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Multifaceted counter-APOBEC3G mechanisms employed by HIV-1 Vif

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Abstract

In the absence of HIV-1 Vif protein, the host antiviral deaminase APOBEC3G (A3G) restricts the production of infectious HIV-1 by deamination of dC residues in the negative ssDNA produced by reverse transcription. The Vif protein averts the lethal threat of deamination by precluding the packaging of A3G into assembling virions by mediating proteasomal degradation of A3G. In spite of this robust Vif activity, residual A3G molecules that escape degradation and incorporate into newly assembled virions are potentially deleterious to the virus. We hypothesized that virion-associated Vif inhibits A3G enzymatic activity, and therefore prevents lethal mutagenesis of the newly synthesized viral DNA. Here we show that: (i) Vif-proficient HIV-1 particles released from H9 cells contain A3G with lower specific activity compared with Δ vif virus associated A3G; (ii) Encapsidated HIV-1 Vif inhibits the deamination activity of recombinant A3G, and (iii) Purified HIV-1 Vif protein and the Vif-derived peptide Vif25-39 inhibit A3G activity *in vitro* at nanomolar concentrations in an uncompetitive manner. Our results manifest the potentiality of Vif to control the deamination threat in virions or in the pre-integration complexes (PICs) following entry to target cells. Hence, virion-associated Vif could serve as a last line of defense, protecting the virus against A3G anti-viral activity.

Keywords

Deaminase; peptides; enzyme kinetics; virion; innate immunity

INTRODUCTION

Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) is a member of a cellular cytidine deaminase family encoded within a cluster of seven APOBEC3 (A3) genes, designated A3A, B, C, D-E, F, G and H, on human chromosome 22^{1; 2; 3}. Other members of the cytidine deaminase family include APOBEC1, an mRNA editor in gastrointestinal cells important in the metabolism of Apolipoprotein B¹, activation-induced deaminase (AID), a lymphoid-specific DNA deaminase involved in B cell maturation and antibody diversification^{4; 5} and APOBEC2, whose expression is restricted to heart and skeletal muscle⁶ and is important to normal muscle development and function^{7; 8}.

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A3 proteins catalyze the deamination of cytidine through a catalytic domain containing the conserved zinc-coordinating motif (H/C)XE(X)₂₃₋₂₈CXXC^{1; 9}. A3G, as well as A3B and A3F, contains two zinc-coordinating motifs which contribute unequally to the biological functions of this enzyme^{10; 11; 12; 13; 14; 15; 16; 17}. APOBEC proteins are involved in the restriction of endogenous retro-transposition, as they inhibit the transposition of murine IAP and MusD retro-elements in HeLa cells^{18; 19}, Ty1 retro-elements in yeast²⁰ and L1-dependent retro-transposition of Alu and small hY elements in human cells^{21; 22}.

In the absence of Vif, A3G provides the host with intrinsic immunity against infection through lethal editing of nascent viral reverse transcripts. This A3G activity takes place inside the virion or post-entry to target cells and involves the deamination of deoxy-cytidine residues on the newly synthesized DNA minus strand during viral reverse transcription^{23; 24; 25; 26}. The induced dC→dU hypermutation occurs preferentially in the 5' (T/C)CC 3' sequence context^{24; 26; 27; 28; 29; 30} and consequently transcribes into dG→dA substitutions on the DNA plus strand. The hypermutated viral DNA is either subjected to degradation due to reduced stability, inability to complete reverse transcription and integration, or further negates HIV-1 replication due to altered viral open reading frames and inappropriate translation termination codons^{23; 24; 26; 27}.

HIV-1 and other lentiviruses overcome the peril of deamination by precluding the packaging of A3G into assembling virions. This is mostly carried out by Vif, which mediates the proteasomal degradation of A3G through coordinative interaction with Cullin5-ElonginB-ElonginC that forms an E3 ubiquitin ligase complex^{27; 31; 32; 33; 34}. Albeit the robust potential of Vif to limit A3G encapsidation, we demonstrated that purified wild-type virions produced in restrictive H9 cells contain A3G molecules with deaminase activity³⁵. Based on the deamination activity, we calculated that wt particles contain 12-20 fold lower than the amounts associated with Δvif viruses, averaging to 0.3-0.8 A3G molecules per wt virion. Accumulating data show that patient-derived proviral DNA sequences often contain G- to A mutations within the A3G 5'-GG-to-AG dinucleotide contexts^{36; 37; 38; 39}, providing indirect evidence that A3G mutates wt HIV-1 *in vivo*. Deletion, insertion and stop-codon mutations were identified in gene sequences of HIV-1 derived from slow progressor AIDS patients⁴⁰ and in humanized mice⁴¹. These observations were corroborated experimentally by demonstrating that cellular deaminases are responsible for lethal and sub-lethal mutations intensively inserted into Vif-proficient and deficient HIV-1 viruses produced in cultured cells and humanized mice^{41; 42; 43}. Others and we therefore speculated that the encapsidated A3G may not only inactivate the viral DNA, but might also contribute to viral diversity and fitness^{41; 44; 45; 46}.

Vif is naturally incorporated into virions^{47; 48}, but the biological significance of this observation is not fully understood. Previously it was demonstrated that Vif inhibits the deaminase activity of A3G and AID in bacteria^{49; 50}. We were therefore interested to determine whether the encapsidated Vif plays a role in the regulation of the virion-associated A3G. Here we report that the specific activity of A3G deaminase associated with *vif*-proficient viruses is lower than that of the enzyme entrapped in *vif*-deficient particles, suggesting that Vif inhibits virion-associated A3G. Encapsidated Vif also inhibits the enzymatic activity of recombinant A3G, further supporting this notion. Purified HIV-1Vif inhibited A3G mediated deamination *in vitro* in an uncompetitive manner with a K'_i of 8.7×10^{-9} M. Screening a battery of Vif-derived peptides covering the full-length Vif protein has revealed that inhibitory sequences are scattered throughout the Vif protein. However, two sequences were implicated in efficient inhibition of A3G deaminase activity, corresponding to Vif25-39 and Vif105-119. Our data show that HIV-1-encapsidated and recombinant Vif molecules directly inhibit the enzymatic activity of A3G, suggesting a

complementary mechanism for A3G neutralization by Vif inside progeny virions or in the pre-integration complex (PIC) formed post-entry to target cells.

RESULTS

1. Native HIV-1-associated A3G has reduced specific activity

In the absence of Vif, A3G deaminates dC residues in the viral negative-strand DNA synthesized by reverse transcription post-entry to the target cell. As Vif is packaged in the virion^{47; 48}, we were interested to reveal whether it directly inhibits A3G enzymatic activity. Figure 1A shows that wt virions released from H9 cells contain reduced amounts of A3G molecules compared to that associated with Δvif particles. Quantification of the bands revealed that the wt particles encapsidated 5.8 times lower (approx. 17%) A3G protein than in Δvif particles (Table I). Figure 1B shows that wt particles released from H9 cells contain reduced amounts of Vif compared to particles released from the permissive Sup T1 cells, which do not express A3G.

The deaminase activity associated with the wt and Vif-deficient viruses released from H9 cells correlates to the amounts of A3G molecules entrapped in the particles (Fig. 1C). These results are well correlated with our published data³⁵. However, calculation of the efficacy of the A3G enzymes packed in the wt and Δvif particles revealed that the deaminase activity of A3G in the wt particles is significantly lower than expected (Table II) and is only 6.42% of the deaminase activity in Δvif particles. Normalizing the virion-associated deaminase activity to A3G protein content indicates that the specific activity of wt HIV-1-associated A3G enzyme is 36.5% of the enzyme associated with the Δvif particles ($6.42/17.58 \times 100$, see Tables I and II). A plausible explanation of these results is that Vif molecules associated with the HIV-1 wt particles inhibit the intrinsic deamination activity of A3G.

2. Inhibition of A3G deaminase activity by HIV-1 Vif

To determine whether Vif molecules associated with HIV-1 particles inhibit A3G activity, we performed exogenous reactions using purified recombinant A3G. A3G alone deaminated 20% of the dC in the oligonucleotide substrate. Adding particles released from wt HIV-1 infected Sup T1 cells, which contain Vif (see Fig. 1B) decreased A3G activity, while adding the same amount of Δvif particles to the reaction had no effect, or even slightly amplified the deaminase activity, probably because of increased concentration of proteins in the mixture (Fig. 2A). These results indicate that Vif molecules associated with HIV-1 are able to inhibit A3G activity *in vitro*.

Next, we performed in-depth biochemical analysis of Vif-mediated inhibition of A3G enzymatic activity. To this end we expressed and purified recombinant Vif protein and examined A3G activity in the presence of Vif. The purification of recombinant His-tagged Vif from bacteria yielded an over 90% purified protein, as revealed by SDS-PAGE analysis and verified by Western blotting (not shown). Purified A3G was incubated with increasing amounts of purified Vif and the effect on A3G-mediated deamination levels of the ss-deoxyoligonucleotide substrate was measured. Figure 2B clearly shows that Vif inhibits A3G deaminase activity in a dose-dependent manner (lanes 5-10). An equal amount of an elution fraction from non Vif-expressing bacteria (lane 4) or 10 μ M BSA (lane 3) used as controls did not inhibit A3G activity. The inhibitory effect exerted by Vif was observed at Vif concentrations ranging down to 10 nM.

In order to ensure that the Vif/A3G interaction is essential for efficient inhibition of A3G enzymatic activity, we compared Vif mediated dose-dependent inhibition of wt A3G and A3G D128K mutant, which is abortive for Vif binding^{49; 51; 52; 53; 54}. A3G D128K deaminase activity was only moderately inhibited in all Vif concentrations used (ranging

from 0.5 μM to 2 μM), indicating that binding of Vif to A3G is essential for A3G inhibition (Figure 2C).

3. Identifying the Vif-derived domains which inhibit A3G deaminase

In order to identify the inhibitory domains in Vif, a battery of 15-mer 46 Vif-derived peptides (Table III) with 11aa overlaps covering the full-length protein was screened for the inhibition of A3G-mediated deamination. Peptides corresponding to sequences throughout the Vif protein exerted a vast inhibitory effect at a concentration of 100 μM (not shown). At a lower concentration of 10 μM , a distinct inhibitory pattern was observed by peptide sequences corresponding to Vif N-terminus (1-51aa) and to a central region (101-127aa), previously characterized as a novel zinc-binding domain^{30; 55} (Fig. 3A). Specifically, six peptides inhibited the A3G deaminase activity at a lower concentration of 1 μM , mapping the inhibitory sequences to Vif9-23, Vif25-39 and Vif37-51 at the N-terminal region, and Vif101-115, Vif105-119 and Vif113-127 at the central region (Fig. 3B). These peptides were further analyzed for the inhibition of A3G activity at lower concentrations ranging down to 40 nM. As shown in Fig. 3C, the peptides Vif25-39 and especially Vif105-119, significantly reduced the A3G activity at 1 μM and 0.2 μM with an IC_{50} of approximately 0.6 μM and 0.1 μM , respectively. To exclude the possibility that the Vif-derived peptides decrease overall PCR efficiency, these experiments were verified by real-time PCR assays that showed no differential amplification rate of A3G reaction products with or without the Vif-derived peptides (data not shown).

4. Determining the mode of Vif-mediated inhibition of A3G enzyme

Next, we determined the mode of inhibition employed by Vif and the Vif-derived peptides corresponding to residues 25-39 and 105-119. A3G initial deamination rates were measured in the presence of 10 and 20 nM Vif (Fig. 4A), 0.1 and 0.2 μM Vif105-119 (Fig. 4B) or 0.5 and 1 μM Vif25-39 (Fig. 4C). The double-reciprocal plot of A3G inhibition by Vif and Vif25-39 reveals an uncompetitive inhibition mode, whereas Vif105-119 inhibits A3G in a mixed mode. Accordingly, the K'_I values of Vif and Vif25-39 are approximately $8.7 \cdot 10^{-9}$ M and $2.5 \cdot 10^{-7}$ M, respectively. The K'_I and K_I values of Vif105-119 are approximately $9.2 \cdot 10^{-8}$ M and $6.8 \cdot 10^{-8}$ M, respectively.

DISCUSSION

Santa-Marta and colleagues^{49; 50} demonstrated that HIV-1 Vif inhibits A3G-mediated cytidine deamination without mediating protein degradation in bacteria. We therefore hypothesize that Vif molecules incorporated into newly assembled virions produced by restrictive cells directly inhibit the enzymatic activity of A3G during viral reverse-transcription in target cells. The present report provides evidence manifesting the potentiality of Vif as a deaminase inhibitor acting at a post entry phase in target cells.

The biological significance of Vif-activity *in vivo* has been implied before. Mehle and colleagues³⁴ reported that mutations of the conserved phosphorylation sites T96 and S144 in Vif impair viral replication without affecting the ability of these mutants to induce A3G degradation, suggesting that Vif plays additional role(s) beside inducing proteasomal degradation of A3G. Furthermore, the production of infectious HIV-1 is not dependent on the depletion of A3G from virus-producing HeLa cells^{56; 57}. In fact, wild-type virions produced in A3G-expressing 293T cells⁴³ or even in restrictive Hut78 cells⁵⁸ contain A3G protein despite Vif expression. Interaction between Vif and A3G molecules in virions was recently demonstrated by Yamashita and his co-workers⁵⁹. These findings are in agreement with our own data showing that infective HIV-1 particles produced in 293T cells expressing exogenous A3G, and in H9 cells expressing endogenous A3G, contain measurable levels of

A3G³⁵. Other groups, however, reported undetectable levels of A3G in HIV-1 virions^{32; 33; 60; 61}. Finally, it was shown that reduced Vif incorporation into virions produced by chronically infected cells decreases its counteracting effects on A3G-mediated deamination²⁶. The reduced levels of Vif in these virions, however, are sufficient for maintaining viral infectivity⁴⁸.

Vif-proficient particles released from H9 cells contain 0.3 to 0.8 molecules of A3G per virion³⁵, while the exact number of virus-associated Vif molecules remains to be determined^{47; 62; 63; 64}. However, it could be larger than the number of A3G in the virion.

It was previously shown that virion encapsidation of a ubiquitination-resistant A3G mutant⁶⁵, Vif-binding-deficient D128K A3G^{51; 52; 53; 54} or A3G fused to virion-targeting polypeptides derived from HIV-1 Vpr or Nef^{66; 67}, caused efficient antiviral effect despite the presence of Vif. It is plausible, however, that high stoichiometry of virion-associated A3G in these systems, which is well above the physiological levels, averted the Vif-mediated inhibition observed with endogenous A3G virion incorporation (Figure 2A).

By using a cell free system we showed that purified Vif inhibits A3G-mediated deamination of an ss-oligonucleotide substrate at sub-micromolar concentrations, with an IC₅₀ of approximately 20 nM (Fig. 2). Moreover, Vif inhibits A3G-deamination in an uncompetitive manner (Fig. 4), suggesting that Vif affects the substrate-bound enzyme. As purified A3G and HIV-1 Vif proteins were used in these assays, we conclude that Vif directly inhibits the deamination activity mediated by A3G and no other molecules are required for this inhibition. Using an array of 15-mer synthetic Vif-derived peptides, we mapped the observed inhibition to two sequences corresponding to Vif25-39 and Vif105-119 (Fig. 3). Although large molar excess of Vif was required for A3G inhibition *in vitro*, it does not exclude the possibility that Vif plays a similar physiological role in virions and/or PICs at a post entry phase, because: (i) proteins purified from bacterial cells could have a much lower specific activity than proteins isolated from mammalian cells³⁵, and (ii) Vif is a structurally disordered protein difficult to maintain in solution^{68; 69; 70}. It can be argued that the observed inhibition of A3G is due to binding of Vif to the DNA substrate rather than to the enzyme⁷¹. Our observation that Vif does not efficiently inhibit the A3G D128K deaminase activity supports the notion that the A3G/Vif interaction is essential for inhibition of A3G deamination⁴⁹. However, since Vif still inhibits 50% of the mutated A3G activity, we cannot exclude the possibility that under these conditions Vif binds the DNA substrate, or alternatively that Vif binds A3G in additional sites, inducing partial inhibition.

The most highly conserved sequences in Vif molecules of HIV-1, HIV-2 and SIV_{MAC} are residues 21-30, 103-115 and 142-150⁷². It was demonstrated that (i) deletions of these sequences reduced viral infectivity⁷³; (ii) substitutions of residues 38W₄₀F, 105Q₁₀₇L, 111Y₁₁₂F or C114 to Ala led to a 93%-98% reduction in the relative viral infectivity, rendering these residues indispensable for viral replication⁷⁴; (iii) the Vif W38A mutant produced a Δ vif phenotype and T32Q mutation specifically affecting A3G-Vif interaction⁷⁵; (iv) complementation assays showed that the H108D/N and C114S mutations in Vif severely impaired Vif function without abolishing its incorporation into virions^{47; 55}, and (v) substitution of lysine residues for arginine showed that of the 16 lysine residues in Vif, only K22 or K26 are required for the productive infectious HIV-1⁷⁶. The Vif105-119 sequence resides within a characterized novel zinc-binding domain that encompasses the conserved sequence 108H-x₅-C-x₁₇₋₁₈-C-x₃₋₅-H139^{30; 55; 77}. This domain presumably coordinates one zinc ion by forming a symmetric loop using the four HCCH residues and is important for the interaction with Cullin5-E3 ligase. Comprised of a sequence containing the upstream HC residues of the loop, it is possible that the Vif105-119 peptide causes steric interference within A3G catalytic zinc-coordinating domains. Accordingly, it is possible that

Vif uses its zinc-binding domain, or a part of it, to hinder the spatial structure of A3G catalytic domains. The Vif105-119 peptide inhibited A3G in a mixed mode, indicating that it binds either to the free or substrate-bound enzyme. On the other hand, the Vif25-39 peptide inhibited A3G deamination in an uncompetitive mode similar to the Vif protein, indicating that they bind directly to the enzyme–substrate complex. The mechanisms facilitating the inhibition of A3G-mediated deamination by Vif and its correlation to the above loss-of-function mutations, as well as the susceptibility of different N' or C'-terminal active site A3G mutants to Vif-mediated inhibition, require further investigation.

As few as 1-2 A3G molecules incorporated into virion could be detrimental to HIV-1 infectivity⁷⁸, highlighting the importance of the putative Vif role in directly inhibiting virion-associated A3G. Importantly, this interplay between A3G mediated lethal hypermutation and the inhibitory effect of Vif during the replication of the viral genome could potentially contribute to the genetic diversity of HIV-1 populations. The *in vitro* experiments presented here demonstrate the potentiality of Vif to control the deamination in the virion or in PICs, pointing at this Vif activity as the last line of defense. However, demonstrating this viral Vif function as crucial toward production of infectious virus would sustain the view of an A3G anti-viral mechanism that obliges its deaminase activity. Studying the existence of such a mechanism and estimating its significance to viral distribution *in vivo* is currently under way in our laboratory. These studies may yield new prospects for violating the suppression of A3G antiviral function by Vif.

MATERIALS AND METHODS

Cells

Human T lymphoblastoid H9 and SupT1 cell lines, provided by the NIH AIDS Reagent Program (Division of AIDS, NIAID, NIH, USA), were grown in RPMI 1640 medium (Biological Industries, Beit Haemek, Israel). The adherent 293T human embryonic kidney (HEK) cell line was kindly supplied by Dr. E. Bacharach (Tel Aviv University, Israel) and the TZM-bl cell line (contributed by Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.) was obtained through the NIH AIDS Reagent Program. The adherent cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM) as a sub confluent monolayer. RPMI 1640 and DMEM media were supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 U/ml streptomycin and 2 mM L-glutamine (Biological Industries, Beit Haemek, Israel).

Preparation of virus stocks

Wild type HIV-1 and HIV-1 Δ vif were generated by transfection of 293T cells with pSVC21 plasmid containing full length HIV-1_{HXB2} or Δ vif viral DNA^{79; 80}. Viruses were harvested 48 and 72 h post transfection and stored at – 80 °C until infection of cultured H9 and SupT1 cells.

Infection of cultured cells

Cultured human T lymphoblastoid H9 and SupT1 cells (5×10^6) were centrifuged for 5 min at 500 g, the supernatant was aspirated and cells were re-suspended in 30 μ l of medium containing wt or HIV-1 Δ vif. Sup T1 cells were infected with wt or Vif-deficient virus at MOI 0.02, whereas H9 cells were infected with wt virus at MOI 0.02, or with Vif-deficient virus at MOI 1. Cells were infected by spinoculation at 1200 rpm for 100 min at room temperature. Following infection, cells were re-suspended in fresh RPMI medium supplied with 10% FCS, and incubated for additional 5-7 days at 37°C. Culture media were harvested daily starting at 5 d.p.i. and viruses were tittered by HIV-1 p24 antigen capture test. The harvests were further centrifuged to remove cells and cell debris (10,000 g for 10 min) and

concentrated by ultracentrifuge for 1.5 h at 100,000 *g* through a 20% sucrose cushion. Pelleted viruses were re-suspended in small volume of phosphate buffered saline (PBS) and stored at -80°C until use.

HIV-1 titration

HIV-1 was titered by the MAGI assay, as described by Kimpton and Emerman ⁸¹.

Quantification of p24

HIV-1 p24 antigen capture assay kit (SAIC, AIDS Vaccine Program, Frederic, MD) was used to determine the amounts of p24 in the culture medium, according to the standards and instructions supplied by the manufacturers.

Expression and purification of A3G

A3G and A3G D128K containing a C-terminal His₆ tag were expressed in 293T cells and purified as previously described³⁵. Briefly, 293T cells were transfected with pcDNA-APO3G ³² or pcDNA-APO3G D128K ⁵⁴. Cells (3×10^8) were harvested 48 h after transfection, washed three times in PBS and suspended in lysis buffer (50 mM Tris, pH 8.0, 1 mM PMSF (Sigma-Aldrich), 10% (v/v) glycerol and 0.8% (v/v) NP-40), to a final concentration of 20,000 cells/ μl . Following 10 min incubation in ice, cell debris and nuclei were pelleted by centrifugation at 10,000 *g* for 20 min. The soluble fraction was adjusted to 0.8 M NaCl and treated with 50 $\mu\text{g}/\text{ml}$ RNase A (Sigma-Aldrich) for 30 min at 37°C . Treated lysates were then added to 50 μl of nickel–nitrilotriacetic acid (Ni-NTA) agarose beads (QIAGEN), mixed on an end-over-end shaker for 1 h at 4°C and loaded onto a standard chromatography column (Bio-Rad Laboratories). Following extensive washing with wash buffer (50 mM Tris, pH 8.0, 0.3 M NaCl, 10% (v/v) glycerol) containing 30–50 mM imidazole, bound proteins were eluted seven times in wash buffer containing 120 mM imidazole. Protein samples were resolved by SDS-PAGE and stained with Imperial protein stain (Pierce Biotechnology). A3G concentration and purity was assessed by densitometry and scanning of stained gels, comparing band-intensity to that of a predetermined protein marker, and by a Bradford assay.

Expression and purification of Vif

The pD10-Vif-His₆ plasmid ⁸⁰, kindly provided by Dr. Dana Gabuzda (Dana-Farber Cancer Institute, Boston, MA), was used to express an N-terminal His₆ tagged HIV-1_{HXBII} Vif protein in *E. coli* MC-1061. Vif was purified as previously described ⁸⁰, with the following exceptions: after induction of Vif expression with 0.5 mM IPTG (Sigma-Aldrich) for 1 h at 37°C , bacteria were pelleted at 4,000 *g* for 15 min, washed with PBS and suspended in lysis buffer containing 50 mM phosphate buffer (pH 8.0), 0.3 M NaCl, 25 $\mu\text{g}/\text{ml}$ DNase, 1 mM PMSF (Sigma-Aldrich), 5 mM imidazole and 0.8% NP-40. Following sonication, insoluble cell debris and inclusion bodies were removed by centrifugation at 10,000 *g* for 20 min and the soluble fraction was subjected to Ni²⁺ affinity chromatography. Briefly, 4 ml of the sample corresponding to 200 ml bacterial culture were incubated with 1 ml 50% Ni-NTA slurry for 1 h at 4°C . Following extensive washing in wash buffer [50 mM phosphate buffer (pH 8.0), 0.3 M NaCl] containing 10–40 mM imidazole, Vif was eluted in the same buffer containing 110 mM imidazole. The eluted sample was dialyzed against A3G reaction buffer for 6 h at 4°C . The concentration and purity of the Vif preparation were assessed as described above for A3G.

Western blot

Transfected or infected cells were harvested, washed once in PBS, re-suspended in SDS-gel loading buffer and boiled for 10 min. Samples of 5×10^4 cells were analyzed by SDS - 12%

polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer of the proteins to polyvinylidene fluoride (PVDF) membrane. HIV-1 proteins were detected by using rabbit anti-Vif and monoclonal anti-Ca-p24 antibodies kindly provided by Dr. D. Gabuzda⁷³ and Dr. B. Chesebro⁸², respectively. A3G protein was identified by rabbit polyclonal anti-A3G provided by Dr. J. Lingappa through the NIH AIDS Research and Reference Reagent Program.

Deamination assay

Deaminase activity of purified enzyme and virion-associated A3G protein was examined as previously described³⁵. Briefly, A3G deamination reactions were performed in a total volume of 10 µl in 25 mM Tris, pH 7.0, and 0.01-1 fmol single-stranded (ss) deoxyoligonucleotide substrate (Integrated DNA Technologies) at 37°C. The reaction was terminated by heating to 95°C for 5 min. One µl of the reaction mixture was used for PCR amplification performed in 20 µl buffer S (Pqlab), using the following program: 1 cycle at 95°C for 3 min, followed by 30 cycles of annealing at 61°C for 30 s and denaturing at 94°C for 30 s. Aliquots of the PCR products (10 µl) were incubated with Eco147I restriction enzyme (Fermentas) for 1 h at 37°C. Completion of the restriction reaction was verified by using positive-control substrate containing CCU instead of CCC. Restriction-reaction products were loaded onto 14% gels and separated by PAGE. Gels were stained with SYBR gold nucleic acid stain (Molecular Probes, Invitrogen) diluted 1:10,000 in 0.5 × Tris-Borate-EDTA buffer (TBE, pH 7.8), visualized by UV light (312 nm), captured by an Olympus C-5050 charge-coupled device (CCD) camera and analyzed by optical density (OD) scan using the TINA2.0 densitometry software (Raytest).

Assessment of A3G entrapped in virions was carried out with concentrated viruses (stocks of 3-5 pg of p24/µl) suspended in PBS containing Triton X-100 at final concentration of 0.1% (v/v) and 50 µg/ml RNase A (Sigma-Aldrich). Deamination reactions were incubated for 1 h at 37°C.

Synthetic oligonucleotides and peptides

The sequence of the 80-mer ss-deoxyoligonucleotide substrate used in the deamination assays is:

5'GGATTGGTTGGTTATTTGTTTAAGGAAGGTGGATTAAAGGCCCAATAAGGTGATGG AAGTTATGTTTGGTAGATTGATGG 3' (A3G target site is underlined and the preferentially deaminated dC residue is bold-face-type). The positive control ss-deoxyoligonucleotide bears the same sequence, but has a dU instead of the target dC residue. The following primers were used for PCR amplification of the substrate and positive control oligonucleotides:

Forward 5' GGATTGGTTGGTTATTTGTTTAAGGA3';

Reverse 5' CCATCAATCT ACCAAACATAACTTCCA 3'.

Vif-derived peptides were obtained through the NIH AIDS Research and Reference Reagent Program as lyophilized powder and dissolved in water. Table 1 shows the amino acid sequences of the Vif-derived peptides.

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ABBREVIATIONS

ss	single-stranded
wt	wild type
IC50	the half maximal inhibitory concentration
PBS	phosphate buffered saline
FCS	foetal calf serum
MAGI	multinuclear activation of a galactosidase indicator
d.p.i.	days post infection
v/v	volume per volume
PMSF	phenylmethylsulfonyl fluoride
Ni-NTA	nickel–nitrilotriacetic acid
IPTG	isopropyl-1- β -D-galactopyranoside
SDS-PAGE	sodium dodecyl sulfate – polyacryl amide gel electrophoresis
PVDF	polyvinylidene fluoride
TBE	Tris-Borate-EDTA
OD	optical density
nt	nucleotide
RT	room temperature

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Fig. 1. HIV-1 produced by H9 cells incorporates enzymatically active A3G protein

A. Western blots of wild-type or HIV-1 Δvif viruses produced by H9 cells. Equal amounts of viral proteins, (20 ng of p24, as measured by p24 antigen capture test) were loaded onto each slot of SDS-PAGE. Endogenous A3G and viral p24 CA proteins (upper and lower panels, respectively) were detected by using specific antibodies. Virus harvested from 293T cells transiently co-transfected with HIV-1 Δvif DNA and pcDNA3-A3G-MycHis was used as positive control (PC). **B.** 30 ng of p24 of HIV-1 wt and Δvif virions produced by H9 and SupT1 cells were loaded into the slots of SDS-gels. The presence of A3G and Vif proteins in those virus preparations was determined by using polyclonal anti-A3G and anti-Vif antibodies. **C.** Deamination of synthetic ss-deoxynucleotide substrate by virus-associated A3G. Equal amounts of HIV-1 wt and Δvif viruses (1.25 ng of p24) were added to the reactions containing increasing amounts of the substrate (ranging from 0.01 to 0.2 fmol), as indicated. S, an 80 nt-long substrate used for the deamination assay; P, a 40 nt-long product of the restriction reaction.

Fig. 2. Inhibition of A3G activity by Vif

A. Recombinant A3G protein (rec. A3G) (0.75 fmol) was mixed with wt HIV-1 or Δ vif virus from SupT1 cells (2.5 ng of p24 per reaction). Reactions were carried out on 1 fmol of A3G oligonucleotide substrate. As negative controls, deamination reactions were loaded with viruses from SupT1 (no A3G). All reactions contained 2.5 ng of p24 (as measured by p24 CA antigen capture test). **B.** Purified A3G (0.35 fmol) was incubated with the ss-deoxy-oligonucleotide substrate in the presence of purified Vif for 15 min. Lane 1, positive control (dU containing oligonucleotide); Lane 2, negative control (no A3G); Lane 3, sample containing 10 μ M BSA; Lane 4, sample containing the elution fraction of Ni-NTA purification from non Vif-expressing bacteria (amount equal to lane 10); Lanes 5-10, dose-dependent inhibition of A3G deamination by increasing Vif concentrations, as indicated. A graphic representation of the Vif-mediated inhibition is shown on the bottom. Values represent the average of triplicates; SD values were less than ± 0.8 . **C.** Purified wt and D128K A3G enzymes (0.2 fmol) were incubated with purified Vif protein at indicated concentrations for 10 min at RT, followed by addition of 10 fmol ss-deoxy-oligonucleotide substrate for 30 min. The plot presents the relative deaminase activity, while 100 percent of activity was adjusted to deamination without Vif.

Fig. 3. Inhibition of A3G deaminase activity by Vif-derived peptides

Fifteen-mer Vif-derived peptides covering the complete Vif sequence were assessed for the inhibition of A3G. The standard deamination reaction was carried out in the presence of 10 μM (A) or 1 μM (B) of each peptide, or an RSV-derived peptide (P.C., positive control). PAGE analyses of the cleaved deamination products are shown above the charts. C. the effect of the Vif-derived peptide concentration on A3G mediated deamination. Values represent the average of triplicates; SD values were less than ± 0.5 where not indicated.

Fig. 4. Determination of the Vif and Vif-derived peptides mode of inhibition

Deamination of an ss-deoxyoligonucleotide as function of the substrate concentration in the presence of Vif (A) and the Vif-derived peptides Vif105-119 (B) and Vif25-39 (C) was determined and is shown by double-reciprocal (double-inverse) plot. The Vif and Vif-derived peptides concentrations used are indicated. Values represent the average of triplicates; SD values were less than ± 0.4 .

Table 1

A3G protein content in HIV-1wt vs HIV-1Δvif virions

Virus	Exp.	p24 ng/slot	A3G/slot, (OD)	A3G(OD)/ ng of p24	A3G in HIV-1wt vs HIV-1Δvif, per ng p24, (%)
HIV-1 wt	#1	58.58	98592	1683	21.60
HIV-1 Δvif		33.68	262441	7792	
HIV-1 wt	#2	58.58	27949	477	15.71
HIV-1 Δvif		33.68	102255	3036	
HIV-1 wt	#3	29.29	25501	871	15.04
HIV-1 Δvif		16.84	97458	5787	
HIV-1 wt	#4	29.29	6667	228	17.98
HIV-1 Δvif		16.84	21321	1266	
A3G _{wt} vs A3G _{Δvif}					17.58 ± 2.96

Table II

Deaminase activity (DA) of A3G in HIV-1wt vs HIV-1 Δ vif virions Each reaction was loaded with equal amounts (1.25 ng of p24) of HIV-1wt or HIV-1 Δ vif purified virus.

	[S], fmol/reaction	Deaminase activity (DA), [P]/([P]+[S])	DA in HIV-1wt vs HIV-1 Δ vif - %
HIV-1 wt	0.02	0.0135	5.81
HIV-1 Δ vif		0.2320	
HIV-1 wt	0.05	0.0185	6.79
HIV-1 Δ vif		0.2727	
HIV-1 wt	0.2	0.0194	5.99
HIV-1 Δ vif		0.3243	
HIV-1 wt	0.5	0.0228	7.08
HIV-1 Δ vif		0.3224	
A3G_{wt} vs A3G_{Δvif}			6.42 $\pm$ 0.62

Table III

Vif-derived peptides^a

NIH S.#	FROM	TO	PEPTIDE SEQUENCE	NIH S.#	FROM	TO	PEPTIDE SEQUENCE
6018	1	15	MENRWQVMVWQVDR	6041	93	107	RYSTQVDPDLADQLI
6019	5	19	WQVMVWQVDRMRIR	6042	97	111	QVDPDLADQLIHLYY
6020	9	23	IVWQVDRMRIRTWKS	6043	101	115	DLADQLIHLYYFDCF
6021	13	27	VDRMRIRTWKSLVKH	6044	105	119	QLIHLYYFDCFSESA
6022	17	31	RIRTWKSLVKHHMYI	6045	109	123	LYYFDCFSESAIRNA
6023	21	35	WKSLLVKHHMYISGKA	6046	113	127	DCFSESAIRNAILGH
6024	25	39	VKHHMYISGKAKGWF	6047	117	131	ESAIRNAILGHIVSP
6025	29	43	MYISGKAKGWFYRHH	6048	121	135	RNAILGHIVSPRCEY
6026	33	47	GKAKGWFYRHHYEST	6049	125	139	LGHIVSPRCEYQAGH
6027	37	51	GWFYRHHYESTHPRI	6050	129	143	VSPRCEYQAGHNKVG
6028	41	55	RHHYESTHPRISSIEV	6051	133	147	CEYQAGHNKVGSLQY
6029	45	59	ESTHPRISSIEVHIPL	6052	137	151	AGHNKVGSLQYLALA
6030	49	63	PRISSEVHIPLGDAR	6053	141	155	KVGSLQYLALALIT
6031	53	67	SEVHIPLGDARLVIT	6054	45	59	QYLALALITPKKI
6032	57	71	IPLGDARLVITTYWG	6055	149	163	ALALITPKKIKPPL
6033	61	75	DARLVITTYWGLHTG	6056	153	167	LITPKKIKPPLPSVT
6034	65	79	VITTYWGLHTGERDW	6057	157	171	KKIKPPLPSVTKLTE
6035	69	83	YWGLHTGERDWHLGQ	6058	161	175	PPLPSVTKLTEDRWVN
6036	73	87	HTGERDWHLGQGVSI	6059	165	179	SVTKLTEDRWVNKPQK
6037	77	91	RDWHLGQGVSIWRK	060	169	183	LTEDRWVNKPQKTKGH
6038	81	95	LGQGVSIWRKKRYS	6061	173	187	RWNKPQKTKGHRGSH
6039	85	99	VSIEWRKKRYSTQVD	6062	177	191	PQKTKGHRGSHMTMG
6040	89	103	WRKKRYSTQVDPDLA	6063	181	192	KGHRGSHMTMNGH

^a Fifteen-mer peptides derived from HIV-1 Vif (sequence accession: #AAZ14773) covering the full-length protein with 11 aa overlaps.