm that the APOBEC3G protein is stably expressed in nonpermissive cells, and reveal that APOBEC3G enzyme levels are moderately upregulated after PHA stimulation of primary PBLs.

Next, we analyzed the subcellular localization of APOEC3G by immunoblotting nuclear and cytoplasmic fractions isolated from nonpermissive H9 cells and permissive SupT1 cells (Figure 1B). Tubulin and histone H3 were employed as cytoplasmic and nuclear markers, respectively, to monitor the purity of the subcellular fractions. Although endogenous APOBEC3G was not evident in permissive SupT1 cells, it was readily detected in the cytoplasm of nonpermissive H9 cells. In addition, small amounts of APOBEC3G were present in the nuclear lysate. Since expression of tubulin was exclusively restricted to the cytoplasmic fraction and histone H3 to

the nuclear fraction, the small amount of nuclear APOBEC3G detected does not appear to reflect crosscontamination of the nuclear lysate with cytoplasmic proteins during the purification. Therefore, APOBEC3G is principally expressed in the cytoplasm although small amounts of APOBEC3G are also present in the nucleus.

Vif Prevents Incorporation of Endogenous APOBEC3G into HIV-1 Virions

Two reports have appeared that disagree as to whether Vif prevents APOBEC3G from being incorporated into virions (Mariani et al., 2003; Sheehy et al., 2002). Both groups produced virus from transiently transfected 293T cells to evaluate whether APOBEC3G is incorporated into virions. However, one group found that Vif had no effect on virion incorporation of APOBEC3G (Sheehy et al., 2002), while the second group reported that Vif excludes APOBEC3G from virions (Mariani et al., 2003). Because of the unnaturally high levels of APOBEC3G produced in transfected 293T cells, this system may not faithfully recapitulate events occurring with the endogenous APOBEC3G enzyme. Therefore, we examined the levels of native APOBEC3G in virions produced from

fraction: 1 3 5 7 9 11 13 15

NL4-3 wt — APOBEC3G
— p24 Gag

NL4-3 ΔVif — APOBEC3G
— p24 Gag
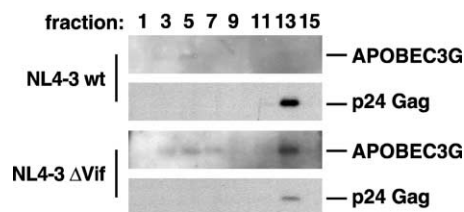
Figure 2. Vif Prevents Incorporation of Endogenous APOBEC3G into Virions

HIV-1 and HIV-1 ΔVif virions were prepared by spinoculation infection of H9 cells with VSV-G pseudotyped-HIV-1 or HIV-1 ΔVif viruses. After 48 hr, virus-containing supernatants were collected, and virions were purified on discontinous step iodixanol gradients. Gradient fractions were immunoblotted with anti-p24 Gag antibody to identify virions (fraction 13 of both gradients). The gradients were also probed with anti-APOBEC3G. Note that APOBEC3G was present in HIV-1 ΔVif virions but was undetectable in HIV-1 wt virions.
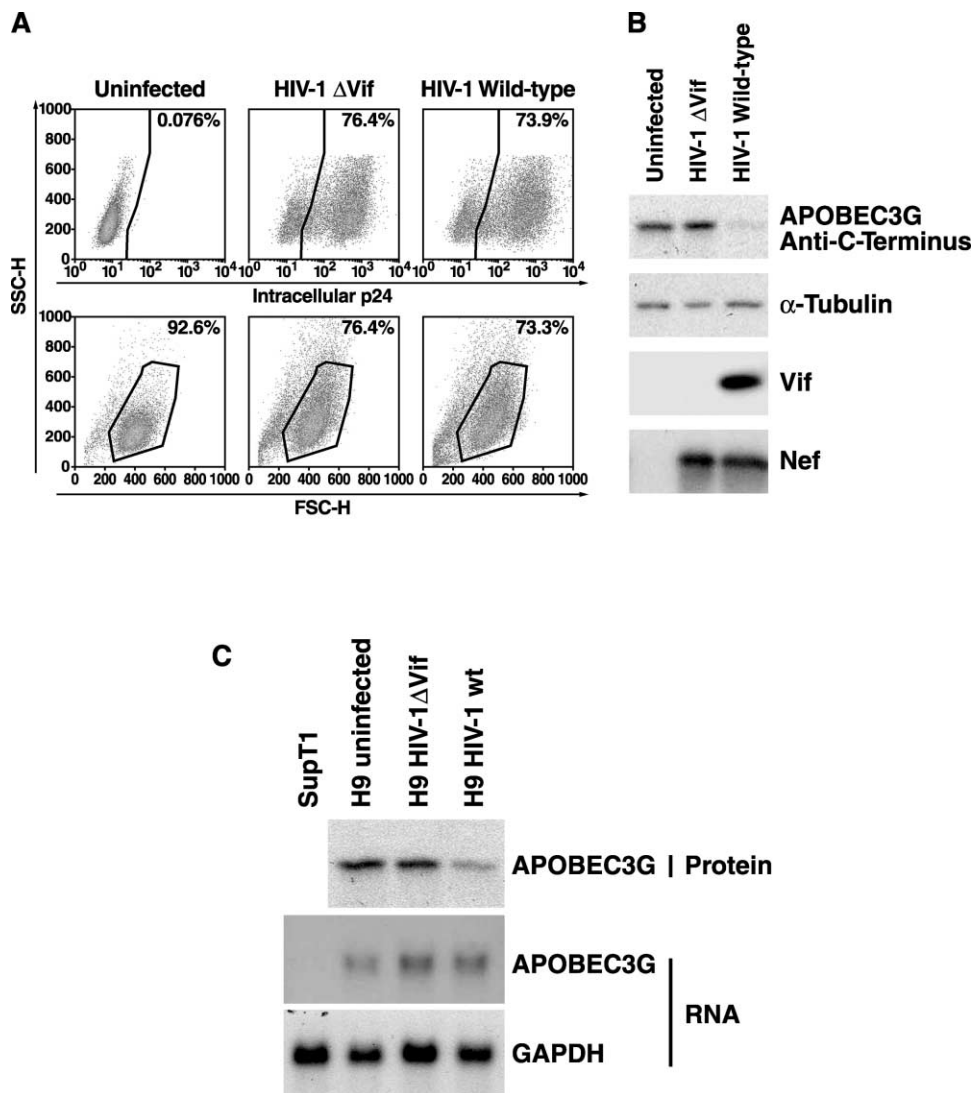
A

B

C

Figure 3. Endogenous APOBEC3G Is Nearly Eliminated from H9 Cells Infected with HIV-1 wt but Not from Cells Infected with HIV-1 ΔVif

(A) H9 cells were infected with VSV-G pseudotyped HIV-1 wt or HIV-1 ΔVif virus by spinoculation and analyzed by flow cytometry using intracellular Gag expression to monitor the extent of viral infection and using forward and side scattering of light (FSC/SSC) to identify live cells. H9 cells infected with HIV-1 wt and HIV-1 ΔVif were comparably infected and displayed equivalent numbers of cells in the live cell gate.

(B) Analysis of endogenous APOBEC3G expression in uninfected H9 cells or H9 cells infected with HIV-1 wt and HIV-1 ΔVif viruses. Cellular lysates from the cultures in (A) were immunoblotted with anti-C-terminal APOBEC3G antiserum and with anti-α-tubulin, anti-Vif, or anti-Nef antibodies. The bands were quantified using Scion Image software (version 1.62), which showed a sharp decline in the intensity of the HIV-1 wt-infected sample (0.24) when compared to that of the HIV-1 ΔVif (1.0).

(C) Vif does not alter APOBEC3G mRNA expression or integrity. Total RNA was extracted from lysates of H9 cells that were either uninfected or infected with VSV-G pseudotyped HIV-1 or HIV-1 ΔVif viruses and then subjected to Northern blot analysis using a ^{32}P-labeled APOBEC3G cDNA probe. Note that APOBEC3G protein expression is decreased in H9 cells proportionally to the fraction of these cells (47%) infected with HIV-1 wt, but the level of APOBEC3G mRNA in these same cells is equivalent to that in cells infected with HIV-1 ΔVif. Radiolabeled GAPDH probes were used as a control for mRNA loading and integrity.

infected H9 T cells. These cells were infected with vesicular stomatitis protein G (VSV-G) pseudotyped HIV-1$_{NL4-3}$ wt or VSV-G-HIV-1$_{NL4-3}$-ΔVif viruses. To isolate highly purified virions, supernatants from the infected H9 cells were pelleted through a 5% iodixanol cushion and then applied to discontinuous iodixanol gradients. Sequential fractions from the gradients were analyzed for p24-Gag content as a marker of HIV-1 wt and Δvif virions (Figure 2, fraction 13). Immunoblotting of these fractions with the APOBEC3G-specific antiserum revealed that endog-

enous APOBEC3G is incorporated in HIV-1 ΔVif virions (Figure 2, fraction 13, third panel). Conversely, no full-length APOBEC3G protein was detected in the HIV-1 wt virions (Figure 2, fraction 13, top panel). Small quantities of APOBEC3G were detected in fractions that did not contain p24-Gag (fractions 3 through 7), likely corresponding to APOBEC3G residing in microvesicles. However, these contaminants were well separated from the virion fractions. These findings demonstrate that the presence of Vif in infected T cells prevents the native
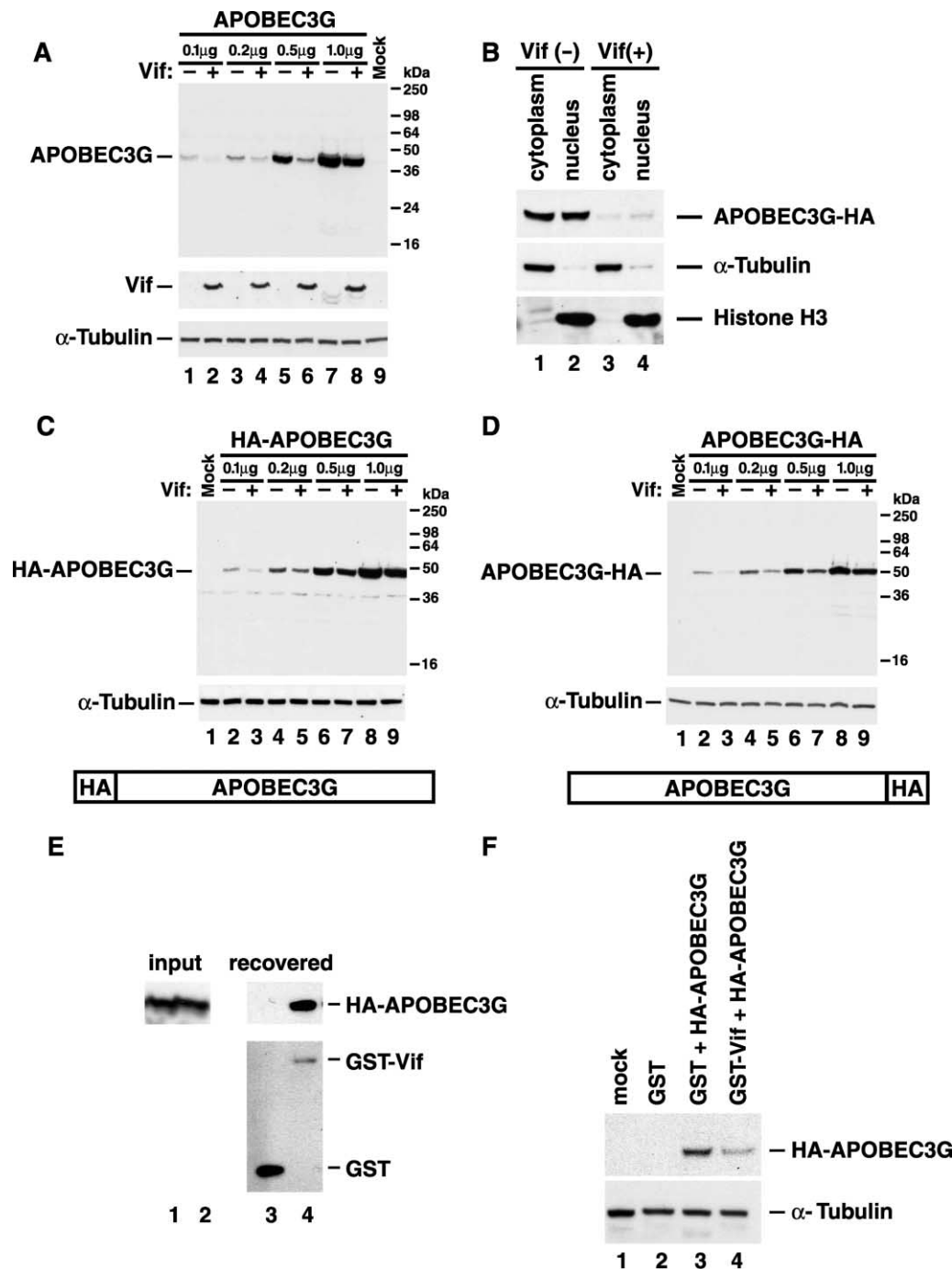
Figure 4. Vif Does Not Require the Participation of Other Viral Proteins to Deplete Intracellular APOBEC3G, the Disappearance of APOBEC3G Occurs at Both Ends of the Protein and Is Not Due to Its Translocation into the Nucleus, and Vif Physically Interacts with APOBEC3G

(A) Vif alone is sufficient to deplete APOBEC3G from transfected 293T cells. 293T cells were transfected with graded amounts of APOBEC3G expression vector DNA (0.1–1 μg) in the presence or absence of a constant amount (2 μg) of Vif expression vector. Cellular lysates from these cultures were immunoblotted with anti-APOBEC3G antiserum. Vif alone induced a decline in APOBEC3G expression over the range of APOBEC3G inputs, although the effect is most pronounced at the lower levels of APOBEC3G expression.

(B) Nuclear and cytoplasmic fractionation. Nuclear and cytoplasmic proteins were purified from transfected 293T cells and probed with anti-HA (to detect APOBEC3G-HA), anti-tubulin, or anti-histone H3 antibodies. Note that Vif induced APOBEC3G depletion in both the nuclear and cytoplasmic fractions.

(C and D) Vif promotes degradation or extensive proteolysis of APOBEC3G as reflected by the loss of both N- and C-terminal immunoreactive epitopes. The experiment in (A) was repeated using N- or C-terminally HA-tagged APOBEC3G. Cellular lysates were analyzed by immunoblotting with anti-HA antibodies. Vif expression induced a loss of anti-HA immunoreactivity regardless of whether the HA epitope was positioned at the N or C terminus of APOBEC3G.

(E) APOBEC3G interacts with GST-Vif. Lysates from 293T cells transfected with HA-APOBEC3G expression vector and either GST or GST-Vif

APOBEC3G enzyme from being encapsidated into newly formed virions.

Infection of Nonpermissive T Cells with HIV-1-Expressing Vif Results in the Apparently Complete Depletion of Intracellular APOBEC3G

To further elucidate the mechanism by which Vif prevents the incorporation of APOBEC3G into HIV-1 virions, we analyzed the intracellular expression of the endogenous APOBEC3G enzyme in H9 T cells 2 days after infection with either HIV-1 wt or HIV-1 ΔVif. For these studies, we employed H9 cell cultures displaying comparable levels of viral infection (76.4% for HIV-1 ΔVif versus 73.9% for HIV-1 wt assessed by intracellular anti-p24-Gag immunostaining) (Figure 3A, top panel) and equivalent viability as assessed by forward and side light scattering (Figure 3A, bottom panel). Equivalent amounts of endogenous APOBEC3G were detected in uninfected H9 cells and H9 cells infected with HIV-1 ΔVif (Figure 3B, top panel). However, APOBEC3G expression was markedly reduced in H9 cells infected with HIV-1 wt. The intensity of the APOBEC3G band from the HIV-1 wt-infected sample was 24% of that from the uninfected sample. Considering that approximately 26% of the cells present in the HIV-1 wt-infected sample were uninfected (p24-Gag-negative), the small amount of APOBEC3G present in the HIV-1 wt sample likely derives from the uninfected cells. Therefore, a likely interpretation of this data is that the endogenous APOBEC3G is nearly completely depleted in the virally infected subset of cells. This conclusion is further supported by the absence of detectable APOBEC3G encapsidation into virions produced by H9 T cells infected with HIV-1 wt viruses (Figure 2).

α-Tubulin levels were comparable in each of the cell lysates, indicating equal loading of lysate proteins (Figure 3B). Moreover, Vif expression was observed in the H9 cells infected with HIV-1 wt but not in the cells infected with HIV-1 ΔVif, and equivalent levels of Nef were detected in both of the virally infected samples, further demonstrating comparable infection of these cultures (Figure 3B). Accordingly, infection of nonpermissive cells with HIV-1 expressing Vif leads to the apparently complete depletion of native APOBEC3G protein from the infected subset of cells.

APOBEC3G mRNA Levels Are Not Altered following Infection of Nonpermissive Cells with HIV-1 Expressing Vif

To further dissect the mechanism underlying the intracellular depletion of APOBEC3G in HIV-1 wt-infected H9 cells, we considered the possibility that Vif alters the expression or integrity of APOBEC3G mRNA. RNA and protein were simultaneously prepared from uninfected and infected nonpermissive H9 T cells, and the levels of APOBEC3G mRNA and protein were assessed (Figure

3C). As expected, APOBEC3G protein expression was decreased in the H9 cells infected with HIV-1 wt in comparison to the HIV-1 ΔVif-infected samples. Quantification of these immunoblots revealed that the band corresponding to APOBEC3G in the HIV-1 wt sample was 48% of the intensity of the APOBEC3G bands in the uninfected and HIV-1 ΔVif-infected samples (data not shown). Since intracellular p24-Gag immunostaining revealed that 47% of the H9 cells were infected with HIV-1 wt in this experiment (data not shown), we again conclude that Vif induced complete or almost complete disappearance of APOBEC3G within the infected subset of cells. Despite these changes in APOBEC3G protein expression, there was no apparent difference in the level of expression of APOBEC3G mRNA among the three samples (Figure 3C). Equivalent RNA loading was confirmed by hybridization with a radiolabeled DNA probe specific for GAPDH, and RNA extracted from the permissive cell line SupT1 was used as a negative control (Figure 3C). These findings indicate that Vif does not impair APOBEC3G protein expression by interfering with the production or stability of APOBEC3G mRNA.

Vif Alone Is Sufficient to Deplete Intracellular APOBEC3G

We next examined whether Vif requires the participation of other viral components to cause the depletion of intracellular APOBEC3G. 293T cells were transiently transfected with increasing doses of untagged APOBEC3G expression plasmid DNA (0.1–1.0 μg) in the presence of a fixed amount of Vif expression vector DNA (2 μg) (Figure 4A). The expression of Vif alone reduced the intracellular levels of the APOBEC3G protein (Figure 4A, lanes 5 and 6), although this effect was less apparent as APOBEC3G input levels increased. Vif expression was comparable in each of the cellular lysates transfected with the Vif expression plasmid. Since these studies were performed with cytoplasmic extracts, we considered the possibility that the Vif-induced depletion of APOBEC3G was caused by translocation of the APOBEC3G enzyme into the nucleus. However, analysis of subcellular fractions revealed that Vif virtually eliminated APOBEC3G from both the cytoplasm and the nucleus (Figure 4B). In this experiment, we observed more APOBEC3G in the nucleus than we did in the previous experiment (Figure 1B). The increase in nuclear APOBEC3G is likely due to protein overexpression as a consequence of transient transfection. These results indicate that Vif does not require participation of other viral components to impair APOBEC3G protein expression. In addition, the observed depletion is not due to translocation of cytoplasmic APOBEC3G into the nuclear compartment.

Vif-Induced Depletion of APOBEC3G Involves Disappearance of the Entire Protein

Next, we investigated whether the apparent loss of APOBEC3G in Vif-expressing cells could be explained by

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expression vectors were incubated with glutathione-Sepharose beads. Immunoblotting with anti-HA antiserum revealed that HA-APOBEC3G was precipitated by GST-Vif but not GST.

(F) GST-Vif is biologically active. Lysates from 293T cells cotransfected with HA-APOBEC3G expression plasmid and either GST or GST-Vif expression plasmid were analyzed by immunoblotting. Expression of GST-Vif but not GST induced a decline in intracellular HA-APOBEC3G levels.

proteolytic clipping of the C terminus, which would result in a loss of the epitope used for immunodetection. When 293T cells were cotransfected with graded doses of HA-APOBEC3G or APOBEC3G-HA expression vector DNA in the presence or absence of Vif expression plasmid DNA, both the N- and C-terminally tagged versions of the APOBEC3G enzyme were diminished in the presence of Vif (Figures 4C and 4D). These findings indicate that Vif targets the entire APOBEC3G protein for elimination rather than inducing proteolysis only at the C terminus.

Vif Physically Assembles with APOBEC3G

To evaluate whether HIV-1 Vif physically interacts with APOBEC3G, 293T cells were cotransfected with HA-APOBEC3G and GST-Vif or control GST expression plasmid DNA (Figure 4E). Cellular lysates were prepared and incubated with glutathione-Sepharose beads. Immunoblotting of proteins bound to these beads demonstrated recovery of HA-APOBEC3G by GST-Vif but not by GST (Figure 4E). To assess whether the GST-Vif protein retained biological activity, we monitored HA-APOBEC3G levels in the presence and absence of GST-Vif and observed lower levels of this enzyme in the presence of GST-Vif (Figure 4F). These findings indicate that Vif is able to assemble with the APOBEC3G protein in living cells and further that the chimeric GST-Vif protein retains the ability to impair intracellular APOBEC3G expression.

Vif Shortens the In Vivo Half-Life of APOBEC3G

We hypothesized that Vif might deplete the intracellular levels of APOBEC3G by accelerating its degradation. To investigate this possibility, [^{35}S]methionine/cysteine pulse-chase radiolabeling studies were performed in HEK293 cells cotransfected with APOBEC3G-HA and pNL-A1 (which expresses wild-type Vif) or pNL-A1-ΔVif. After incubation in starvation medium followed by a 30 min pulse, the cultures were chased for up to 7 hr in media containing excess unlabeled methionine and cysteine. APOBEC3G-HA was then immunoprecipitated from lysates of cells harvested at different intervals (Figure 5A). In the absence of Vif, the radiolabeled APOBEC3G protein was relatively stable, and about 80% of the protein was present even after 7 hr of chase. However, in the presence of Vif, the radiolabeled APOBEC3G disappeared more rapidly, displaying a $T_{1/2}$ that consistently was less than 2 hr (Figure 5A). These findings demonstrate that Vif shortens the half-life of APOBEC3G.

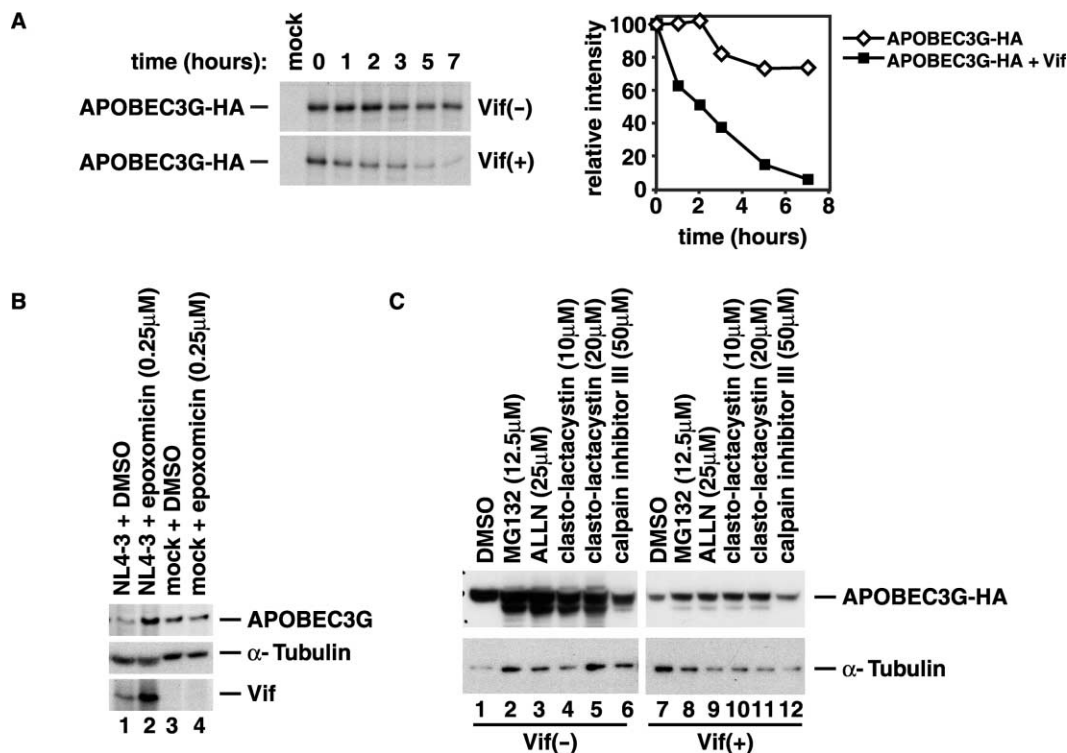## Proteasome Inhibitors Partially Block Vif-Mediated Depletion of APOBEC3G

We hypothesized that the 26S proteasome might mediate the Vif-induced degradation of APOBEC3G. To explore this possibility, H9 cells were infected with HIV-1 wt or were mock infected. Forty-four hours after infection, the cells were treated for 16 hr with either DMSO (0.1%) or 0.25 μM epoxomicin, a specific inhibitor of three proteolytic activities in the proteasome. Whole-cell lysates were subsequently prepared by boiling the cells in Laemmli lysis buffer, followed by protein separation by SDS-PAGE and immunoblotting. As expected, APOBEC3G levels were much lower in the infected sample when compared to the uninfected sample (Figure

5B, lanes 1 and 3). However, APOBEC3G levels were elevated in infected cells treated with epoxomicin when compared to DMSO-treated cells (Figure 5B, lanes 1 and 2), while epoxomicin did not appear to cause a similar increase in the level of APOBEC3G in the uninfected cultures (Figure 5B, lanes 3 and 4). Interestingly, epoxomicin also caused an increase in Vif levels. We then performed a similar experiment using 293T cells transiently transfected with an APOBEC3G-HA expression vector and either a Vif expression vector or a control vector. The cells were then treated with a panel of different proteasome inhibitors for 15 hr. We observed that the proteasome inhibitors MG132 (12.5 μM) and ALLN (25 μM) as well as the more specific proteasome inhibitor *clasto*-lactacystin β-lactone (10 μM, 20 μM) enhanced the levels of APOBEC3G-HA in the presence of Vif when compared to the diluent control sample (0.1% DMSO) (Figure 5C, lanes 7–12). In contrast, calpain inhibitor III (50 μM), which inhibits a separate proteolytic pathway (Figure 5C, lane 12), did not cause an increase in APOBEC3G levels. We also observed that proteasome inhibitors enhanced APOBEC3G levels (including a lower molecular weight APOBEC3G band) even in the absence of Vif (Figure 5C, lanes 2–6). Taken together, our results indicate that the Vif-mediated depletion of APOBEC3G is at least partially mediated by the proteasome, or, alternatively, that Vif accelerates the natural turnover of APOBEC3G by the proteasome.

Vif Also Impairs Translation of APOBEC3G mRNA

In the course of the in vivo pulse-chase radiolabeling experiments, we observed that 15%–40% less radiolabeled APOBEC3G was recovered after the pulse-labeling phase in cells expressing Vif as compared to cells not expressing Vif. (This finding was not presented in the graph in Figure 5A since the time = 0 samples were normalized for better comparison of the relative degradation rates.) These results raised the possibility that Vif might inhibit APOBEC3G expression by impairing the translation of APOBEC3G mRNA. To further investigate this possibility, we performed shorter pulse-labeling experiments (15 min) in the presence of increasing amounts of Vif (0–8 μg) (Figure 6A, left panel). These studies revealed that Vif impaired [^{35}S]methionine/cysteine radiolabeling of APOBEC3G-HA in vivo in a dose-related manner, consistently causing a 30%–50% decline in APOBEC3G translation during the pulse-labeling period. Immunoblotting analysis of the same samples revealed an 80% decline in the steady-state levels of APOBEC3G-HA (Figure 6A, right panel). Since a 30%–50% decline in translation does not fully account for the 80% decline in the steady-state levels of APOBEC3G-HA, Vif must function both by partially blocking the synthesis of the APOBEC3G protein and by inducing APOBEC3G degradation after translation.

To further examine the effects of Vif on APOBEC3G mRNA translation, we employed an in vitro-coupled transcription/translation system. Rabbit reticulocyte lysates were programmed with APOBEC3G DNA in the presence of pretranslated Vif or GST control protein (Figure 6B, left panel). In this in vitro system, Vif impaired APOBEC3G translation by approximately 70%–75%. To confirm that the effect was due to a translational impair-

Figure 5. Vif Shortens the Half-Life of APOBEC3G, and Proteasome Inhibitors Partially Block the Intracellular Depletion of APOBEC3G-HA

(A) Pulse-chase radiolabeling. HEK293 cells transiently transfected with APOBEC3G-HA and pNL-A1 or pNL-A1ΔVif DNA were pulse-labeled and then incubated with chase media for the indicated time points (left panel). After immunoprecipitation with anti-HA antibody, the proteins were separated by SDS-PAGE, and the gels were subjected to autoradiography. The relative intensity of the bands was quantified, and the Vif (+) and Vif (−) samples were normalized to 100 and graphed (right panel).

(B) Proteasome inhibitor studies using infected H9 cells. Mock or HIV-1 wt-infected H9 cells were treated with epoxomicin (0.25 μM) or DMSO (0.1%) 44 hr after infection. Whole-cell lysates were prepared by boiling the cells in Laemmli lysis buffer. Note that epoxomicin caused an increase in endogenous APOBEC3G levels in the infected sample (compare lane 2 with lane 1).

(C) Proteasome inhibitor studies using transiently transfected 293T cells. After transfection of 293T cells with 0.1 μg APOBEC3G and 2 μg Vif or control vector DNA, the cells were treated with the indicated concentrations of inhibitor for 15 hr. APOBEC3G-HA levels (including a minor molecular weight band derived from APOBEC3G) were elevated in the presence of the proteasome inhibitors MG132, ALLN, and *clasto*-lactacystin β-lactone (lanes 8–11), but not in the presence of calpain inhibitor III (lane 12).

ment, we performed a similar experiment in the presence or absence of 5 μM epoxomicin, a potent proteasome inhibitor (Figure 6B, right panel). Although epoxomicin did slightly increase APOBEC3G levels in both samples, the Vif-mediated translation inhibition was not diminished.

Together, these results demonstrate that Vif depletes intracellular APOBEC3G in a bimodal manner both by inhibiting translation of APOBEC3G mRNA and by accelerating proteasome-mediated degradation of the APOBEC3G enzyme. In combination, these two mechanisms cause the essentially complete elimination of the APOBEC3G enzyme from infected T cells.
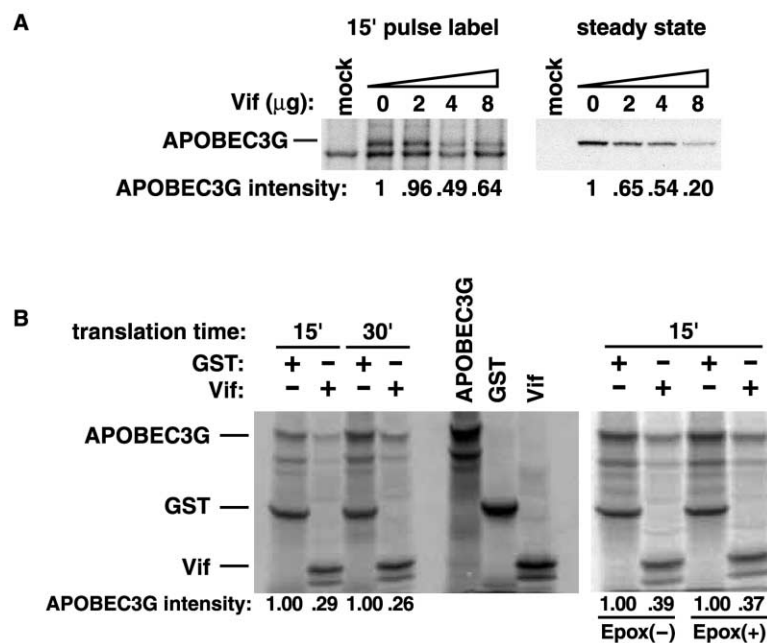
Discussion

APOBEC3G mediates extensive hypermutation of viral DNA during reverse transcription but has no apparent effect on the full-length genomic RNA packaged into the virion (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Zhang et al., 2003). It follows that APOBEC3G must be incorporated into the virion in order to mutate DNA in the subsequent target cell during viral

DNA synthesis. Our results demonstrate that HIV-1 Vif effectively prevents native full-length APOBEC3G from being incorporated into HIV-1 virions. These results agree with the recently published work of Mariani and colleagues (Mariani et al., 2003) who made similar observations in transiently transfected cultures.

Our studies are also most consistent with the conclusion that Vif prevents virion incorporation of endogenous APOBEC3G by causing the near-complete depletion of the intracellular levels of this enzyme in HIV-1-infected T cells. We suggest that the dramatic reduction in the native APOBEC3G levels that occurs in HIV-1-infected T cells lies at the heart of Vif action and explains why APOBEC3G is not encapsidated in progeny HIV-1 virions. This conclusion is further supported by our finding that the intracellular levels of APOBEC3G are not altered in cells comparably infected with HIV-1 ΔVif viruses and that the virions derived from these cells contain readily detectable APOBEC3G.

Mariani et al. observed only a modest decrease in cellular APOBEC3G levels in the presence of Vif. We also found that in transiently transfected cells, moderate expression of APOBEC3G can diminish the Vif effect.

A

B

Figure 6. Vif Impairs the Translation of APO-BEC3G-HA mRNA

(A) In vivo pulse label studies. HEK293 cells transiently transfected with 1 μg of APOBEC3G-HA expression vector DNA and 0–8 μg Vif expression vector DNA were pulsed with radiolabeled methionine and cysteine for 15 min and then immediately harvested. After immunoprecipitation with anti-HA antibody, the samples were either (1) subjected to SDS-PAGE and analyzed by autoradiography (left panel) or (2) subjected to SDS-PAGE and analyzed by immunoblotting (right panel). Higher concentrations of Vif caused a significant (36%–50%) impairment in the in vivo translation of APOBEC3G-HA (left panel). Vif caused an even greater (80%) reduction in the steady-state levels of APOBEC3G-HA (right panel).

(B) In vitro transcription/translation studies. Vif or control GST protein was initially transcribed and translated for 90 min using the Promega TNT T7-Coupled Reticulocyte Lysate System according to the manufacturer's instructions. The presynthesized Vif or GST was then added to a new transcription/translation mix programmed with APOBEC3G DNA on ice in the presence or absence of 5 μM epoxomicin. The reactions were allowed to proceed for 15 or 30 min. The proteins were then separated by SDS-PAGE, and the gel was analyzed by autoradiography. The relative intensity of the bands was quantified, and all of the Vif (−) samples were normalized to 1. Note that Vif produced up to a ∼70%–75% impairment in the in vitro synthesis of APOBEC3G (left panel) even in the presence of epoxomicin (right panel).

However, at lower doses of APOBEC3G, Vif caused a marked reduction in the intracellular levels of APOBEC3G even in this system. Mariani and colleagues have suggested that Vif assembly with APOBEC3G somehow prevents the incorporation of this enzyme into the virion (Mariani et al., 2003). We similarly find that Vif physically assembles with APOBEC3G. However, our studies reveal that the biological consequences of the Vif-APOBEC3G interaction are not simply to block virion incorporation. Rather, we find that Vif targets the APOBEC3G enzyme for essentially complete elimination in HIV-1-infected T cells. We also show that Vif does not require the participation of other viral components to reduce the levels of APOBEC3G.

Intriguingly, our studies indicate that Vif-mediated depletion of intracellular APOBEC3G involves bimodal inhibitory effects with Vif both triggering the accelerated degradation of the APOBEC3G protein and impairing the translation of APOBEC3G mRNA (summarized in Figure 7). Pulse-chase radiolabeling studies revealed that Vif markedly shortens the half-life of APOBEC3G, which is relatively stable in the absence of Vif. Our pulse-chase results differ from those of Mariani et al. One potential explanation is that we employed different transfection conditions than Mariani et al., who used a viral plasmid-to-APOBEC3G plasmid ratio of 2:1. Since we had previously observed that the levels of APOBEC3G and Vif achieved under these conditions resulted in negligible Vif-induced depletion of APOBEC3G and that the same 2:1 ratio of plasmids in the studies of Mariani et al. similarly did not rescue full viral infectivity, we performed additional experiments in which the Vif-to-APOBEC3G plasmid ratio was increased. Under these conditions, we consistently observed that Vif markedly shortened the intracellular half-life of APOBEC3G.

Our studies further support the notion that Vif-induced degradation of APOBEC3G involves the proteasome since addition of epoxomicin, a drug that specifically inhibits each of the three proteolytic activities present in the 26S proteasome, partially blocks the ability of Vif to deplete APOBEC3G in the cell. Since we also observed some effect of proteasome inhibitors on APOBEC3G levels in the absence of Vif, it is also possible that Vif accelerates the normal turnover of APOBEC3G by the 26S proteasome. Such targeted degradation of a host factor by a viral protein is not without precedent. For example, both the STAT1 transcription factor and the Rb tumor suppressor are targeted for proteasome degradation by the simian virus 5 V protein and human papilloma virus (HPV) E7 proteins respectively (Boyer et al., 1996; Didcock et al., 1999).

However, Vif-induced degradation of APOBEC3G by the proteasome does not appear to be the sole mechanism by which Vif depletes APOBEC3G in the infected host cell. Pulse-labeling studies and in vitro translation experiments support an additional inhibitory effect of Vif occurring at the level of APOBEC3G mRNA translation. In the in vitro translation system, Vif produced up to a 70%–75% reduction in APOBEC3G mRNA translation while in the pulse-labeling experiment, a 30%–40% impairment in translation was observed. Interestingly, an even greater decline (80%) in the steady-state level of APOBEC3G was observed in samples from the in vivo pulse-labeling experiment. These findings further support the bimodal nature of Vif action since the impairment of translation is not sufficient to account for the even greater decline in steady-state levels of APOBEC3G. Thus, Vif targets two different steps in the APOBEC3G cycle, one producing inhibition of APOBEC3G mRNA translation and the second increasing turnover

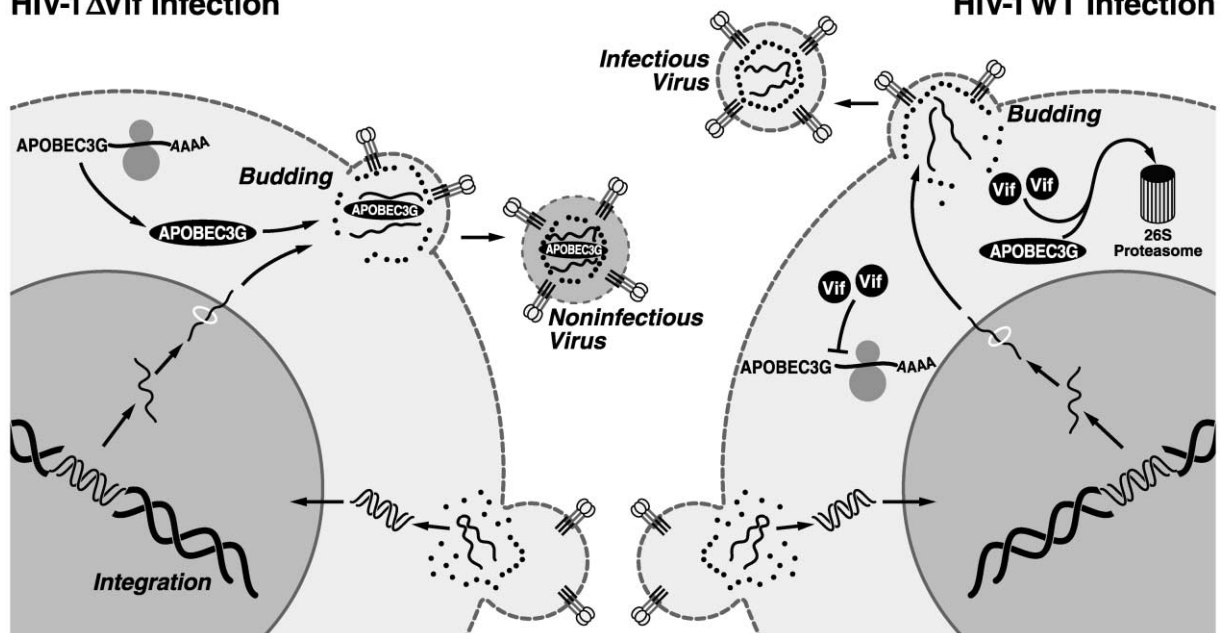## HIV-1 ΔVif Infection

HIV-1 WT Infection

Figure 7. Schematic Summary Depicting How Vif Both Impairs the Translation of APOBEC3G mRNA and Targets the Remaining APOBEC3G Protein for Degradation by the Proteasome

The combined effect of these two activities of Vif effectively eliminates APOBEC3G from the cell leading to a failure of APOBEC3G encapsidation in virions. Through these combined effects, Vif prevents APOBEC3G from inducing devastating mutations in the viral DNA synthesized during reverse transcription in the subsequent target cell.

of the synthesized APOBEC3G protein. Whether the accelerated turnover of the APOBEC3G protein could be the result of Vif-mediated inhibition of translation of a second cellular factor required for stability of the APOBEC3G protein remains a possibility that is under further study. Nevertheless, such a two-pronged strategy allows HIV-1 to maintain genetic economy while ensuring the essentially complete elimination of the potent antiviral activity of APOBEC3G within the HIV-1-infected host cell.

Other viruses also attack key intracellular proteins in a bimodal manner. For example, the HPV type 16 E6 oncoprotein inhibits the p53 tumor suppressor by two distinct mechanisms (Zimmermann et al., 1999). Specifically, the E6 protein abrogates p53 function both by binding to CBP/p300, which represses the transcriptional activity of p53, and by directly targeting the p53 protein for degradation by the 26S proteasome (Zimmermann et al., 1999).

It remains unknown whether the impairment of translation produced by Vif is mediated through binding to APOBEC3G mRNA or to the APOBEC3G primary translation product. An impairment before translation is unlikely in view of our finding that Vif does not alter the levels or stability of APOBEC3G mRNA. However, it is possible that Vif targets a more general process such as the initiation of translation. In this regard, poliovirus (PV) induces marked inhibition of host cell cap-dependent protein synthesis by cleaving several components necessary for efficient translation (Etchison et al., 1982; Kuyumcu-Martinez et al., 2002). Interestingly, PV-induced cleavage of an essential translation initiation factor, eIF4GI, results in only a 50% decrease in the translation of cellu-

lar proteins (Bonneau and Sonenberg, 1987; Bovee et al., 1998). Whether a similar mechanism could underlie the partial impairment of APOBEC3G mRNA translation induced by Vif is currently under study.

Taken together, our findings shed new light on the mechanism by which HIV-1 Vif defeats the function of APOBEC3G, a powerful innate antiretroviral factor. It might be possible in the future to exploit these mechanistic insights for the development of a new class of antiviral agents that interfere with the ability of Vif to reduce APOBEC3G levels in the cell. Such small molecules would lead to increased virion encapsidation of APOBEC3G and allow this cytidine deaminase to induce debilitating mutations during viral DNA synthesis in the subsequent target cell. By suppressing the activity of Vif, this class of antiviral drugs would restore an essential feature of the innate immune system and enable host cells to utilize endogenous APOBEC3G to inhibit HIV replication.

Experimental Procedures

Plasmids

The pNL4-3 and pNL4-3 ΔVif (also known as pNL-ND) expression vectors have been described (Adachi et al., 1991; Sakai et al., 1993). The pNL-A1 and pNL-A1-ΔVif plasmids were a generous gift from Dr. K. Strebel (Khan et al., 2001). The APOBEC3G-HA expression vector was produced by subcloning an APOBEC3G-HA cassette, isolated from the NG/C15 retroviral vector (a generous gift from Dr. M. Malim) (Sheehy et al., 2002) into pcDNA3.1 (Invitrogen). A stop codon was introduced at the end of the APOBEC3G cDNA to produce the untagged pcDNA3.1 APOBEC3G vector. To prepare the pCMV4-HA-APOBEC3G vector, APOBEC3G cDNA was amplified by PCR from an H9 cDNA library and then inserted into the HindIII and

Xbal sites of the pCMV4 vector. pCMV4-Vif and pBC-GST-Vif were prepared by PCR amplification of the Vif coding region present in a pNL4-3 vector followed by insertion of the amplicon into either the KpnI and Xbal sites of the pCMV4-his vector or the EcoRI and NheI sites of the pBC vector (a gift of C. Kedinger) (Chatton et al., 1995), respectively.

Antibodies

Anti-APOBEC3G antibody was prepared by immunizing and boosting rabbits with a synthetic peptide corresponding to the C-terminal 16 amino acids of human APOBEC3G ([C]-QDLSGRLRAILQNQEN). Serum was obtained after 42 days and tested for immunoreactivity with H9 cellular lysates. As a negative control, the same lysates were tested for immunoreactivity with preimmune serum isolated from the same rabbits (Antibody Solutions, Palo Alto, CA). Polyclonal anti-Vif antiserum was a gift from Dr. D. Gabuzda through the AIDS Research and Reference Reagent Program (Goncalves et al., 1994). Rabbit anti-Nef antiserum has been previously described (Bresnahan et al., 1998). Mouse monoclonal anti-p24 Gag ascites was a generous gift from Beckman Coulter. Other antibodies used included rabbit anti-HA (Santa Cruz Biotechnology) and mouse anti-HA (12CA5, Roche) (262K, Cell Signaling Technology). FITC-KC57 anti-p24 antibody (Coulter) was used in the intracellular anti-p24 Gag immunostaining studies.

Cell Lines, Spinoculation, and Preparation of Highly Purified Virions

H9, Supt-1, Jurkat, and 293T cells were maintained using standard tissue culture techniques. Fresh human PBLs were maintained in complete RPMI medium (10% FBS) supplemented with IL-2 (5 μg/l) (Roche Diagnostics). A portion of PBLs was activated with phytohemagglutinin (PHA) (5 μg/ml) for 24 hr before use. HIV-1 wt and HIV-1 ΔVif viruses were pseudotyped with the VSV-G envelope by calcium phosphate-mediated cotransfection of 293T cells with expression vector DNAs encoding VSV-G and pNL4-3 or pNL4-3 ΔVif. After 48 hr of culture, virion-containing supernatants were harvested, clarified by centrifugation at 500 $\times$ g for 5 min, and filtered through 0.2 μm filters. The virus-containing supernatant was then incubated with 0.4 $\times$ 10^6 H9 cells/well in 48-well plates and centrifuged at low speed for 90 min at 34°C. The cells were then washed, incubated in complete medium for 40 hr at 37°C, pelleted by centrifugation at 500 $\times$ g for 5 min, washed, and lysed for immunoblotting or Northern blotting studies. Highly purified virions were obtained from the virus-containing supernatant using the discontinuous iodixanol step gradient purification method previously described (Dettenhofer and Yu, 1999).

Protein Expression Assays

293T cells were transfected with the indicated amounts of expression vector DNA using the calcium phosphate method. Lysis buffers used for immunoblotting were (1) 100–400 mM NaCl, 50 mM HEPES, 0.2% NP40, 0–5 mM EDTA, 0.1 mM PMSF, plus complete protease inhibitor cocktail (Roche) (1 tablet/50 ml buffer) or (2) 50 mM HEPES (pH 7.2), 135 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 10% glycerol, 1 mM EDTA, 1$\times$ protease inhibitor cocktail (Calbiochem). Samples were analyzed using standard SDS-PAGE techniques.

For the proteasome inhibitor experiments in transiently transfected cells, 293T cells were transiently transfected with 0.1 μg of APOBEC3G-HA DNA, 2 μg of control vector DNA, and 2 μg of either pcDNA3.1 Vif DNA vector or an additional 2 μg of control vector DNA. Twenty-four hours after transfection, the cells were treated with the indicated inhibitor and harvested 15 hr later. For the proteasome inhibitor experiments using infected H9 cells, H9 cells were infected by spinoculation as described above. Forty-four hours after infection, the cells were treated for 16 hr with either DMSO (0.1%) or 0.25 μM epoxomicin. All of the inhibitors (ALLN, MG132, epoxomicin, calpain inhibitor III, and clasto-lactacystin β-lactone) were purchased from CalBiochem and were dissolved in DMSO.

Intracellular anti-24 Gag immunostaining of H9 cells was performed as described (Eckstein et al., 2001). In brief, H9 cells were harvested, washed, and permeabilized by incubation at room temperature for 30 min in PermeaFix (Ortho Diagnostic Systems, Raritan, NJ). After two additional washes, the cells were incubated with

FITC-conjugated anti-p24-Gag antibody (Coulter) for 30 min at room temperature. After additional washes, the cells were analyzed by flow cytometry.

To analyze subcellular distribution, H9 or transfected 293T cells were washed and suspended in swelling buffer (10 mM HEPES [pH 7.9], 1.5 mM MgCl$_2$, 10 mM KCL, protease inhibitor cocktail and PMSF) for 10 min on ice before being vortexed in the presence of 0.4% NP-40. The lysate was layered on top of 1 ml of 1% sucrose in swelling buffer and then centrifuged at 3150 RPM in a Beckman GS-6R centrifuge.

Northern Analysis

RNA was extracted from 10^7 infected or uninfected H9 or Supt-1 cells using the Qiagen RNAeasy RNA extraction kit. The RNA was then separated on a 1% formaldehyde-agarose gel, transferred to a nitrocellulose membrane, and probed with a ^{32}P-labeled APOBEC3G cDNA. Hybridizing bands on the membrane were then visualized using autoradiography.

Pulse-Chase and Pulse Radiolabeling Experiments

HEK293 cells were cotransfected with 1 μg of APOBEC3G-HA expression vector and 8 μg of either pNL-A1 or pNL-A1ΔVif. The transfected HEK293 cells were preincubated for 1 hr in labeling media (DMEM without methionine and cysteine [Cellgro] plus 10% dialyzed FBS). Each sample was subsequently pulse-labeled for 30 min with 250 μCi EasyTag EXPRESS ^{35}S Protein Labeling Mix (PerkinElmer). The initial pulse-labeled (t = 0) samples were harvested. The remaining radiolabeled samples were incubated with chase media (DMEM plus 10% FBS with 4.02 mM methionine [20$\times$] and 3 mM cysteine [15$\times$]) and harvested at various time points. For the pulse-label experiment, each sample was pulse-labeled for 15 min and then harvested immediately.

The harvested cell pellets were lysed in buffer A (50 mM HEPES, 135 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 10% glycerol, 1 mM EDTA [pH 7.2] plus protease inhibitor cocktail set I [Calbiochem]) for 15 min on ice and clarified at 14,000 $\times$ g, at 4°C for 10 min. Immunoprecipitations (IPs) were set up at equal protein concentration/volume in the presence of anti-HA monoclonal antibodies (262K) and incubated on ice. The IPs were washed four times with buffer A and eluted. The samples were analyzed by SDS-PAGE, and the gels were fixed before treatment with Amplify fluorographic reagent (Amersham Biosciences). The dried gels were subjected to autoradiography and the scanned images were quantified using Scion Image software (version 1.62).

In Vitro Translation

Vif or control GST protein was initially transcribed and translated for 90 min using pcDNA-Amp Vif and pBC vectors and the Promega TNT T7-coupled reticulocyte lysate system according to the manufacturer's instructions. The presynthesized Vif or GST was then added to a new transcription/translation mix programmed with APOBEC3G DNA on ice in the presence or absence of 5 μM epoxomicin. The reactions were then transferred to a 30°C water bath and allowed to proceed for 15 or 30 min. The proteins were separated by SDS-PAGE, and the gel was analyzed by autoradiography.

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