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Species-Specific Exclusion of APOBEC3G from HIV-1 Virions by Vif

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Summary

The HIV-1 accessory protein Vif (virion infectivity factor) is required for the production of infectious virions by CD4$^+$ lymphocytes. Vif facilitates particle infectivity by blocking the inhibitory activity of APOBEC3G (CEM15), a virion-encapsidated cellular protein that deaminates minus-strand reverse transcript cytosines to uracils. We report that HIV-1 Vif forms a complex with human APOBEC3G that prevents its virion encapsidation. HIV-1 Vif did not efficiently form a complex with mouse APOBEC3G. Vif dramatically reduced the amount of human APOBEC3G encapsidated in HIV-1 virions but did not prevent encapsidation of mouse or AGM APOBEC3G. As a result, these enzymes are potent inhibitors of wild-type HIV-1 replication. The species-specificity of this interaction may play a role in restricting HIV-1 infection to humans. Together these findings suggest that therapeutic intervention that either induced APOBEC3G or blocked its interaction with Vif could be clinically beneficial.

Introduction

The genome of retroviruses that infect rodents and birds consist of *gag*, *pol*, and *env* genes that encode virion structural proteins. The more complex genome of lentiviruses, which includes the simian (SIV) and human immunodeficiency viruses (HIV), encode six or seven additional proteins (Nef, Rev, Tat, Vif, Vpr, Vpu, and Vpx) that serve regulatory or accessory roles in virus replication (Emerman and Malim, 1998; Trono, 1995). Vif (virion infectivity factor) is a 190–240 amino acid protein that is encoded by all of the lentiviruses except for equine infectious anemia virus. The amino acid sequence of Vif is relatively well conserved between HIV-1 strains, but is only 30% identical to Vif from rhesus macaque SIV (SIVmac) (Oberste and Gonda, 1992). SIVmac239 that is deficient in *vif* (Δ*vif*), replicates to low titers in infected macaques and does not cause disease (Desrosiers et al., 1998).

Δ*vif* HIV-1 does not replicate in primary T cells, macrophages or some CD4$^+$ transformed T cell lines (termed "nonpermissive"). In contrast, several transformed T cell lines (termed "permissive") support the replication of Δ*vif* HIV-1 (Gabuzda et al., 1992; Strebel et al., 1987; von Schwedler et al., 1993). Δ*vif* HIV-1 that is derived from permissive cells can infect nonpermissive cells in a single round of replication. The cells then produce virus, but the infectivity of the virions is reduced 1000–2500-fold compared to wild-type (Chowdhury et al., 1996; Strebel et al., 1987). Expression of Vif in trans in transfected cells complements the infectivity defect in the virus producer cells, but not in the target cells (Simon et al., 1995; von Schwedler et al., 1993).

Analyses of Δ*vif* HIV-1 virions derived from permissive and nonpermissive cells revealed no differences in protein, RNA composition, or reverse transcriptase activity (Gaddis et al., 2003; Simon and Malim, 1996; von Schwedler et al., 1993). Yet, Δ*vif* HIV-1 virus derived from nonpermissive cells fails to complete a single cycle of replication. The virus appeared to initiate reverse transcription but failed to complete the synthesis of double-stranded cDNA and failed to reach the proviral stage (Goncalves et al., 1996; Simon and Malim, 1996; von Schwedler et al., 1993). Whether Vif is present in significant quantity in virions is controversial (Camaur and Trono, 1996; Dettenhofer and Yu, 1999; Fouchier et al., 1996; Khan et al., 2001; Liu et al., 1995; Simon et al., 1998). In somatic cell fusion experiments, heterokaryons formed by the fusion of permissive to nonpermissive cells were found to be nonpermissive (Madani and Kabat, 1998; Simon et al., 1998). This finding suggested that nonpermissiveness is caused by an inhibitory factor. Sheehy et al. (2002) identified the factor and termed it CEM15, a protein that is identical to apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G). Expression of APOBEC3G in permissive cells rendered them nonpermissive (Sheehy et al., 2002).

APOBEC3G is a member of a family of RNA editing enzymes (Jarmuz et al., 2002; Wedekind et al., 2003) that deaminate specific cytosines to uracil in mRNA or DNA (Anant et al., 1995; Lau et al., 2001; Yang et al., 2000). In humans, the family consists of APOBEC1, APOBEC2, APOBEC3A-G, and the activation-induced deaminase (AID) (Jarmuz et al., 2002; Muto et al., 2000; Wedekind et al., 2003). APOBEC2 and APOBEC3 have features in common with APOBEC1, including a conserved intron/exon organization, tissue-specific expression, homodimerization, and zinc and RNA binding (Jarmuz et al., 2002). APOBEC1 and APOBEC3G also have activity as DNA mutators that can trigger the cytosine deamination of target genes when expressed in *E. coli* (Harris et al., 2002). Recently, Lecossier et al. (Lecossier et al., 2003) reported that in infected nonpermissive cells, Δ*vif* HIV-1 minus-strand reverse transcripts contained frequent G→A mutations, suggesting a role for cytosine deamination.

We report here that HIV-1 Vif forms a complex with human APOBEC3G, preventing its encapsidation. HIV-1 Vif did not form a complex with mouse APOBEC3G, and as a result, did not prevent its encapsidation. Consequently, mouse APOBEC3G and APOBEC3Gs from AGM and rhesus macaque, were potent inhibitors of HIV-1 replication that were resistant to HIV-1 Vif. These findings provide a molecular mechanism for Vif function

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and have implications regarding the restriction of HIV-1 infection to humans and the host factors that influence disease progression in infected individuals.

Results

Rodent APOBEC3Gs Prevent Wild-Type HIV-1 Replication

Lentiviruses such as HIV-1 and SIV require Vif for replication in primary cells but simpler retroviruses, such as murine leukemia virus (MLV), do not. The reason for this difference is not known. One possible explanation would be the absence of a functional APOBEC3G ortholog in the host species. The *Mus musculus* genome contains a single gene with similarity to human APOBEC3G (for simplicity, provisionally termed in this study "mouse APOBEC3G"). The predicted protein differs from human APOBEC3G by 70% but conserves the two Zn^{2+} coordination domains that are characteristic of cytosine deaminases (Wedekind et al., 2003). To determine whether rodent APOBEC proteins retain the antiviral activity of human APOBEC3G, rodent APOBEC1, APOBEC2, and APOBEC3G cDNAs were derived from C57BL/6 splenocytes. In addition, APOBEC3G cDNAs were cloned from NIH-3T3, EL4 thymoma cells, L1.2 B lymphoma cells, Chinese hamster ovary cells (CHO), *Mus dunni* tail fibroblast (MDTF), and Rat2 rat fibroblasts (Sequences are shown in the Supplemental Data available at http://www.cell.com/cgi/content/full/114/1/21/DC1). A hemagglutinin (HA) epitope tag was linked to the C terminus of each cDNA and these were transferred to plasmid (pcDNA3.1) and retroviral (pBABE-*neo*; Morgenstern and Land, 1990) expression vectors. Nearly all of the rodent APOBEC3G cDNAs were derived from an alternatively spliced mRNA that lacked exon V. The deletion removed the 99 nucleotides that encode amino acids 199 to 232. Only a single full-length cDNA, which was derived from NIH 3T3, was found.

To measure Vif and APOBEC3G function, we developed an assay based on the single-cycle luciferase reporter virus pNL-LucR^-E^-(Connor et al., 1995; Deng et al., 1996). In the assay, wild-type and Δ*vif* luciferase reporter viruses were produced by cotransfection of 293T cells with pNL-LucR^-E^-or pNL-LucR^-E^-Δ*vif*, APOBEC3G expression vector and JR.FL envelope glycoprotein or VSV-G expression vector to pseudotype the particles (Connor et al., 1995; Deng et al., 1996). HOS.CD4.R5 cells were then infected with equivalent amounts of each virus (Deng et al., 1996). Infectivity of the viruses was determined by quantitation of intracellular luciferase activity three days later. The ability of the assay to measure APOBEC3G function was demonstrated by the dramatically reduced infectivity of Δ*vif* reporter virus that had been produced in the presence of human APOBEC3G (Figure 1A). Mouse APOBEC3G was also active against Δ*vif* HIV-1. Unexpectedly, the mouse enzyme was equally active against the wild-type virus, reducing its infectivity >150-fold in some experiments. Mouse APOBEC1 and APOBEC2 were inactive (Figure 1A) as were human APOBEC3A-F (not shown). The full-length mouse APOBEC3G (NIH-3T3 cDNA) and the more common exon V-deleted (e.g., C57BL/6 cDNA) were similarly active (Figure 1B). These findings demonstrated that the antiviral property of APOBEC3G is conserved by the mouse protein but that the ability to functionally interact with Vif is species-restricted, probably reflecting in vivo selective pressure on Vif to adapt to the human enzyme.

Most of the other rodent APOBEC3Gs shared the property of Vif-resistant inhibition of HIV-1 infectivity (Figure 1B) including those from L1.2, CHO, and 3T3. In contrast, EL4, Rat2, and MDTF APOBEC3G were inactive, raising the possibility of strain and species differences in antiviral function. EL4 APOBEC3G was identical to C57BL/6 APOBEC3G with the exception of a change of S281G in the second Zn^{2+} coordination domain, suggesting the importance of catalysis in the antiviral phenotype. Each of the APOBEC3Gs contained a C-terminal HA epitope tag. The HA tag did not interfere with function and allowed detection of the proteins in the cell lysates (Figure 1C).

To determine whether the mouse APOBEC3G gene was expressed in vivo, the abundance of APOBEC family member mRNAs was measured by real-time quantitative PCR (Figure 1D). APOBEC3G mRNA was most abundant in splenic lymphocytes. APOBEC3G mRNA was also detected in lymph node lymphocytes and lung, a tissue rich in macrophages. The mRNA was not detected in muscle, kidney, heart, brain, liver, or thymus and its pattern of expression differed from that of APOBEC1 and APOBEC2. The absence of the mRNA in thymus, which is rich in immature T cells, suggested that the gene is not activated until late in T cell development. Primers that distinguished the full-length and exon V-deleted mRNA detected only the latter in all of the tissues (data not shown). This form was therefore used in the subsequent experiments. Taken together, the findings suggested that APOBEC3G is mainly expressed in lymphocytes and monocytes of the mouse, cell-types that are targeted by retroviruses.

To quantitatively compare the activities of mouse and human APOBEC3G, wild-type, and Δ*vif* reporter viruses were prepared in cells expressing a range of APOBEC3G concentrations (Figure 2). Human APOBEC3G caused a relatively small, 3-fold decrease in infectivity of the wild-type virus and a more pronounced >80-fold decrease in infectivity of the Δ*vif* virus. The mildly inhibitory effect of human APOBEC3G at the highest concentrations on wild-type virus suggested that Vif could become limiting under some conditions. Mouse APOBEC3G reduced the infectivity of wild-type and Δ*vif* viruses to a similar extent over the range of concentrations. The similarity of the responsiveness of the Δ*vif* virus to the mouse and human enzymes confirmed that the two proteins were of similar potency but that the mouse enzyme was resistant to HIV-1 Vif. Immunoblot quantitation of the amount of mouse and human APOBEC3G expressed in the cells demonstrated that the resistance of mouse APOBEC3G to HIV-1 Vif was not due to overexpression of the mouse enzyme (Figure 2). Lower molecular weight bands that appeared to be degradation products of human APOBEC3G were faintly visible on immunoblots on lysates of cells in which Vif had been expressed.

To determine whether mouse APOBEC3G would protect cells from productive HIV-1 replication as a result of its Vif-resistant phenotype, HOS.CD4.X4 cells were prepared that stably produced human or mouse APOBEC3G and then challenged with wild-type and Δ*vif*

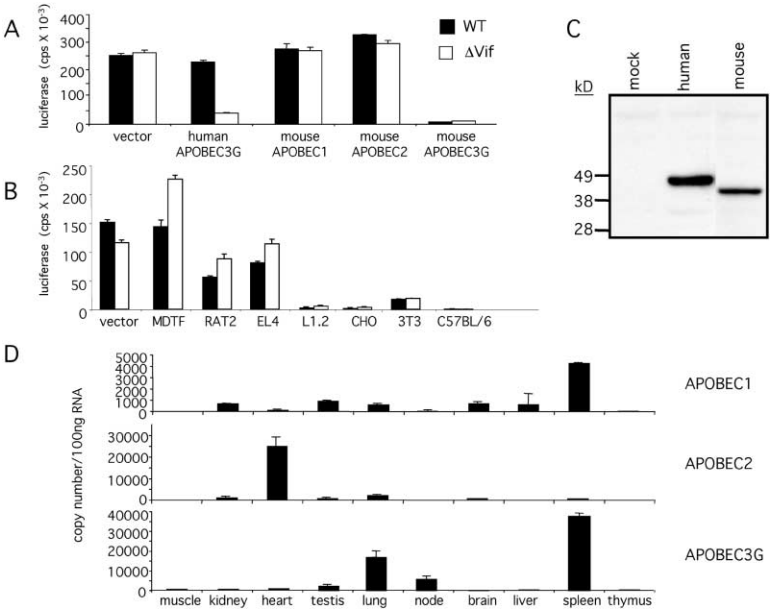

Figure 1. Rodent APOBEC3G Inhibits Wild-Type and Δ*vif* HIV-1 and Is Expressed in Lymphoid Tissues

(A) Wild-type and Δ*vif* HIV-1 luciferase reporter viruses were produced in 293T cells in the presence or absence of human APOBEC3G, or mouse APOBEC1, APOBEC2, or APOBEC3G. cDNAs were derived from C57BL/6 spleen. Infectivity of the viruses on HOS cells was determined three days postinfection by quantitation of intracellular luciferase activity.

(B) WT and Δ*vif* HIV-1 luciferase reporter viruses were produced in the presence of the indicated rodent APOBEC3G.

(C) 293T cells were transfected with human or mouse APOBEC3G-HA expression vector and the protein expressed was detected on immunoblots probed with anti-HA monoclonal antibody (mAb).

(D) APOBEC1, APOBEC2, and APOBEC3G mRNA was quantitated in mouse tissues by real-time PCR. The results are normalized to GAPDH.

NL4-3 HIV-1. Control HOS.CD4.X4 cells were susceptible to both viruses which spread rapidly in the culture and killed the cells in 5–7 days. Six independent HOS.CD4.X4 cell clones that expressed a similar amount of mouse APOBEC3G were tested (Figure 3A). None of the cell lines supported detectable Δ*vif* or wild-type NL4-3 replication (a representative clone is shown in Figure 3B). HOS.CD4.X4 cell clones were found to express variable amounts of human APOBEC3G (Figure 3A). Clones 2 and 4 expressed the largest amount of human APOBEC3G while clones 1, 3, 5, and 6 expressed 2–5 fold less. Measurement of virus replication kinetics showed that clones 1 and 3 were nonpermissive (supported replication of wild-type but not Δ*vif* virus); clones 5 and 6 were semipermissive and, interestingly, clones 2 and 4 were resistant to wild-type and Δ*vif* replication (Figure 3C). Thus, moderate levels of human APOBEC3G can limit wild-type HIV-1 replication, a finding that has potential clinical relevance.

Macaque and AGM APOBEC3G Block Wild-Type HIV-1 Infectivity

HIV-1 does not replicate productively in nonhuman primates with the exception of the chimpanzee. The Vif-

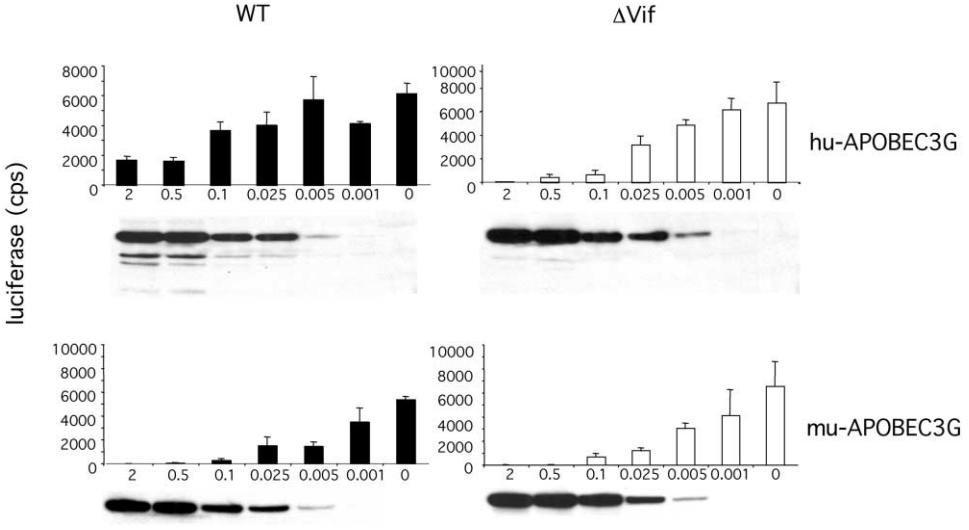

Figure 2. Inhibition by Mouse APOBEC3G Is Vif-Resistant

Wild-type and Δ*vif* luciferase reporter viruses pseudotyped by JR.FL glycoprotein were produced in 293T cells transfected with decreasing amounts of human (hu-) or mouse (mu-) APOBEC3G-HA expression vector. The amount of expression vector plasmid transfected is shown in μg on the X-axis. Infectivity of the viruses was determined on HOS.CD4.R5 cells by intracellular luciferase quantitation. Below each histogram an immunoblot probed with anti-HA mAb of the proteins present in the cell lysates is shown. The lower molecular weight proteins in the upper left panel are putative degradation products of human APOBEC3G.

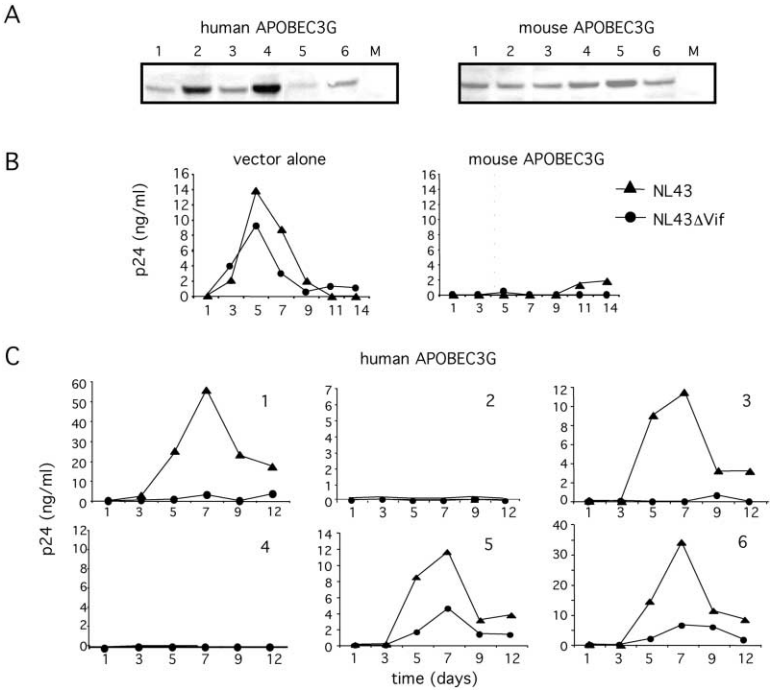

Figure 3. Virus Replication Kinetics in Cells Expressing Mouse or Human APOBEC3G

(A) Human and mouse APOBEC3G expressed by HOS.CD4.X4 cell clones derived by retro viral vector transduction was detected on immunoblots probed with anti-HA mAb. The parental cells (M) lack endogenous APOBEC3G.

(B) HOS.CD4.X4 and mouse APOBEC3G expressing cell lines were infected with wild-type and Δ*vif* NL4-3 HIV-1. Supernatant p24 was measured over two weeks. Representative data for one clone is shown.

(C) Individual cell clones that stably expressed different amounts of human APOBEC3G were infected as in B.

resistant inhibition of HIV-1 by mouse APOBEC3G raised the question of whether this phenomenon could be a more general property of nonhuman APOBEC3G and could account for species-specific restrictions to HIV-1. To determine whether simian APOBEC3G could restrict HIV-1, AGM, rhesus macaque and chimpanzee APOBEC3G were cloned, sequenced, and tested against wild-type and Δ*vif* HIV-1, SIVagm, and SIVmac luciferase reporter viruses. Sequences of primate, human, and mouse APOBEC3G are aligned in Figure 4A. Chimpanzee APOBEC3G was most similar to human, differing in amino acid sequence by 5%. AGM and macaque APOBEC3G were more divergent, differing from human by 23% and 25%, respectively, but were 88% identical to one another. The reporter virus analysis showed that the three primate APOBEC3Gs were active against Δ*vif* HIV-1 (Figure 4B). AGM and macaque APOBEC3G, like the mouse enzyme, were resistant to HIV-1 Vif, blocking wild-type HIV-1. Only the chimpanzee APOBEC3G was sensitive to HIV-1 Vif. Each of the APOBEC3Gs was active against Δ*vif* SIVmac and was sensitive to SIVmac Vif. AGM and macaque APOBEC3G were sensitive to SIVagm Vif. Interestingly, human and chimpanzee APOBEC3G were resistant to AGM Vif. Thus, these enzymes are expected to block SIVagm but not SIVmac infection of humans and chimpanzees.

Mouse APOBEC3G Induces G→A Changes in Minus-Strand cDNA Similar to Those Induced by Human APOBEC3G

Nucleotide sequences of the viral genomic RNA in wild-type and Δ*vif* viruses formed in the presence or absence of human APOBEC3G were determined. Sequences were determined of a 200 bp region that included the primer binding site (PBS) and 5′-LTR of 12 clones from each virus. There was no difference in mutation frequency among the four classes of virions which each differed by 0.01% from the parental NL4-3 (not shown). In contrast, sequences of a 100 bp region 5′ to the PBS of viral cDNA from newly infected cells showed pronounced differences in mutation frequency that were APOBEC3G and Vif-dependent (Figure 5A). Reverse transcripts that were synthesized by wild-type and Δ*vif* virus produced in the absence of APOBEC3G were rarely mutated (0.064%–0.027%). The mutation frequency was slightly higher for wild-type virus produced in the presence of APOBEC3G (0.6%), consistent with incomplete protection by Vif. The mutation rate was higher for Δ*vif* virus produced in the presence of human APOBEC3G (1.23%) and for wild-type (1.53%) and Δ*vif* (0.92%) viruses produced in the presence of mouse APOBEC3G. Two hot spots for mutation by the mouse and human enzymes contained the sequence TGG. Of the nucleotides changed in the presence of human or mouse APOBEC3G, 78% and 84% were G→A, respectively. Such changes are consistent with cytosine deamination to uracil in the viral minus-strand cDNA that is subsequently copied as A in the plus strand.

APOBEC3G Deamination Blocks Integration by Inducing Degradation of Newly Synthesized Reverse Transcripts

We next investigated the effect of cytosine deamination on the fate of the viral reverse transcripts. Three mechanisms could account for the block to replication caused by uracil in the viral cDNA: (1) uracil-containing minus-strand cDNA might be an inefficient template for plus-strand synthesis; (2) full-length cDNA could be generated but then degraded by DNA repair enzymes such as uracil DNA glycosylase and apurinic endonuclease (Krokan et al., 2002); and (3) double-stranded viral cDNA could integrate to form a provirus but then fail to encode

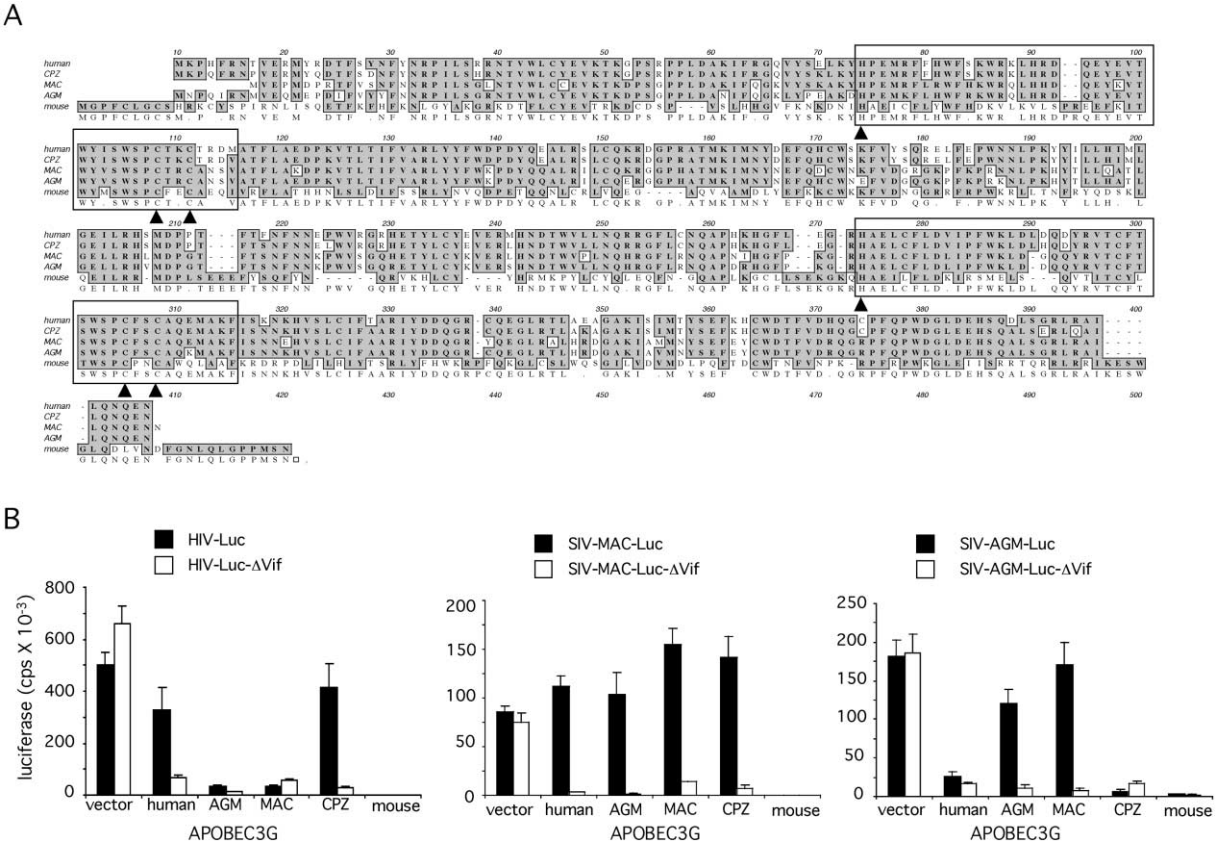

Figure 4. Simian APOBEC3Gs Are Vif-Resistant Inhibitors of HIV-1

(A) Aligned sequences of primate and mouse APOBEC3G are shown. The Zn^{2+} coordination domains are boxed. Arrows indicate conserved cysteine and histidine residues.

(B) Wild-type and Δ*vif* HIV-1, SIVmac, and SIVagm luciferase reporter viruses were prepared as VSV-G pseudotypes in the presence of human, AGM, macaque, chimpanzee, or mouse APOBEC3G. The viruses were produced in 293T cells transfected with equal quantities of reporter virus, APOBEC3G expression vector and pcVSV-G plasmids. Infectivity of the viruses normalized for capsid (CA) was measured on HOS.CD4 cells.

functional viral proteins because of frequent inactivating mutations. To distinguish these possibilities, viral cDNA synthesis was analyzed by quantitative real-time PCR in a single cycle of replication using primers specific for early reverse transcripts, late reverse transcripts, and integrated proviruses (Figure 5B). Early reverse transcripts peaked at 10 hr and then decreased in abundance over time and this was not influenced by APOBEC3G. Late reverse transcripts peaked between 9–12 hr and were similar for each of the viruses until 24 hr at which the Δ*vif*/APOBEC3G virus decreased to undetectable levels. Although degradation of the cDNA occurred for all of the viruses, consistent with Butler et al. (2001), the decrease was most pronounced for the Δ*vif*-defective virus. Quantitation of integrated proviruses revealed a more pronounced defect. The Δ*vif* defective viruses generated about 7-fold fewer proviruses than the active viruses (Figure 5B). Taken together, these data suggested that deamination of the minus-strands by APOBEC3G during reverse transcription does not interfere with completion of cDNA synthesis but that the presence of uracil results in degradation of the molecules prior to integration.

Vif Binds to APOBEC3G Blocking Encapsidation of Human but Not Mouse or AGM APOBEC3G

APOBEC3G is encapsidated in Δ*vif* HIV-1 virions (Sheehy et al., 2002) and upon infection, deaminates newly synthesized viral reverse transcripts. In light of the species-specificity of the Vif:APOBEC3G interaction, we hypothesized that Vif binds to APOBEC3G, preventing the enzyme from causing cytosine deamination. We first tested whether Vif would influence the amount of APOBEC3G encapsidated into virions. Wild-type and Δ*vif* virions produced in the presence or absence of human, mouse, or AGM APOBEC3G were pelleted through sucrose. The amount of encapsidated APOBEC3G was quantitated on immunoblots (Figures 6A and 6B). Human APOBEC3G was detected as an intense band in Δ*vif* virions, but was reduced 140-fold in wild-type virions (Figure 6A). In contrast, mouse and AGM APOBEC3G were present in virions regardless of whether Vif was expressed. In fact, wild-type virions reproducibly contained 2–3-fold more mouse and AGM APOBEC3G than Δ*vif* particles (Figure 6A). In cell lysates, Vif caused a modest 3-fold reduction in human but not mouse or AGM APOBEC3G (Figure 6B).

The most straightforward explanation of these results

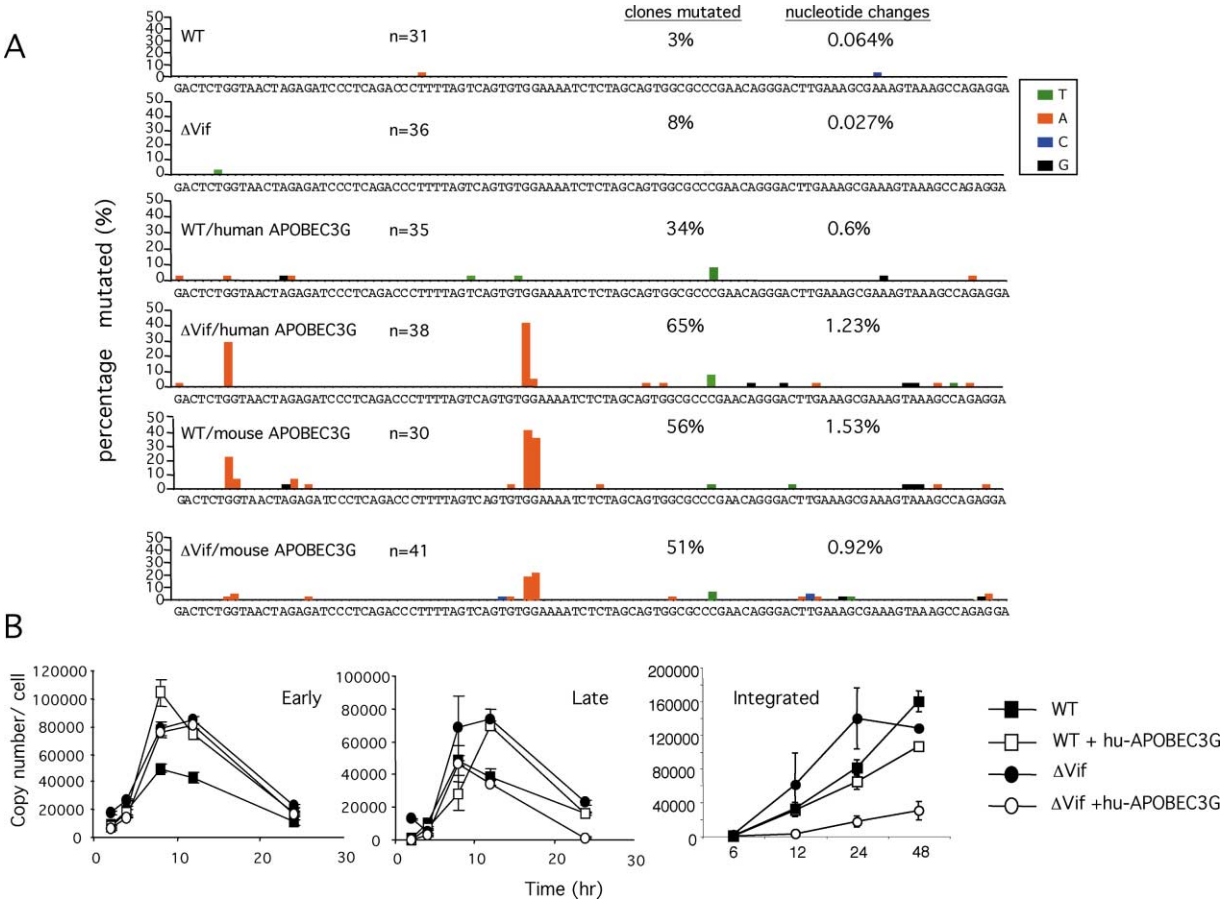

Figure 5. Mouse and Human APOBEC3G Induce Cytosine Deamination of Minus-Strand Reverse Transcripts that Results in Degradation of the Viral DNA

(A) HOS.CD4.X4 cells were infected with DNase-treated virions produced by transfected 293T cells. Cellular DNA was prepared 12 hr postinfection and a 140 base pair sequence containing the PBS, U5, and R was amplified, cloned, and sequenced. The parental NL4-3 sequence is shown below. The height of the bars corresponds to the percentage of clones in which the underlying nucleotide was changed. The color of the bar indicates the nucleotide that was introduced. "n" indicates the number of independent clones sequenced.

(B) HOS cells lacking receptors were infected in triplicate with VSV-G pseudotyped NL4-3 or Δ*vif* NL4-3 produced in transfected 293T cells in the presence or absence of human APOBEC3G. Cultures were lysed at the specified times postinfection and DNA was prepared. Newly synthesized cDNA was measured by quantitative real-time PCR with primers specific for early, late reverse transcripts or integrated proviruses (Butler et al., 2001). The results are representative of four independent repetitions of the experiment.

was that Vif physically bound to APOBEC3G, preventing its encapsidation. To detect a complex containing the two proteins, APOBEC3G-HA was immunoprecipitated from 293T cells cotransfected with wild-type or Δ*vif* NL4-3 and lysed in mild detergent. Coimmunoprecipitated Vif was then detected on immunoblots probed with anti-Vif serum (Figure 6C). This analysis detected a complex of HIV-1 Vif with human but not mouse APOBEC3G.

The detection of faint degradation products of APOBEC3G in Figure 2 and the modest decrease in lysate human APOBEC3G caused by Vif suggested that Vif might exclude human APOBEC3G by causing its rapid degradation. To test this possibility, the half-life of APOBEC3G was measured by pulse-chase metabolic labeling (Figure 6D). Vif was found to cause a 4.6-fold decrease in the amount of APOBEC3G initially synthesized, but did not affect the half-life of the enzyme. Thus, Vif does not primarily exclude APOBEC3G from virions by a degradative mechanism. The effects of Vif on APOBEC3G levels are likely to be secondary effects.

Because MLV does not require Vif for its replication, we expected that it would not encapsidate APOBEC3G. Surprisingly, MLV virions were found to contain large quantities of human, mouse, and AGM APOBEC3G (Figure 6A). In light of this finding, we tested the effect of human and mouse APOBEC3G on MLV infectivity. MDTF and HOS cells were infected with single-cycle, MLV-EGFP at an MOI that would be maximally sensitive to changes in infectivity (Figure 6E). Encapsidated APOBEC3G was detected on immunoblots (Figure 6E, right). Mouse and human APOBEC3G caused a minimal reduction (<2-fold) in the number of $EFGP^{+}$ cells. Thus, MLV is relatively resistant to APOBEC3G, regardless of APOBEC3G in the virions.

Discussion

We report here that the antiviral activity of APOBEC3G is maintained across diverse species in spite of extensive amino acid sequence divergence and regardless of

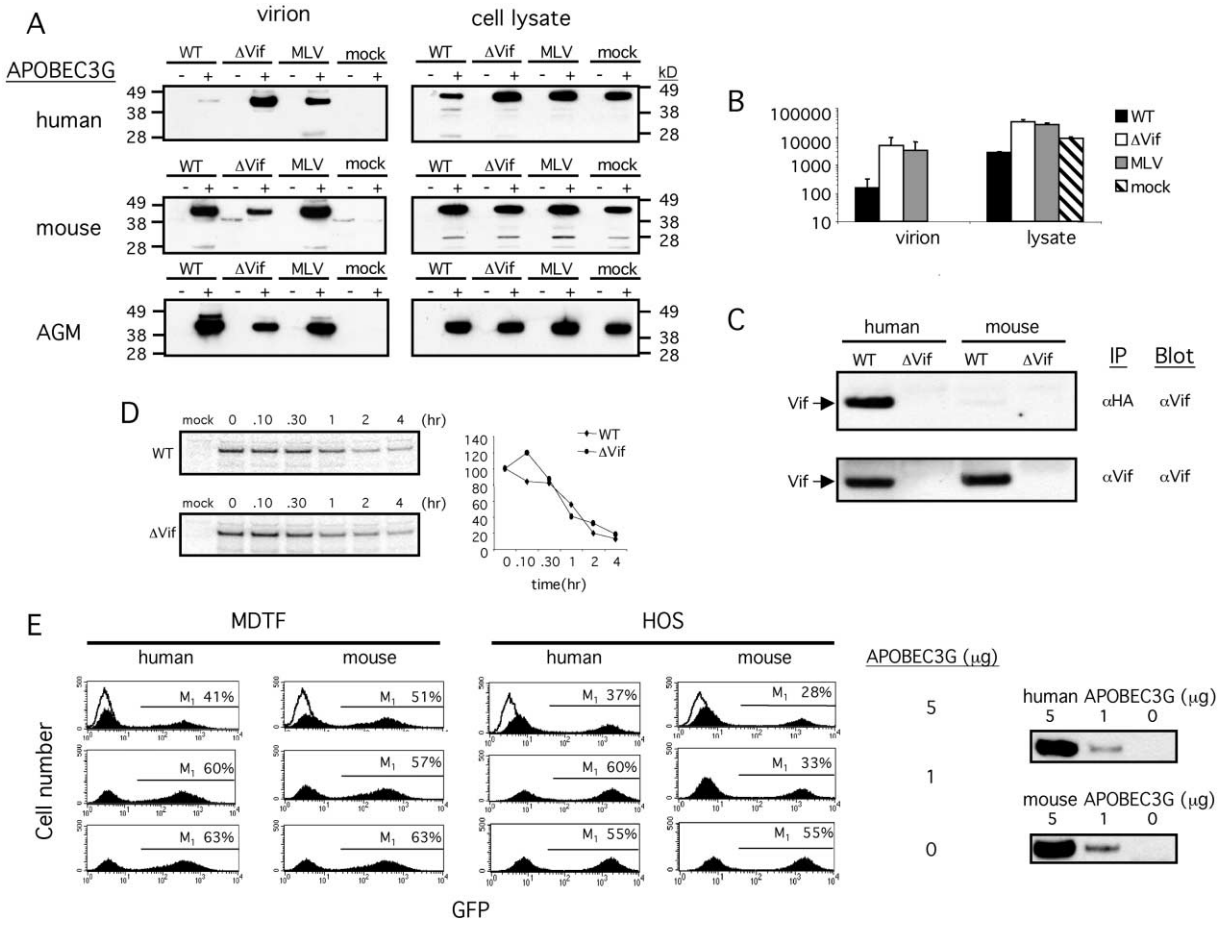

Figure 6. HIV-1 Vif Prevents Virion Encapsidation of Human but Not Mouse or AGM APOBEC3G and Binds Specifically to Human APOBEC3G

(A) Wild-type and Δ*vif* NL4-3 HIV-1 virions and MLV virions prepared in 293T cells cotransfected with the indicated APOBEC3G expression vector were pelleted through 20% sucrose. The virions, normalized for CA, and cell lysates, normalized for protein, were analyzed on immunoblots probed with anti-HA mAb. The MLV virions were prepared by transfection of 293T cells with pMX-EGFP (Onishi et al., 1996), pHIT60, pcVSV-G, and the indicated APOBEC3G expression vector. The results are representative of three independent experiments. Viral DNA was omitted in "mock". The presence (+) or absence (−) of APOBEC3G is indicated above each lane.

(B) A repetition of the experiment in (A) was quantitated and the results for the human APOBEC3G are shown as the average of triplicates.

(C) 293T cells were cotransfected with wild-type and Δ*vif* pNL4-3 and human or mouse APOBEC3G-HA expression vector. Lysates were immunoprecipitated with anti-HA mAb or with anti-Vif. Precipitated Vif was detected on immunoblots probed with anti-Vif serum.

(D) The half-life of human APOBEC3G in the presence or absence of HIV-1 Vif was measured by pulse-chase metabolic labeling. The bands were quantitated and the cpm at t = 0 was normalized to 100 (shown on the right). At t = 0 wild-type was 60,000 cpm and Δ*vif* was 13,000 cpm.

(E) HOS.CD4 and MDTF were infected with MLV-EGFP. The infected cells were quantitated by FACS and the percent $EGFP^+$ cells is indicated in the upper right of each quadrant. The data shown are representative of three independent repetitions of the experiment. Infection with 10-fold more virus resulted in >96% $EGFP^+$ cells and similar results (not shown). An immunoblot to quantitate the APOBEC3G content of the MLV virions is shown at right.

whether lentiviruses infect the species. Mouse, macaque, and AGM APOBEC3Gs were potent inhibitors, not only of Δ*vif* HIV-1, but also of wild-type virus. The nonhomologous APOBEC3Gs, like the human enzyme, were virion encapsidated and induced cytosine deamination to uracil of the viral minus-strand reverse transcripts. Vif efficiently prevented human APOBEC3G from becoming virion-encapsidated but failed to prevent the encapsidation of nonhomologous APOBEC3G, which was found in virions at high levels regardless of Vif. HIV-1 Vif formed a complex with human but not mouse APOBEC3G. Thus, Vif-resistant inhibition is caused by a failure of HIV-1 Vif to interact with nonhomologous APOBEC3G to prevent its encapsidation.

Our findings suggest a model for APOBEC3G and Vif function in HIV-1 replication (Figure 7). In nonpermissive cells infected with Δ*vif* HIV-1, APOBEC3G is packaged into the virions during virus assembly. When APOBEC3G-containing Δ*vif* virions infect a new cell, minus-strand cDNA reverse transcription is initiated and the encapsidated APOBEC3G deaminates cytosines in the newly synthesized DNA. The incorporated uracil does not interfere with further reverse transcription but destabilizes the DNA, causing it to degrade before integration. In a nonpermissive cell infected with wild-type virus, Vif binds to APOBEC3G, blocking its encapsidation, and preventing the subsequent cytosine deamination. In cells that express a nonhomologous APOBEC3G, Vif fails to block

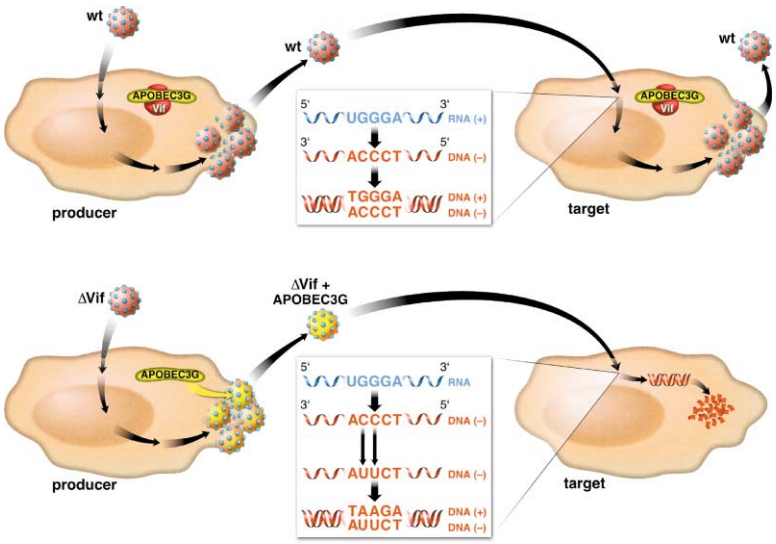

Figure 7. Proposed Model for APOBEC3G and Vif function

In nonpermissive cells infected with Δ*vif* HIV-1, the assembling virion encapsidates APOBEC3G. The encapsidated APOBEC3G does not alter the viral genomic RNA or production of virions. Upon infection of a new cell, minus-strand cytosines are deaminated to uracil at TGG sequences during reverse transcription. The uracil-containing DNA is degraded, causing a block to integration. In cells infected with wild-type virus, Vif binds to APOBEC3G preventing its encapsidation. HIV-1 Vif does not bind tightly with mouse or simian APOBEC3G, and thus fails to prevent their encapsidation.

encapsidation of the deaminase. Minus-strand cytosines are deaminated resulting in uracil-containing reverse transcripts that are poor substrates for integration.

Some features of the model are supported by the data but not fully established. Efficient completion of reverse transcription of the uracil-containing minus-strand cDNA was suggested by the real-time PCR measurements and is consistent with findings of Gaddis et al. (2003). Degradation of the uracil-containing viral DNA prior to integration was supported by the decreased abundance of completed cDNA of Δ*vif* virus at the late time point and by the 7-fold decrease in the number of integrated proviruses. Cellular DNA repair enzymes such as uracil DNA glycosylase and apurinic endonuclease could play a role in degradation of the uracil-containing viral cDNA (Krokan et al., 2002). APOBEC3G did not completely prevent integration of the viral cDNA. However, the proviruses that escaped degradation are likely to be heavily mutated and not competent for further replication. Taken together, the data suggest that there is a small effect on preintegrative DNA, a larger effect on the number of proviruses that integrate and a much larger effect on functional capacity of the integrated viral DNA. G→A changes are expected to introduce frequent translational stop codons in the open reading frames of the viral genes.

The coimmunoprecipitation findings demonstrated that Vif forms a complex with APOBEC3G. However, the mechanism by which Vif prevents APOBEC3G encapsidation is not clear. The presence of low molecular weight APOBEC3G fragments in the presence of Vif and a modest reduction in cellular APOBEC3G were suggestive of Vif-induced degradation. However, pulse-chase analysis showed that Vif did not affect the half-life of human APOBEC3G. Its only effect was a modest (4.6-fold) decrease in APOBEC3G synthesis that could have been caused by Vif binding to nascent APOBEC3G, interfering with the completion of its synthesis. Vif-induced degradation of APOBEC3G, although induced to a small extent, did not appear to be the primary mechanism by which it blocked APOBEC3G encapsidation. One possibility is that Vif prevents APOBEC3G encapsidation by masking a domain on APOBEC3G that mediates the interaction with the assembling virion. Alternatively, Vif could direct APOBEC3G away from sites of virus assembly.

Vif formed a complex with human but not mouse APOBEC3G. This suggested that the species-specificity of Vif:APOBEC3G complex formation accounts for the failure of HIV-1 Vif to exclude nonhomologous APOBEC3G from virions. This specificity probably originated both from evolutionary selection for Vif to interact with the homologous APOBEC3G and the sequence divergence of APOBEC3G in the various species. Interestingly, Δ*vif* virions encapsidated more mouse and AGM APOBEC3G than wild-type virions. Perhaps this is because Vif excludes other, unidentified cellular proteins from virions, allowing for more efficient encapsidation of nonhomologous APOBEC3G.

The predominance of G→A sequence changes in the reverse transcripts was consistent with the recent findings of Lecossier et al. (2003), Harris et al. (2003), Mangeat et al. (2003), and Zhang et al. (2003) and is consistent with preferential deamination of the minus-strand cDNA. The preference for minus-strand degradation could be caused by a requirement to deaminate single-stranded DNA generated by the RNaseH activity of reverse transcriptase. By analogy, AID deaminates cytosine in the single-stranded cellular DNA that forms at R loops in the switch regions adjacent to immunoglobulin isotype genes (Honjo et al., 2002).

It is not clear whether APOBEC3G evolved in mammals to counteract retroviral replication or whether the antiviral activity of APOBEC3G is an inadvertent consequence of its physiological function. Rodents are not known to be infected by lentiviruses and thus were under no selective pressure to evolve an antilentiviral activity or to maintain this activity through evolution, yet their genomes encode an APOBEC3G that blocks lentivirus replication. It is possible that APOBEC3G is incorporated into virions because it has affinity for a virion component or because it localizes to cellular sites of virus assembly. But whether the property of virion encapsidation was fortuitous or whether it was evolutionarily se-

lected for its protective quality, remains to be determined.

Our findings differ significantly from those recently reported by Harris et al. (2003) who found Vif-sensitive inhibition of MLV by human APOBEC3G. The reason for this difference is unclear but could be related to differences in the amount of APOBEC3G encapsidated into the virions or to differences in the packaged RNA genomes used in the two studies. Nevertheless, it is clear that MLV has a means of replicating in the presence of APOBEC3G, which as we demonstrated, is enzymatically active, expressed in mouse leukocytes, and encapsidated into MLV virions. A scan for the APOBEC3G target sequence TGG in MLV shows that these are abundant in the virus genome and have not been removed by evolutionary selective pressure. Thus, MLV has a yet undetermined mechanism of resisting APOBEC3G-mediated deamination.

The species-specificity of the Vif:APOBEC3G interaction probably plays a role in restricting HIV-1 infection to humans. The Vif-resistant inhibitory activity of rhesus macaque and AGM APOBEC3G would prevent replication of HIV-1 in these species. Such restrictions impose an obstacle to the development of animal models for AIDS. Development of an animal model in which HIV-1 infects nonhuman primates or rodents will require removal of the APOBEC3G block. Transgenic rats and rat cells are more susceptible than mice to HIV-1 infection (Keppler et al., 2001, 2002), a finding that may be related to the relatively weak antiviral activity of rat APOBEC3G. In addition, some strains of mice may be more permissive than others to HIV-1 due to differences in the activity of their APOBEC3G.

The finding that a moderate amount of human APOBEC3G also imposes a degree of Vif-resistant inhibition of HIV-1 replication has potential implications for AIDS progression. In vivo, some cell types may express quantities of APOBEC3G sufficient to limit wild-type virus replication. In addition, APOBEC3G, like the related AID, is likely to be regulated by cellular activation signals and these could increase expression to limiting levels. Furthermore, polymorphisms analogous to those of the CCR5 promoter could affect APOBEC3G expression and contribute to differential rates of disease progression. Limiting levels of APOBEC3G in vivo are suggested by previous reports of G→A hypermutation in patient-derived HIV-1 isolates (Borman et al., 1995; Vartanian et al., 1991, 1994, 2002). If this is the case, then therapeutic intervention that either induced APOBEC3G or blocked its interaction with Vif could be clinically beneficial.

Experimental Procedures

Cloning of Rodent, Human, and Primate APOBECs

Rodent and human RNA was prepared from C57BL/6 mouse spleen and thymus, NIH 3T3, *Mus dunni* tail fibroblasts (MDTF), CHO (American Tissue Culture Collection CCL-61) (Puck, 1958), Rat2, EL4 (Ralph and Nakoinz, 1973), L1-2 (Jutila et al., 1994), and CEMSS (Foley et al., 1965; Nara and Fischinger, 1988). Chimpanzee (CPZ, *Pan Troglodytes*), rhesus macaque (MAC, *Macaca mulatta*), and African green monkey (AGM, *Chlorocebus aethiops*) RNA was prepared from PHA-activated PBMC. cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen) primed by oligo-dT (Invitrogen). Rodent (GenBank #BC003314) and human (GenBank #NM_021822) APOBEC3G were amplified with oligonucleotide primers containing EcoRI and XhoI restriction sites. Mouse APOBEC1 and APOBEC2 cDNAs were amplified with primers containing BamHI and XhoI restriction sites. Chimpanzee, Rhesus macaque, and AGM APOBEC3G were amplified with primers containing EcoRI and XhoI sites. Antisense primers encoded the influenza hemagglutinin (HA) sequence YPYDVPDYA. Amplicons were cleaved at the restriction sites and ligated to similarly cleaved pcDNA3.1 (Invitrogen) or pBABE-*neo* (Morgenstern and Land, 1990). See Supplemental Data (available at http://www.cell.com/cgi/content/full/114/1/21/DC1) for oligonucleotide and rodent APOBEC3G cDNA sequences.

HIV-1, SIV, and MLV Plasmid Constructs

Replication-competent Δ*vif* HIV-1 (pNL4-3-Δ*vif*) was generated by introducing stop codons near the 5′ end of *vif* at nt 4472 and nt 4474 by overlapping PCR. A 5′ *vif* fragment was amplified with the primers gag671Xho (5′-CGA CGC TCG AGG CCA GAG GAG ATC TCT C) and 3′Δ*Vif* (5′-CCT TGA AAT ATA CTA TTA TAC TAA TCT TTT CC) and a 3′ fragment was generated with primers 5′Δ*Vif* (5′-GGA AAA GAT TAG TAT AAT AGT ATA TTT CAA GG) and 3′-*vpr* (5′-CGC GGA TCC TCC TGG ATG CTT CCA). The 5′ and 3′ amplicons were mixed, denatured, and amplified with the external primers gag671Xho and 3′-*vpr*. The amplicon was cloned into the SphI (nt 1447) and EcoRI (nt 5743) sites of pNL4-3. To generate Δ*vif* SIVmac239, a 5′ *vif* fragment was amplified from pSIVmac239 with primers F-SIV5330 (5′-GCA TGA ATT TTA AAA GAA GGG GAG G-3′) and R-ΔStop7 (5′-GGG CAC ATA GCA AAC CTT TTG CAA GTT TTA TAT TTC AGA TAT TTT ATG AG) and a 3′ fragment was amplified with the primers F-ΔStop7 (5′-CTC ATA AAA TAT CTG AAA TAT AAA ACT GAC AAA AGG TTT GCT ATG TGC CC) and R-SIV6300 (5′-GCC TTT TGT ATT AAA CAC AAG TAT CTG). The two amplicons were mixed, denatured, and amplified with the external primers F-SIV5330 and R-SIV6300. The amplicon was then cloned into the unique PacI (nt 5386) and BstBI (nt 6132) sites of pSIVmac239. Virus stocks were prepared by transfection of 293T cells.

Single-cycle Δ*vif* HIV-1 luciferase reporter virus pNL-Luc-E$^-$R$^-$-Δ*vif* was generated by T4 polymerase fill-in of the NdeI site (nt 4501) near the 5′ end of *vif* in pNL-Luc-E$^-$R$^-$ (Connor et al., 1995; Deng et al., 1996). SIVmac luciferase reporter virus pSIV-Luc-E$^-$R$^-$Δ*vif* was generated by replacing the *vif*-containing PacI-BstBI fragment of pSIV-HSA with that from pSIVmac239-Δ*vif*. The HSA gene was removed by cleaving with NotI and XhoI and replaced with the firefly luciferase gene. SIVagm luciferase reporter virus was generated by fill-in of the AgeI site (nt 5337) near the 5′ end of *vif* in SIVagm TAN-1 (Soares et al., 1997). The luciferase gene was inserted in-frame with the *nef* initiation codon at the BsiWI site (nt 8814). Virus stocks were produced as JR.FL or VSV-G pseudotypes by cotransfection of 293T using Lipofectamine 2000 (Invitrogen) and were quantitated by p24 or p27 ELISA.

To generate single-cycle MLV encoding EGFP (MLV-EGFP), EGFP was cloned into pMX (Onishi et al., 1996) at the EcoRI-NotI sites. VSV-G pseudotyped MLV-EGFP was prepared by cotransfection of 293T cells with pHIT-60 Gag/Pol expression vector (Soneoka et al., 1995) and pcVSV-G and normalized by quantitation on immunoblots probed with anti-MLV serum.

Real-Time PCR Quantitation of APOBEC3G mRNA

Total cellular RNA was prepared using Triazol (Gibco/BRL) and treated with 20 U RNase-free DNase for 1 hr at 37°. Oligo-dT primed cDNA was synthesized with Superscript II reverse transcriptase using 500 ng RNA template and amplified in an Applied Biosystems Prism 7700. Amplification was monitored by SYBR green fluorescence. Standard curves were derived by serial dilution of pc-mu-APOBEC3G and pcGAPDH. Mouse APOBEC3G was amplified with F-mu-CEM15.309 and R-mu-CEM15.359. RNAs were normalized by amplification of GAPDH with specific primers.

Assays for Vif/APOBEC3G Function and Encapsidation

Reporter viruses were prepared in the presence or absence of APOBEC3G by cotransfection of 293T with 2 μg APOBEC3G expression vector or control empty vector; 2 μg wild-type or Δ*vif* reporter virus plasmid; and 1 μg of pSV-JRFL env or pcVSV-G. Virus-containing supernatant was harvested two days posttransfection and quantitated by p24 or p27 ELISA. HOS.CD4.CCR5 cells (1×10^4)

were infected in 96-well plates in triplicate with 5 ng p24/p27 JR.FL pseudotype or 1 ng p24/p27 VSV-G pseudotype. Luciferase was quantitated three days later using Luc-Lite Plus reagent (Packard) and expressed as the average counts per second (cps). Data are the average of the triplicates, +/− standard deviation. APOBEC3G expression was quantitated on immunoblots probed with anti-HA mAb 16B12 (Covance) and developed with horseradish peroxidase-conjugated sheep antimouse immunoglobulin and ECL reagents (Amersham).

The effect of APOBEC3G on virus replication was determined using cells that stably expressed APOBEC3G. HOS.CD4.X4 were infected with VSV-G pseudotpyed pBABE-*neo* retroviral vector and the cells were selected two days later in medium containing 0.5 mg/ml G418. Drug-resistant clones were isolated and expanded. Cell clones (1×10^5) were infected with wild-type or Δ*vif* NL4-3 at an MOI = 0.2. Supernatant p24 was measured over 14 days.

Encapsidated APOBEC3G was detected by pelleting the supernatant of 293T cells transfected with viral DNA and APOBEC3G expression vector at 35,000 RPM in an SW40.1 rotor for 1.5 hr. P24 in the solubilized virions was quantitated and 100 ng was subjected to immunoblot analysis with anti-HA mAb. Band intensities were quantitated on a Molecular Dynamics Phosphoimager.

Pulse-Chase Metabolic Labeling and Coimmunoprecipitation

293T cells were transfected with wild-type or Δ*vif* pNL4-3 and human pcAPOBEC3G-HA at a 2:1 ratio. Two days posttransfection, the cells were pulse-chase labeled as described previously (Lenburg and Landau, 1993). The cells were starved for 30 min in methionine-cysteine-free medium, labeled for 30 min in medium containing 100 μCi ^{35}S-methionine after which the medium was removed and replaced with complete medium supplemented with unlabeled methionine and cysteine. The cells were harvested and lysed at the indicated intervals and APOBEC3G-HA was immunoprecipitated with anti-HA mAb. The proteins were separated by SDS-PAGE and quantitated on a phosphorimager.

For coimmunoprecipitation, 293T cells were transfected with wild-type or Δ*vif* pNL4-3 and mouse or human APOBEC3G-HA expression vector. Two days later, the cells were lysed in buffer containing 5.0 mM CHAPS/50 mM NaCl/20 mM Tris, [pH 7.5]. The lysates were immunoprecipitated with anti-HA mAb and analyzed on immunoblots with rabbit anti-Vif serum.

Sequencing of Newly Synthesized Viral cDNA

HOS.CD4.X4 cells were infected as in the real-time analysis. A 140 bp fragment 5′ to the PBS (nt 560–700) was amplified with high fidelity polymerase using the U5 forward primer MH531 and reverse primer 46 bp 3′ to the PBS, MH532 (Butler et al., 2001) through 40 cycles (95°, 5 min; 95°, 15 s; 60°, 1 min). Amplicons were agarose gel purified, cloned into pCR-XL-TOPO (Invitrogen), and sequenced using the flanking M13 forward primer.

Real-Time PCR Quantitation of Newly Synthesized HIV-1 cDNA

HOS.CD4.X4 (1×10^6) were infected with DNase-treated viruses derived by 293T transfection. Cultures were lysed and DNA was prepared 0.5 to 24 hr postinfection. Early HIV-1 reverse transcripts were quantitated with primers ert2f (5′-GTG CCC GTC TGT TGT GTG AC) and ert2r (5′-GGC GCC ACT GCT AGA GAT TT) and the probe ERT2 [5′-(FAM)-CTA GAG ATC CCT CAG ACC CTT TTA GTC AGT GTG G-(TAMRA)-3′]. Late reverse transcripts were quantitated with primers MH535, MH532, and the probe LRT-P (Butler et al., 2001). Integrated proviruses were quantitated using MH531 and the AluI repeat primer, MH704. AZT added before infection abolished the signal.

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Accession Numbers

GenBank accession numbers for AGM, chimpanzee, and rhesus macaque APOBEC3G are AY331714, AY331715, and AY331716, respectively.