Virion-associated Uracil DNA Glycosylase-2 and Apurinic/Apyrimidinic Endonuclease Are Involved in the Degradation of APOBEC3G-edited Nascent HIV-1 DNA*

Bin Yang 1,2, Keyang Chen 1, Chune Zhang, Sophia Huang, and Hui Zhang 3

From the Center for Human Virology, Division of Infectious Diseases, Department of Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Cellular cytidine deaminases APOBEC3 family is a group of potent inhibitors for many exogenous and endogenous retroviruses. It has been demonstrated that they induce G to A hypermutations in the nascent retroviral DNA, resulting from the cytosine (C) to uracil (U) conversions in minus-stranded viral DNA. In this report, we have demonstrated that the result of C to U conversion in minus-stranded DNA of human immunodeficiency virus type 1 (HIV-1) could trigger a degradation of nascent viral DNA mediated by uracil DNA glycosylases-2 (UNG2) and apurinic/apyrimidinic endonuclease (APE). Since antiviral activity of APOBEC3G is partially affected by UNG2 inhibitor Ugi or UNG2-specific short-interfering RNA in virus-producing cells but not target cells, the virion-associated UNG2 most likely mediates this process. Interestingly, as APE-specific short-interfering RNA can also partially inhibit the anti-HIV-1 activity of APOBEC3G in virus-producing cells but not in target cells and APE molecules can be detected within HIV-1 virions, it seems that the required APE is also virion-associated. Furthermore, the in vitro cleavage experiment using uracil-containing single-stranded DNA as a template has demonstrated that the uracil-excising catalytic activity of virion-associated UNG2 can remove dU from the uracil-containing viral DNA and leave an abasic site, which could be further cleaved by virion-associated APE. Based upon our observations, we propose that the degradation of APOBEC3G-edited viral DNA mediated by virion-associated UNG2 and APE during or after reverse transcription could be partially responsible for the potent anti-HIV-1 effect by APOBEC3G in the absence of vif.

APOBEC3 is a family of cytidine deaminases that has been identified as the host factor to restrict various retroviruses, endogenous retroviruses, and long interspersed nucleotide element (LINE) elements (1–11). It is closely related to APOBEC1, a cytidine deaminase that causes a specific cytosine to uracil change in the apolipoprotein B mRNA, and to an activation-induced deaminase (AID) enzyme that causes hypermutation of immunoglobulin genes (12). The similarities of the catalytic domains in these proteins strongly suggest that APOBEC3 edits the nucleic acids of various retrotransposons. Among these cytidine deaminases, APOBEC3G is the most extensively studied enzyme. It can be efficiently incorporated into human immunodeficiency virus type 1 (HIV-1)4 particles and causes extensive cytosine to uracil conversion in the viral minus-stranded DNA during reverse transcription (2, 3, 13). The significant C to U conversion in minus-stranded DNA is apparently correlated with the decreased viral infectivity. Two consequences could occur after the C to U conversion. First, uracil in minus-stranded DNA could be excised by recruited uracil DNA glycosylase-2 (UNG2), a host DNA repair enzyme. The resulting abasic site could be further cleaved by apurinic/apyrimidinic endonucleases (APE). The vif-defective viruses generated from the restrictive cells are unable to effectively process reverse transcription, or the completed reverse transcripts are readily subjected to degradation in the target cells, which is consistent with this hypothesis (3, 14–17). Second, C to U conversion in minus-stranded DNA could lead to G to A hypermutation in the plus-stranded DNA. These double-stranded DNA harboring G to A hypermutation would encode viral proteins containing aberrant premature stop codons or mutated proteins and would lead to accumulated genomic damage in viral replication (13). In this report, we have designed a series of experiments to test the first hypothesis. We have shown that in the presence of inhibitors of cellular UNG2 or APE, respectively, the antiviral activity of APOBEC3G can be decreased. Our findings suggest that DNA repair enzymes encapsidated within the virions directly participate in the degradation of uracilated-viral DNA induced by APOBEC3G and that this degradation process is correlated to the antiviral activity of APOBEC3G.

EXPERIMENTAL PROCEDURES

Plasmid Construction—An infectious HIV-1 clone with a deletion in the vif region (234 bp deletion), pNL4-3Δvif, and APOBEC3G expressing vectors pcDNA3-APOBEC3G and pSLX-GFP were constructed previously (2). The DNA of Ugi was PCR-amplified from pZWtac1 with a 5′-primer containing

* This work was supported by National Institutes of Health Grants AI047720 and AI058798 (to H. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Both authors contributed equally to this work.

2 Present address: Indiana University-Bloomington, Department of Chemistry, 800 E Kirkwood Ave., Bloomington, IN 47405.

3 To whom correspondence should be addressed: Thomas Jefferson University, JAH334, 1020 Locust St., Philadelphia, PA 19107. Tel: 215-503-0163; E-mail: hui.zhang@jefferson.edu.
UNG2 and APE Degrade APOBEC3G-edited HIV-1 DNA

a MluI site and a 3′-primer containing a XhoI site and a c-Myc tag encoding EQKLISEEDL (18). The PCR product (~260 bp) was digested with MluI and XhoI. The resulting DNA fragment was inserted into the pSLX vector to generate pSLX-Ugi-Myc. The APE DNA was amplified by RT-PCR from mRNA extracted from 293T cells with primers containing EcoRI or XhoI sites. The 3′-primer also contains a c-Myc tag. The RT-PCR product was then digested with EcoRI and XhoI followed by insertion into the pSLV vector to generate pSLX-APE-Myc.

RNA Interference—The chemically synthesized siRNAs used in the experiments were purchased from Dharmacon. The UNG2-specific siRNA and APE-specific siRNA were siGENOME SMARTpool. The luciferase-specific siRNA or GFP-specific siRNA served as negative controls. Conversely, the siLentGene U6 cassette RNA interference system (Promega) was used to generate U6 promoter-controlled APE-specific siRNAs. The APE siRNA pool was designed by the Dharmacon siDESIGN program. Based upon the protocol, various oligonucleotides were designed as the downstream primers: oligonucleotide 1A (for antisense RNA), 5′-CAA AAA CTG TAA AAA GCC TAT ECC GTA AGA AAC CGG TGT TTT GTC CTT TCC ACA AGA-3′; oligonucleotide 1S (for sense RNA), 5′-CAA AAA CTG TAA AAA GGT TTA CGG CAT AGG CGG TGT TTT GTC CTT TCC ACA AGA-3′; oligonucleotide 2A, 5′-CAA AAA CTG TAA AAA GGT TTA CGG CAT ACC AGC ACA AAC GAG CGG TGT TTT GTC CTT TCC ACA AGA-3′; oligonucleotide 3S, 5′-CAA AAA CTG TAA AAA GGT TTA CGG CAT ACC AGC ACA AAC GAG CGG TGT TTT GTC CTT TCC ACA AGA-3′; oligonucleotide 2S, 5′-CAA AAA CTG TAA AAA GGT TTA CGG CAT ACC AGC ACA AAC GAG CGG TGT TTT GTC CTT TCC ACA AGA-3′; oligonucleotide 2S, 5′-CAA AAA CTG TAA AAA GGT TTA CGG CAT ACC AGC ACA AAC GAG CGG TGT TTT GTC CTT TCC ACA AGA-3′; oligonucleotide 2S, 5′-CAA AAA CTG TAA AAA GGT TTA CGG CAT ACC AGC ACA AAC GAG CGG TGT TTT GTC CTT TCC ACA AGA-3′; oligonucleotide 4A, 5′-CAA AAA CTG TAA AAA GGT TTA CGG CAT ACC AGC ACA AAC GAG CGG TGT TTT GTC CTT TCC ACA AGA-3′; oligonucleotide 4S, 5′-CAA AAA CTG TAA AAA GGT TTA CGG CAT ACC AGC ACA AAC GAG CGG TGT TTT GTC CTT TCC ACA AGA-3′; oligonucleotide 4S, 5′-CAA AAA CTG TAA AAA GGT TTA CGG CAT ACC AGC ACA AAC GAG CGG TGT TTT GTC CTT TCC ACA AGA-3′. The sequences of APE are labeled bold. These various downstream primers were used for PCR amplification, along with the upstream primer and the template supplied by manufacturer. The PCR products were then inserted into the vectors to generate plasmids expressing various sense and antisense siRNAs, controlled by the U6 promoter. After transfection into 293T cells, these sense and antisense transcripts would complement each other and form various double-stranded siRNAs.

Viral Infection—Viral stocks were produced in 293T cells by transfection with 2 μg of pNL4-3Δvif and 2 μg of pNL4-3Δvif plus 1 μg of pcDNA3-APOBEC3G, respectively, or in combination with the vectors expressing various genes such as Ugi or GFP, as well as various siRNAs, using Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche Applied Science). Viruses in the supernatants were harvested at 48 h after transfection. After treatment with RQ1 DNase to remove input plasmids, the viruses were passed through a 0.45-μm filter and further quantitated with HIV-1 p24 via enzyme-linked immunosorbent assay. The viral infection was then performed with HLC4D-CAT as the target cells, which contain stably integrated, silent copies of the HIV-1 LTR promoter linked to the CAT gene. At 48 h after infection, the infected cells were harvested and subjected to CAT analysis, as described previously (2).

Cytidine Deaminase Assay—HIV-1Δvif viruses were harvested from the supernatant of 293T cells at 48 h after transfection, filtered by a 0.45-μm filter, and purified by rate-zonal sedimentation followed by equilibrium density centrifugation, as described previously (19). The isolated viruses were further washed by cold phosphate-buffered saline and ultracentrifuged for 1 h at 25,000 × g. The viruses were then quantified by p24 enzyme-linked immunosorbent assay detection and mixed with a lysing buffer containing 50 mM Tris (pH 8.0), 40 mM KCl, 50 mM NaCl, 5 mM EDTA, 10 mM dithiothreitol, and 0.1% (v/v) Triton X-100. Conversely, various oligodeoxynucleotides were 5′-32P-labeled through incubation with T4 kinase and [γ-32P]ATP. The sequences for various oligonucleotides were modified from previous designs (13, 20, 21)(see Fig. 4A). The purified deoxyoligonucleotides (106 cpm) were incubated with 1 μg (p24 antigen equivalent) of viral lysate in buffer R (40 mM Tris (pH 8.0), 40 mM KCl, 50 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol). After incubation for 4 h at 37 °C, the reactions were then terminated by extraction with phenol/chloroform/isoamyl alcohol (25:24:1). The extracted deoxyoligonucleotides were then electrophoresized on 15% Tris-borate-EDTA-urea PAGE gel and followed by direct autoradiography. A 32P-labeled oligodeoxynucleotide oligo(U) that contains a dU instead of a dC at position 18 (see Fig. 4A) was treated with 5 units of UNG2 and subsequently 2 N NaOH in parallel to serve as a size marker for the cleaved products.

Virus-associated UNG2 and APE Catalytic Activity Assay—The lysate of HIV-1 virions (1 μg of p24 antigen equivalent) prepared as described above was mixed with 105 cpm 5′-32P-labeled oligodeoxynucleotide oligo(U) with and without recombinant UNG2 or APE (Novus Bio) at various concentrations in a buffer containing 20 mM Tris (pH 8.0), 10 mM MgCl2, 20 mM NaCl, 2 mM dithiothreitol, and 0.5 mM ATP. After incubation for 2 h, the cleaved products were electrophoresized on 15% Tris-borate-EDTA-urea PAGE and followed by direct autoradiography.

Immunoblotting Analysis—The lysate of 293T or HLC4D-CAT (3–5 μg totally protein) or the lysate (100–250 ng of p24 antigen equivalent) of purified HIV-1Δvif viruses were subjected to detecting UNG2, APE, or Ugi-Myc. After electrophoresis in SDS-PAGE gel, rabbit polyclonal anti-UNG2 antibody (a gift of Dr. G. Slupphaug), mouse monoclonal anti-APE antibody (Novus Biologicals), and mouse anti-c-Myc monoclonal antibody (Sigma-Aldrich) were used, respectively, for detecting different proteins.

Quantitative PCR Analysis of HIV-1 Reverse Transcripts—The HIV-1Δvif and HIV-1Δvif/APOBEC3G viruses were produced in 293T cells with and without co-transfected with Ugi-expressing vectors or APE-specific siRNA (SMARTpool) (100 nm). C8166 cells were infected with normalized viruses. After 24 h, cellular DNA was extracted and amplified by PCR with various primer pairs followed by Southern blotting analysis, as described in previous studies (22). The gag region was detected by the primer/probe set SK38-SK39/SK19, and the RU5-PBS-5NC region was detected by the primer/probe set M667-M661/SK31.
FIGURE 1. Expression of UNG2 inhibitor Ugi affects antiviral activity of APOBEC3G. A, detection of virion-associated UNG2 and Ugi. The 293T cells were transfected with Ugi-Myc expressing vectors (pSLX-Ugi-Myc) and pNL4-3Δvif. The cell lysate and lysate from purified viruses were analyzed with immunoblotting, via anti-UNG2 (top), anti-Myc(Ugi) (middle), and anti-β-actin/anti-HIV-p24 (bottom), respectively. B, transfection of Ugi-expressing plasmid into virus-producing cells (293T cells). HIV-1 vif or HIV-1 vif/APOBEC3G viruses were generated from 293T cells by transfecting pNL4-3Δvif with and without pcDNA3-APOBEC3G or pSLX-Ugi-Myc. Viral supernatants were harvested at 48 h. After normalization with HIV-1 p24 antigen, the viruses were allowed to infect HLC4-CAT cells. At 48 h after infection, the viral infectivity was analyzed by CAT assay. C, transfection of Ugi-expressing plasmid into virus-targeting cells (HLC4-CAT cells). The pSLX-Ugi-Myc plasmid was transfected into the virus-targeting HLC4-CAT cells before virus infection. As controls, vector only (cytomegalovirus) or pSLX-GFP were also transfected. At 48 h after transfection, HIV-1 vif or HIV-1 vif/APOBEC3G viruses generated from 293T cells, after normalization with HIV-1 p24 antigen, were allowed to infect HLC4-CAT cells. At 48 h after infection, the viral infectivity was analyzed by CAT assay. D, quantitative analysis of viral infectivity in the presence of APOBEC3G and Ugi in viral-producing cells or target cells. The experiments were performed six times simultaneously. The multiple data were analyzed and calculated. The infectivity of Δvif viruses without any treatment was set as 100% (lane 1). E, various amounts of pcDNA3-APOBEC3G were transfected into 293T cells with pNL4-3Δvif. The APOBEC3G expression was detected by Western blotting. F, the effect of various amounts of pcDNA3-APOBEC3G transfected in virus-producing cells (293T) upon infectivity of HIV-1 Δvif viruses was examined by CAT assay, as described above. These data represent at least two experiments.
**RESULTS**

Expression of UNG2-inhibitor Ugi or UNG2-specific siRNA in Virus-producing Cells Can Partially Block Antiviral Activity of APOBEC3G—It has been demonstrated that virion-associated APOBEC3G can convert dC to dU in newly synthesized minus-stranded viral DNA. The dU in newly synthesized viral DNA could be removed by virion-associated UNG2 and leave an abasic site in the DNA chain. This abasic site could be further cleaved by APE. If this hypothesis is correct, inhibition of virion-associated UNG2 could lead to less abasic site(s) in newly synthesized viral DNA chains and less degradation of newly synthesized viral DNA, and subsequently, less viral infectivity. Therefore we employed Ugi, a bacteriophage-encoded short polypeptide that can tightly bind to the DNA-binding site of UNG2, to inhibit the catalyzing activity of UNG2 in either virus-producing cells or viral target cells (23–28). We have transfected Ugi-expressing vector in virus-producing 293T cells or viral target cells (HLCD4-CAT), respectively. The Ugi protein can be detected in 293T cells (Fig. 1A, middle left panel). Its expression does not affect the expression of UNG2 in cells and the incorporation of UNG2 into virions (Fig. 1A, upper panel). Ugi can also be found in HIV-1Δvif virions, possibly because Ugi forms a strong complex with UNG2 in the virus-producing cells and subsequently is packaged into the virions (Fig. 1A, middle right panel).

As shown in Fig. 1B, the viruses generated from HIV-1Δvif in the presence of APOBEC3G do not have any infectivity (lane 2). However, when vectors expressing Ugi were transfected to 293T virus-producing cells, the infectivity of HIV-1Δvif in the presence of APOBEC3G can be partially rescued (lane 8). The vector only (cytomegalovirus immediate early promoter) or GFP-expressing vector cannot rescue the virus infectivity (lanes 4 and 6). Conversely, transfection of the vector expressing Ugi-Myc into HLCD4-CAT, the virus target cells, cannot significantly rescue the viral infectivity, indicating that UNG2, which removes dU in the minus-stranded viral DNA, comes from virus-producing cells (Fig. 1C). To verify these observations, we have simultaneously performed similar experiments six times, and the results have been shown in Fig. 1D. The level of viral infectivity rescued by Ugi expression at different ratios to APOBEC3G (1:1, 0.5:1, and 0.25:1) in 293T cells is significantly higher than that without Ugi expression (Fig. 1D, lanes 5, 6, and 7 versus lane 2) (p < 0.001, t test). However, the level of viral infectivity rescued by Ugi expression in HLCD4-CAT cells is not significantly higher than that without Ugi expression (Fig. 1D, lane 8 versus lane 2). These quantitative data further demonstrate that Ugi at various concentrations is able to partially rescue the infectivity of HIV-1Δvif in the presence of APOBEC3G.

To confirm that APOBEC3G protein in our experimental system is not overexpressed, we have compared the APOBEC3G expression that resulted from transfection with various amount of APOBEC3G-expressing plasmid with the concentration of APOBEC3G in “non-permissive” primary CD4 T-cells and “permissive” Supt-1 T-cells. Fig. 1E demonstrates that the APOBEC3G expression in our experiment (1 μg of APOBEC3G-expressing plasmid) is lower than the APOBEC3G expression in primary CD4 T-cells, the native target cells for HIV-1 replication. If a very low amount of APOBEC3G-expressing plasmid (0.01 μg) is transfected into 293T cells to generate APOBEC3G protein (Fig. 1E), we have also found that the expression level of APOBEC3G is as low as that in Supt1 T-cells. This amount of APOBEC3G cannot significantly inhibit the infectivity of HIV-1Δvif viruses (Fig. 1F).

To further verify that the inhibition of UNG2 in virus-producing cells can rescue the infectivity of HIV-1Δvif in the presence of APOBEC3G, we transfected UNG2-specific siRNA (SMARTpool) into the virus-producing cells. As indicated in Fig. 2A, UNG2-specific siRNA can effectively decrease the expression of UNG2 in 293T cells. When transfected into 293T virus-producing cells, it can also significantly rescue the infectivity of HIV-1Δvif in the presence of APOBEC3G (Fig. 2B, lane 6 versus lane 3). However, transfection into HLCD4-CAT, the viral target cells, cannot effectively rescue the viral infectivity (Fig. 2B, lane 9).

**Viral Infectivity Suppressed by APOBEC3G Can Be Partially Rescued by APE-specific siRNA**—The excision of uracils from DNA by UNG2 results in abasic sites in the minus-stranded viral DNA. The abasic sites could be subsequently cleaved by APE. However, there is no direct evidence showing that the cellular APE participates in the degradation of uracilated-DNA in any viruses. To test the possible involvement of APE...
in APOBEC3G-induced cDNA degradation, the APE-specific siRNAs (SMARTpool) was chemically synthesized and transfected to 293T cells, and its potent APE-suppressing activity was confirmed by Western blotting (Fig. 3A). To determine the cells in which APE plays a role, APE-specific siRNA was transfected into 293T virus-producing cells or HLCD4-CAT target cells before viral infection. The viral infectivity was analyzed by CAT assay, as described above. APE-specific siRNA could partially rescue the viral infectivity blocked by APOBEC3G when co-transfected into virus-producing cells (Fig. 3B, lane 6), whereas having much less effect in HLCD4-CAT target cells (Fig. 3C, lane 6). These results indicate that
UNG2 and APE Degrade APOBEC3G-edited HIV-1 DNA

APE is involved in the degradation of APOBEC3G-edited viral DNA. Again, this result was further confirmed by experiments, using either the chemically synthesized SMARTpool of APE-specific siRNA or the U6 promoter-driven APE-specific siRNA pool (Fig. 3D). Our data suggest that APE in virus-producing cells, like UNG2, should be encapsidated in viruses and directly participates in the degradation of newly synthesized viral DNA. With an immunoblotting assay, APE was indeed detected in purified HIV-1 DNA (Fig. 3E).

Uracil-containing DNA Can Be Degraded by Virion-associated UNG2 and APE Enzymes—It has been proven that APOBEC3G deaminates the single-stranded oligonucleotide in vitro (13). To examine the cytidine deaminase activity of APOBEC3G in vitro, we have used a similar system. Oligonucleotides containing cytidine from one to five were incubated with the lysate of Δvif/APOBEC3G viruses (Fig. 4A). We found that the cleaved fragments were clearly detected when 5'32P-labeled single oligonucleotides containing more than two cytidines were incubated with the lysate from HIV-1Δvif viruses containing APOBEC3G (Fig. 4B). This result indicates that all the required elements for dC to dU deamination, removal of dU from the oligonucleotide and cleavage of the abasic site, are all enclosed in the virions. To further determine whether the virion-encapsidated UNG2 and APE are catalytic, we developed an experiment to monitor the virion-associated UNG2 and APE enzymatic activity by the similar method previously described (27). In this experiment, 5'32P-labeled uracil-containing oligodeoxynucleotide (oligo(U)) was treated by the viral lysate. If both the virion-associated UNG2 and the virion-associated APE are catalytically active, we assumed that a short length broken DNA fragment should be separated by denaturing PAGE gel without the addition of UNG2 and APE. As shown in Fig. 4C, when 5'32P-labeled uracil-containing DNA was incubated only with the lysate from Δvif/APOBEC3G viruses or Δvif viruses, the degraded products were clearly detected (lanes 3 and 8), suggesting that the virion-associated UNG2 and APE have the enzymatic ability to degrade the uracil-containing DNA. As additional recombinant UNG2 can enhance the degradation (Fig. 4C, lanes 4, 5, 9, and 10), the virion-associated UNG2 is likely not sufficient to catalyze the removal of dU. However, the addition of APE did not significantly improve the digestion rate (Fig. 4C, lanes 6, 7, 11, and 12), suggesting that virion-associated APE is sufficient to cleave the abasic site. Furthermore, to test whether the uracil excision catalytic activity of virion-associated UNG2 can be inhibited by virion-associated Ugi, viral lysate from Ugi-co-transfected Δvif/APOBEC3G viruses were prepared, and a similar experiment as described in the legend for Fig. 4C was performed. As shown in Fig. 4D, the cleaved products were much less when uracil-containing oligodeoxynucleotide was incubated with the lysate of Δvif/APOBEC3G/Ugi viruses (Fig. 4D, lane 3) than that with the lysate from Δvif/APOBEC3G viruses (Fig. 4D, lane 2).

Inhibition of UNG2 and APE Significantly Decrease APOBEC3G-induced Degradation of Newly Synthesized Reverse Transcripts—It has been shown that the newly synthesized viral DNA by reverse transcription was very low in Δvif viruses from non-permissive cells (3, 14, 16, 17, 29). To examine the effect of UNG2 and APE inhibitors upon the viral DNA synthesis of HIV-1Δvif/APOBEC3G viruses in the target cells, a semiquantitative PCR was used (22, 30). HIV-1Δvif/APOBEC3G viruses were generated from 293T cells with and without various co-transfections. These viruses were then allowed to infect C8166, a CD4 T-cell line. At 24 h after infection, cellular DNA was extracted and amplified by PCR. The late part of minus-
stranded DNA (gag region) and plus-stranded DNA (RUS-PBS-5NC region) of Δvif/APOBEC3G viruses were monitored. Fig. 5 indicates that the DNA accumulation of Δvif/APOBEC3G viruses significantly decreased (lanes 2 and 4). However, Ugi- or APE-specific siRNA can significantly rescue the accumulation of viral DNA (Fig. 5, lanes 6 and 10), indicating that UNG2 and APE are involved in the degradation of APOBEC3G-edited viral DNA.

**DISCUSSION**

To prove the possible involvements of UNG2 and APE in the antiviral function of APOBEC3G, we have blocked the enzymatic activity of UNG2 and APE, respectively, by the UNG2 inhibitor Ugi, UNG2-specific siRNA, or APE-specific siRNAs. Our data have shown that after catalytically enzymatic activity or expression of UNG2 or APE is decreased in virus-producing cells rather than in virus-targeting cells, the antiviral activity of APOBEC3G is partially inhibited. These findings demonstrate that most of the uracil bases in minus-stranded DNA generated by APOBEC3G will be first removed by excision of virion-associated UNG2 and then degraded by virion-associated APE before integration. This hypothesis is supported by the early observations that less full-length viral DNA is synthesized during the reverse transcriptions of Δvif/APOBEC3G viruses (3, 14–17). Our data suggest that the subsequent degradation pathway perhaps plays a role in the antiviral activity of APOBEC3G. The G to A hypermutations observed by our group and others in HIV-1 plus-stranded DNA are supposed to be synthesized, with a few of surviving non-degraded uracil-containing minus-stranded DNA as a template (2, 3, 13, 29).

It is no doubt that UNG2 is encapsidated to virions, although there are different hypotheses for the encapsidation pathway. It has been reported that UNG2 is incorporated to HIV-1 particles by Vpr because of the direct protein-protein interaction between Vpr and UNG2 (27, 31–34). However, it has also been reported that UNG2 is incorporated into HIV-1 virions via HIV-1 integrase but not Vpr (35). Mansky et al. suggest (31) that UNG2 is present in vpr+ but not in Δvpr viruses. A recent report indicates UNG2 and SMUG are both found to be encapsidated in Δvpr HIV-1 viruses with a significantly lower amount than in vpr+ viruses (36). This result could be due to the fact that Vpr induces the degradation of UNG2 and SMUG (36). It has been shown that the knock-out of UNG2 by siRNA in macrophages or expression of Ugi in the virus-producing cells can both generate non-infectious viruses (28). However, recent reports have shown that the inhibition of UNG2 does not appear to significantly affect the viral infectivity (36, 37).

As Ugi expression in the virus-producing cells cannot affect viral DNA synthesis and infectivity of Δvif viruses in the presence of APOBEC3G, Kaiser and Emerman (37) have proposed that the activity of UNG2 is dispensable for the antiviral activity of APOBEC3G. In contrast to this observation, we have found that Ugi can be packaged into virions and can inhibit uracil excision activity of UNG2 in vitro when a uracil-containing single-stranded DNA was incubated with the lysate of virions harboring both UND2 and Ugi. Further, we have found that Ugi is able to partially rescue the infectivity of Δvif/APOBEC3G viruses. This result indicates that the degradation of viral DNA induced by UNG2 is involved in the antiviral activity of APOBEC3G. When UNG2 is inhibited by Ugi, more uracilated cDNA survives and is subsequently integrated. As a result, viral infectivity is partially rescued. We have carefully performed our experiments, and our conclusion still remains the same. Our data are further supported by the experiments using UNG2-specific siRNA (Fig. 2).

To clarify the discrepancy between our data and the findings of Kaiser and Emerman (37), we have compared the expression of APOBEC3G in 293T cells resulting from transfection and the native expression of APOBEC3G from non-permissive activated primary CD4 T-cells, the major HIV-1 target cells in vivo, and a well known permissive cells Supt1 T-cell line. Our data in Fig. 1E have shown that the expression of APOBEC3G that results from the transfection with 1 μg of APOBEC3G-expressing plasmid, a dose we routinely used in our experiments, is less than the APOBEC3G expression in primary CD4 T-cells. This result indicates that the APOBEC3G expression in our experiment is not overexpressed. Conversely, we have also compared the APOBEC3G expression that results from transfection with 0.003 and 0.01 μg of APOBEC3G-ex-
pressing plasmid, two of the doses used in the works of Kaiser and Emerman (37) showing significantly inhibitory effect upon the infectivity of HIV-Δvif viruses. A 90% (1 log) decrease in viral infectivity has been obtained by Kaiser and Emerman (37) when 0.01 μg of APOBEC3G plasmid was co-transfected with HIV-1Δvif clone into 293T cells. Surprisingly, our data show that the APOBEC3G expression level generated from the transfection with 0.003 and 0.01 μg of APOBEC3G plasmid is as low as that in SupT1 permissive T-cells (Fig. 1E, lane 8). Previous experiments using ΔVif viruses to infect SupT1 did not yield infectivity at such low level (38). Our data have also shown that the viral infectivity is almost the same when Δvif viruses are generated with or without transfection with 0.01 μg of APOBEC3G-expressing plasmid (Fig. 1F).

The conversion of dC to dU in newly synthesized HIV-1 DNA by APOBEC3G initiates the subsequent degradation of the uracilated DNA. Besides Ung2 and Ape, there is another cellular uracil base-excision-repair pathway that needs additional polymerase to cleave the 3’-terminus of the abasic site and then place a correct nucleotide. The XRCC1-ligase 3 complex then seals the remaining nick (39, 40). It remains to be clarified whether HIV-1 virions harbor the same base-excision-repair machinery as cells do. It has been proposed that Ape lyase and ligase enzymes are not required for HIV-1 replication (41). HIV-1 RT could contain some Ape enzymatic activity by itself (28). However, this process strictly requires the participation of dNTPs. As the virion-associated Ape activity is a dNTP-independent process (Fig. 4B), it is unlikely that HIV-1 RT plays a major role for virion-associated Ape activity. In our experiments, Ape can be detected in the purified viruses. When Ape was decreased by Ape-specific siRNA in virus-producing cells rather than in viral target cells, the infectivity of Δvif/APOBEC3G virus was partially rescued, indicating that virion-associated Ape takes effect to cleave the DNA abasic site following the uracil excision in newly synthesized viral DNA. In vitro cleavage experiments using uracil-containing single-stranded DNA as a template support this hypothesis.

The observations described in this report provide primary clues that the virion-associated enzymes for base uracil excision and abasic site cleavage are most likely involved in the antiviral mechanism of the virion-associated cytidine deaminase APOBEC3G. Our data support the hypothesis that the subsequent degradation of viral DNA is one of the antiviral mechanisms of virion-associated APOBEC3G. This hypothesis has been largely supported by many research groups (2, 3, 42-44). It is notable that data from Malim’s group have shown that the mutations in the zinc-binding domain of APOBEC3G do not have any influence upon the antiviral activity of APOBEC3G (45–47). Several reports have suggested that antiviral activity of APOBEC3G is unrelated to its cytidine deaminase in other viruses such as human T-lymphoma virus type 1 and hepatitis B viruses (48–50). It could be due to the possible inhibition of APOBEC3G on viral RNA packaging (50). Furthermore, we and others have identified that APOBEC3G in the resting CD4 cells and myeloid dendritic cells can effectively inhibit the incoming HIV-1 viruses (30, 51, 52). This inhibition is also unlikely contributed by its cytidine deaminase activity. It seems that APOBEC3G could have antiviral activity at the multiple steps of viral life cycle and that cytidine deaminase activity of APOBEC3G is not required for all the inhibitory effects. Our current work is related to the anti-HIV-1 mechanism of virion-associated APOBEC3G that is packaged from virus-producing cells. Our previous work has demonstrated that the effect of mutation(s) in the zinc-binding domain of APOBEC3G upon in vitro cytidine deaminase activity is different from the effect upon the anti-viral activity of APOBEC3G in the cell culture system (2). The effect of mutation(s) in the zinc-binding domain of APOBEC3G upon in vitro cytidine deaminase activity is quite significant, whereas the effect upon antiviral activity of APOBEC3G is only partial (2), indicating that other mechanism(s) could also contribute to the anti-viral activity of APOBEC3G. This report is consistent with our previous findings and indicates that the degradation of newly synthesized viral DNA mediated by virion-associated Ung2 and Ape is partially responsible for the potent viral inhibitory effect by virion-associated APOBEC3G upon vif-defective viruses.

Acknowledgments—We thank Dr. Geir Slupphaug at Norwegian University of Science and Technology for supplying anti-UNG2 antibody; Dr. Dale Mosbaugh at Oregon State University for supplying pZWtac1 containing ugi gene; and Jennifer Rosa for proofreading.

REFERENCES

1. Sheehy, A. M., Gaddis, N. C., Choi, J. D., and Malim, M. H. (2002) Nature 418, 646 – 650
2. Zhang, H., Yang, B., Pomerantz, R. J., Zhang, C., Arunachalam, S. C., and Gao, I. (2003) Nature 424, 94 – 98
3. Mangeat, B., Turelli, P., Caron, G., Friedli, M., Perrin, L., and Trono, D. (2003) Nature 424, 99 – 103
4. Jarmuz, A., Chester, A., Bayliss, J., Gisbourne, J., Dunham, I., Scott, J., and Navaratnam, N. (2002) Genomics 79, 285 – 296
5. Bishop, K. N., Holmes, R. K., Sheehy, A. M., Davidson, N. O., Cho, S. J., and Malim, M. H. (2004) Curr. Biol. 14, 1392 – 1396
6. Doeble, B. P., Schafer, A., and Cullen, B. R. (2005) Virology 30, 12102 – 12108
7. Noguchi, C., Ishino, H., Tsuge, M., Fujimoto, Y., Imamura, M., Takahashi, S., and Chayama, K. (2005) Hepatology 42, 626 – 633
8. Yu, Q., Chen, D., Konig, R., Mariani, R., Unutmaz, D., and Landau, N. R. (2004) J. Biol. Chem. 279, 53379 – 53386
9. Zheng, Y. H., Irwin, D., Kurosu, T., Tokunaga, K., Sata, T., and Peterlin, B. M. (2004) J. Virol. 78, 6073 – 6076
10. Elsholtz, C., Heidmann, O., Delebecque, F., Devanne, M., Ribet, D., Hance, A. J., Heidmann, T., and Schwartz, O. (2005) Nature 433, 430 – 433
11. Böger, H. P., Wiegand, H. L., Doeble, B. P., Lueders, M. K., and Cullen, B. R. (2006) Nucleic Acids Res. 34, 89 – 95
12. Navaratnam, N., and Sarwar, R. (2006) Int. J. Hematol. 83, 195 – 200
13. Yu, Q., Konig, R., Pillai, S., Chiles, K., Kearney, M., Palmer, S., Richman, D., Coffin, J. M., and Landau, N. R. (2004) Nat. Struct. Mol. Biol. 11, 435 – 442
14. von Schwedler, U., Song, J., Aiken, C., and Trono, D. (1993) J. Virol. 67, 4945 – 4955
15. Sova, P., and Volsky, D. J. (1993) J. Virol. 67, 6322 – 6326
16. Dornadula, G., Yang, S., Pomerantz, R. J., and Zhang, H. (2000) J. Virol. 74, 2594 – 2602
17. Simon, J. H., and Malim, M. H. (1996) J. Virol. 70, 5297 – 5305
18. Wang, Z. G., Smith, D. G., and Mosbaugh, D. W. (1991) Gene (Amst.) 99, 31 – 37
19. Zhang, H., Zhang, Y., Spencer, T. P., Abbott, L. Z., Abbott, M., and Poiesz, B. J. (1993) AIDS Res. Hum. Retroviruses 9, 1287 – 1296
20. Petersen-Mahrt, S. K., and Neuberger, M. S. (2003) J. Biol. Chem. 278, 19583 – 19586
21. Conticello, S. G., Harris, R. S., and Neuberger, M. S. (2003) Curr. Biol. 13,
UNG2 and APE Degrade APOBEC3G-edited HIV-1 DNA

APRIL 20, 2007 • VOLUME 282 • NUMBER 16
JOURNAL OF BIOLOGICAL CHEMISTRY

11675

22. Zhang, H., Dornadula, G., and Pomerantz, R. J. (1996) J. Virol. 70, 2809–2824
23. Bennett, S. E., and Mosbaugh, D. W. (1992) J. Biol. Chem. 267, 22512–22521
24. Handa, P., Acharya, N., and Varshney, U. (2002) Nucleic Acids Res. 30, 3086–3095
25. Acharya, N., Roy, S., and Varshney, U. (2002) J. Mol. Biol. 321, 579–590
26. Acharya, N., Kumar, P., and Varshney, U. (2003) Microbiology (Reading) 149, 1647–1658
27. Chen, R., Le Rouzic, E., Kearney, J. A., Mansky, L. M., and Benichou, S. (2004) J. Biol. Chem. 279, 28419–28425
28. Priet, S., Gros, N., Navarro, J. M., Boretto, J., Canard, B., Querat, G., and Sire, J. (2005) Mol. Cell 17, 479–490
29. Mariani, R., Chen, D., Schrofelbauer, B., Navarro, F., Konig, R., Bollman, B., Munk, C., Nymark-McMahon, H., and Landau, N. R. (2003) Cell 114, 21–31
30. Chen, K., Huang, J., Zhang, C., Huang, S., Nunnari, G., Wang, F. X., Tong, X., Gao, L., Nikiforov, K., and Zhang, H. (2006) J. Virol. 80, 7645–7657
31. Mansky, L. M., Preveral, S., Selig, L., Benarous, R., and Benichou, S. (2000) J. Virol. 74, 7039–7047
32. Selig, L., Pages, J. C., Tanchou, V., Preveral, S., Berlioz-Torrent, C., Liu, L. X., Erdmann, L., Darlix, J., Benarous, R., and Benichou, S. (1999) J. Virol. 73, 592–600
33. Bouhamdan, M., Benichou, S., Rey, F., Navarro, J. M., Agostini, I., Spire, B., Camonis, J., Slupphaug, G., Vigne, R., Benarous, R., and Sire, J. (1996) J. Virol. 70, 697–704
34. Selig, L., Benichou, S., Rogel, M. E., Wu, L. I., Vodicka, M. A., Sire, J., Benarous, R., and Emerman, M. (1997) J. Virol. 71, 4842–4846
35. Willetts, K. E., Rey, F., Agostini, I., Navarro, J. M., Baudat, Y., Vigne, R., and Sire, J. (1999) J. Virol. 73, 1682–1688
36. Schrofelbauer, B., Yu, Q., Zeitlin, S. G., and Landau, N. R. (2005) J. Virol. 79, 10978–10987
37. Kaiser, S. M., and Emerman, M. (2006) J. Virol. 80, 875–882
38. Gabuzda, D. H., Lawrence, K., Langhoff, E., Terwilliger, E., Dorfman, T., Heseltine, W. A., and Sodroski, J. (1992) J. Virol. 66, 6489–6495
39. Krokan, H. E., Drablos, F., and Slupphaug, G. (2002) Oncogene 21, 8935–8948
40. Hoeijmakers, J. H. (2001) Nature 411, 366–374
41. Hottiger, M., Podust, V. N., Thimmig, R. L., McHenry, C., and Hubscher, U. (1994) J. Biol. Chem. 269, 986–991
42. Watanabe, Y., Takeuchi, H., Strebel, K., and Levin, J. G. (2006) J. Virol. 80, 5992–6002
43. Navarro, F., Bollman, B., Chen, H., Konig, R., Yu, Q., Chiles, K., and Landau, N. R. (2005) Virology 333, 374–386
44. Shindo, K., Takaori-Kondo, A., Kobayashi, M., Abudu, A., Fukenaga, K., and Uchiyama, T. (2003) J. Biol. Chem. 278, 44412–44416
45. Newman, E. N., Holmes, R. K., Craig, H. M., Klein, K. C., Lingappa, J. R., Malim, M. H., and Sheehy, A. M. (2005) Curr. Biol. 15, 166–170
46. Holmes, R. K., Konig, F. A., Bishop, K. N., and Malim, M. H. (2006) J. Biol. Chem. 281, 2587–2595
47. Bishop, K. N., Holmes, R. K., and Malim, M. H. (2006) J. Virol. 80, 8450–8458
48. Sasada, A., Takaori-Kondo, A., Shirakawa, K., Kobayashi, M., Abudu, A., Hishizawa, M., Imada, K., Tanaka, Y., and Uchiyama, T. (2005) Retrovirology 2, 32
49. Rosler, C., Kock, J., Kann, M., Malim, M. H., Blum, H. E., Baumert, T. F., and von Weizsacker, F. (2005) Hepatology 42, 301–309
50. Turelli, P., Mangeat, B., Jost, S., Vianin, S., and Trono, D. (2004) Science 303, 1829
51. Chiu, Y. L., Soros, V. B., Kreisberg, J. F., Stopak, K., Yonemoto, W., and Greene, W. C. (2005) Nature 435, 108–114
52. Pion, M., Granelli-Piperno, A., Mangeat, B., Stalder, R., Correa, R., Steinman, R. M., and Piguet, V. (2006) J. Exp. Med. 203, 2887–2893