Functional Role of HIV-1 Virion-associated Uracil DNA Glycosylase 2 in the Correction of G:U Mispairs to G:C Pairs*

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Stéphane Priet, Jean-Marc Navarro, Nathalie Gros, Gilles Quérat, and Joséphine Sire‡

From the Pathogénie des Infections à Lentivirus, INSERM U372, 163 avenue de Luminy, BP 178, 13276 Marseille Cedex 09, France

Human monocytes/macrophages are target cells for HIV-1 infection. As other non-dividing cells, they are characterized by low and imbalanced intracellular dNTP pool levels and an excess of dUTP. The replication of HIV-1 in this cellular context favors misincorporation of uracil residues into viral DNA because of the use of dUTP in place of dCTP. We have previously reported that the host uracil DNA glycosylase enzyme UNG2 is packaged into HIV-1 viral particles via a specific association with the integrase domain of the Gag-Pol precursor. In this study, we investigated whether virion-associated UNG2 plays a role similar to that of its cellular counterpart. We show that the L172A mutation of integrase impaired the packaging of UNG2 into viral particles. Using a primer-template DNA substrate containing G:U mispairs, we demonstrate that wild-type viral lysate has the ability to repair G:U mismatched pairs to G:C matched pairs, in contrast to UNG2-deficient viral lysate. Moreover, no correction of G:T mispairs by wild-type HIV-1 viral lysate was observed, which argues for the specificity of the repair process. We also show that UNG2 physically associates with the viral reverse transcriptase enzyme. Altogether our data indicate for the first time that a uracil repair pathway is specifically associated with HIV-1 viral particles. However, the molecular mechanism of this process remains to be characterized further.

The control of uracil residue levels in cellular DNA is ensured by a family of uracil DNA glycosylase (UNG) DNA repair enzymes involved in the base excision repair (BER) pathway (for a review see Ref. 1). Uracil in DNA may result from misincorporation of dUTP instead of dTTP or from spontaneous deamination of cytosine. Incorporation into DNA of uracil opposite to guanine is catalyzed by AP endonuclease and by the sugar-phosphate backbone 5′-OH end. The removal of the baseless sugar residue and the creation of a free 3′-OH end enables the AP endonuclease to excise uracil from DNA. The sugar-phosphate backbone 5′-OH end also primes the excision of the uracil residue by the uracil DNA glycosylase enzyme family, the UNG2 enzyme (20). This uracil DNA glycosylase enzyme recognizes and excises uracil residues from DNA, thus initiating the BER pathway by creating an abasic site. The sugar-phosphate backbone 5′ of the abasic site is then cleaved by an AP-endonuclease, leaving a 3′-OH end. The removal of the baseless sugar residue and insertion of the correct nucleotide are performed by the polymerase β via its lyase and polymerase activities, respectively. Finally, the remaining nick is sealed by the XRCC1-ligase complex (2). The nuclei of human cells contain at least five distinct enzymes to excise uracil from DNA, namely UNG2, UNG2, TDG, MBD4, and SMUG1 (3–7). This redundancy of enzymatic activities required to process uracil residues from DNA argues for the importance of this process in the survival of the cell. In addition to UNGs, all free-living organisms also express the deoxyuridine triphosphatase (dUTPase) enzyme that prevents misincorporation of uracil residues into DNA but through a mechanistically different pathway by acting on the pool of intracellular nucleotides to maintain a low ratio of dUTP to dTTP.

In the viral kingdom, genomes of some DNA viruses, namely pox and herpes viruses, encode both UNG and dUTPase enzymatic activities (8, 9). dUTPase and/or UNG minus the mutants of herpes viruses replicate well in cultured dividing cells but are severely impaired in replication, neuroinvasiveness, and reactivation from latency in non-dividing neurons (10–12). Genomes of β-retroviruses such as Mazon Pfizer monkey virus and murine mammary tumor virus and non-primate lentiviruses such as Visna-maedi virus, caprine arthritis-encephalitis virus, feline immunodeficiency virus, and equine infectious anemia virus encode only dUTPase (13). The presence of these virally encoded enzymes in viral particles suggests that they might play an important role in the viral life cycle. Indeed, dUTPase-deficient non-primate lentiviruses replicate well in dividing cells but are impaired in replication in non-dividing macrophages or resting T-cells and display a high level of G-to-A mutations and a loss of invasiveness and pathogenicity in vivo (14–18). These data strongly argue for the necessity to control the incorporation of uracil residues into proviral DNA in the course of infection of non-dividing cells.

Interestingly, genomes of primate lentiviruses such as human immunodeficiency viruses 1 and 2 (HIV-1 and HIV-2) and simian immunodeficiency viruses encode neither UNG nor dUTPase. Because primate lentiviruses infect non-dividing macrophage cells that are characterized by low intracellular dNTP pool levels with a low dCTP/dTTP ratio and a high level of dUTP pool (19), they have a non-negligible probability to misincorporate uracil residues into their genome during viral DNA synthesis. Therefore, it is likely that they have evolved to control uracil misincorporation into viral DNA. Indeed, we have previously demonstrated that HIV-1 viral particles have the ability to package one of the members of the cellular uracil DNA glycosylase enzyme family, the UNG2 enzyme (20). This packaging occurred via a specific association with the integrase (IN) domain of the Gag-Pol precursor. We have proposed that
host UNG2 localized inside HIV-1 viral particles might have a role similar to that played by viral dUTPase encoded by non-primate lentiviruses (i.e. to prevent the fixation of uracil residues in DNA), avoiding the accumulation of G-to-A mutations in the viral genome. The presence of UNG2 in HIV-1 viral particles highly suggests that HIV-1 might have the ability to control the level of uracil residues misincorporated during viral DNA synthesis.

In this study, we investigated the functional role of virion-associated UNG2 by examining the ability of wild-type or UNG2-deficient HIV-1 viral lysate to process uracil residues from a primer-template DNA substrate containing G:U mismatched pairs. For this purpose, we first demonstrated that leucine residue 172 of IN was critical for UNG2 binding and that recombinant HIV-1 viruses carrying the L172A mutation were impaired for UNG2 packaging into viral particles. We then demonstrated that (i) HIV-1 wild-type viral lysate is competent to correct G:U mismatches to G:C pairs from a uracil-containing primer-template DNA substrate, (ii) virion-associated UNG2 is a critical component for this process, and (iii) UNG2 and RT can be physically associated. Our data support the notion that HIV-1 virion-associated UNG2 initiates the processing of uracil residues from DNA and that HIV-1 viruses are able to counteract the mutagenic threat of uracil misincorporation into DNA during viral DNA synthesis.

**EXPERIMENTAL PROCEDURES**

**GST Fusion Plasmid Constructions and GST Pull-down Assays—** The IN open reading frame was amplified by PCR from the HIV-1NL43 molecular clone and subcloned in-frame into the pGEX-5X-2 plasmid vector (Amersham Biosciences) to obtain a resulting plasmid encoding a glutathione S-transferase (GST)-wild-type IN fusion protein. Site-directed mutagenesis was performed by PCR amplification procedures to obtain IN point mutants. The cDNA encoding the UNG2 sequence (21) was amplified by PCR and cloned in-frame with the GST-amino terminus. GST fusion proteins and His-tagged UNG2 were expressed in E.coli BL21 codonPlus RIL (Stratagene). The purification of bacterially expressed GST derivatives was performed as reported previously (23). Experimental procedures for GST pull-down assays were done as described previously (20). GST recombinant derivatives and protein substrates were allowed to interact on a rotating wheel for 2 h at 4°C in 20 mM Hepes, pH 7.6, 250 mM NaCl, 5 mM MgCl₂, 5 mM dithiothreitol, and 0.5% Nonidet P-40 and then were washed four times with 1 ml of incubation buffer. Anti-UNG2 and anti-IN antibody were kind gifts from G. Slupphaug and D. Trono, respectively. Anti-RT and anti-CA antibodies were purchased from Intracel and Aalto BioReagents, respectively. Primary antibody binding was revealed by horseradish peroxidase-conjugated secondary antibody (DAKO). The horseradish peroxidase activity was detected with ECL Western blotting detection reagents (Amersham Biosciences). Recombinant p66/p51 RT heterodimer, p66/p66 RT homodimer, and p51/p51 RT homodimer were purified until near homogeneity as reported previously (24). Recombinant His₄₅-tagged UNG2 was purified until near homogeneity according to Slupphaug et al. (21).

**Molecular Cloning and Purification of Viruses—** Viral molecular clones used in this study were those from HIV-1NL43 and HIV-1AD8. The complete sequence of the 70-mer oligonucleotide containing primer-template DNA substrate (5′-CAGTCTCCCTACTTCAATTCCTCCCTCTC-ACACCTTCTCCCGTCAGTCTTGGCGCCCGTCCCTC-3′). In the duplex, the uracil residue is included opposite to guanine within the palindromic sequence recognized by the SaI restriction enzyme. We also used a 32/70-mer primer-template DNA substrate but with a thymine at position 32 so that a G:T mispair was created. The mismatch-containing substrate processing was assayed as described previously (26). Viral lysate was incubated with 2 ml of labeled uracil-containing DNA substrate in a final volume of 20 μl of a buffer containing 20 mM Hepes, pH 7.6, 50 mM KCl, 10 mM MgCl₂, 10 μM ZnCl₂, 0.05% Triton X-100, 1 mM dithiothreitol, 5% glycerol, and 100 μg/ml bovine serum albumin in the presence of 200 μM of each of the four dNTPs. After incubation at 37°C for 30 min and extraction with phenol/chloroform, DNA was recovered by ethanol precipitation in the presence of 3 μg of tRNA carrier and subjected to SaI digestion (2 units/point) for 60 min at 37°C in a final volume of 4 μl. Four microliters of formamide loading dye was then added, and samples were heated at 75°C for 3 min and resolved by electrophoresis on a 8% urea-containing 15% polyacrylamide gel in 1× TBE buffer. Radiolabeled DNA products were revealed by autoradiography.

**RESULTS**

**Mapping of the UNG2 Epitope within IN—** In a previous study (20), we reported that HIV-1 viruses deficient for the expression of IN (HIV-1 ΔIN) failed to incorporate host UNG2, indicating that IN acts as a carrier to package UNG2 into viral particles. We proposed that virion-associated UNG2 in a manner similar to its cellular counterpart might have a role in controlling misincorporation of uracil residues into DNA during viral DNA synthesis. Because it has been reported that HIV-1 ΔIN was impaired for efficient viral DNA synthesis (27), we decided to analyze the role of virion-associated UNG2 in the processing of uracil residues by using viruses containing point mutations into IN so that the host UNG2 packaging was altered but not the reverse transcriptase activity.

To map residues of IN required for association with UNG2, we generated an affinity matrix consisting of a glutathione S-transferase (GST) fused to HIV-1 N-terminal deletions (Fig. 1A) or C-terminal deletions (Fig. 1B) and immobilized on glutathione-agarose beads. Recombinant purified His₄₅-tagged UNG2 was incubated with matrix, and matrix-bound UNG2 was analyzed by SDS-PAGE followed by Western blotting. Similar amounts of GST derivatives were used as judged by Coomassie Blue staining. Results showed that all of the deleted INs still retained their ability to bind UNG2 with the exception of when the region encompassing residues 170–180 was missing. These data indicate that residues 170–180 are required for UNG2 binding.

To determine what residues within the region 170–180 of IN were critical for the UNG2 binding, we generated IN point mutants by alanine-scanning mutagenesis of residues 170–181. Each of the mutants was expressed as a GST fusion protein, and GST pull-down assays were carried out using recombinant His₄₅-tagged UNG2. Results from Fig. 2, left panel, revealed that the mutation of both leucine 172 and lysine 173 residues of IN failed to retain UNG2. All other mutations did not affect the binding of UNG2. To map precisely the residue of IN critical for UNG2 binding, we introduced single point mu-
tations on residues belonging to the region 170–173 of IN. As shown in Fig. 2, right panel, the mutation of residues 170, 171, and 173 has no effect on UNG2 binding. In contrast, the L172A mutation impaired the binding of UNG2. These data indicate that leucine residue 172 is important for the interaction of IN with UNG2, although it cannot be ascertained that leucine 172 is the binding residue of UNG2.

Incorporation of Host UNG2 into IN Point Mutants—We then analyzed whether L172A IN mutation inserted in the context of the HIV-1NL4.3 molecular clone impaired UNG2 packaging into viral particles. Recombinant viral molecular clones were engineered to express a IN gene with point mutations in the 170–181 region and used to transfect 293T cells. Viruses produced in the cell-free supernatant were highly purified by Optiprep gradient velocity centrifugation. Each of the viral lysates was then assayed by Western blotting for the packaging of host UNG2. As shown in Fig. 3, left panel, UNG2 was undetectable in viral lysate from viruses either deficient for IN (ΔIN) or containing L172A/K173A IN mutations. In contrast, all other point mutant viruses exhibited levels of virion-associated UNG2 similar to that of wild-type viruses. The mutation of leucine residue 172 of IN abrogated the packaging of UNG2 (Fig. 3, right panel). Similar amounts of each of the viral lysates were used as judged by amounts of CA p24 antigen revealed by Coomassie Blue staining of the gel. In addition, Western blotting with anti-CA and anti-IN antibody revealed that Gag and Gag-Pol precursors in viruses carrying IN mutations were processed as well as in wild-type viruses, indicating that mutations did not induce obvious viral protein alterations. These in vivo data are consistent with in vitro data and demonstrate that leucine residue 172 of IN is required to fully package host UNG2 into viral particles.

Correction of G:U Mispairs to G:C Pairs by Wild-type but Not 172/173 IN HIV-1 Viral Lysate—The role of the cellular UNG2
**Fig. 3.** Leucine residue 172 of IN is important for host UNG2 packaging into viral particles. 293T cells were transfected with HIV-1 molecular clones containing point mutations within the 170–181 IN region, and viruses produced in the cell-free supernatant were purified by Optiprep gradient velocity centrifugation. Purified viruses were solubilized, and viral lysate was analyzed by Western blot for the presence of host UNG2 (upper panel). Expression of mutated IN in viral lysate was visualized by using anti-IN antibody (middle panel). Amounts of viral lysate were estimated by amounts of CA p24 antigen revealed with anti-p24 antibody (lower panel). Molecular mass markers are shown in kilodaltons.

DNA repair enzyme is to initiate the BER process by excising misincorporated uracil residues from DNA. We designed experiments to analyze whether virion-associated UNG2 plays a role similar to that of its cellular counterpart. The uracil-containing DNA substrate (32/70-mer) was composed of a 5’ end-labeled 32-mer primer oligonucleotide with a uracil residue at position 32 annealed with the unlabelled complementary 70-mer template oligonucleotide (Fig. 4A). In the primer-template DNA substrate, the uracil residue is included opposite to guanine residue within the palindromic sequence recognized by the SalI restriction enzyme. The presence of uracil residue opposite to guanine residue in the SalI sequence has been reported to prevent DNA cleavage by SalI (26). The restoration of the SalI digest implies that the uracil residue in G:U mismatches was corrected to G:C pairs.

The 32/70-mer DNA substrate was incubated with increasing amounts (0.3, 0.6, and 1.2 µg of CA p24 antigen) of highly purified HIV-1 wild type or L172A/K173A IN mutant viral lysate in the presence of each of the four dNTPs. The substrate was then recovered by ethanol precipitation and subjected to SalI digestion. DNA products were resolved on urea-denaturing gel and analyzed by autoradiography (Fig. 4B). Efficient DNA exonuclease digestion of the primer by increased levels of wild-type or L172A/K173A IN viral lysate was evidenced by the appearance of increased levels of the labeled 70-mer product concomitant with decreased levels of the labeled 32-mer primer. A proportion of the DNA substrate upon incubation with increasing amounts of wild-type viral lysate became susceptible to cleavage by SalI as judged by the appearance of a dose-dependent labeled DNA product with the expected size (27-mer) for the SalI digestion product. This results presumably from a specific repair of G:U mismatched pairs to G:C pairs. In contrast, incubation of the DNA substrate with similar amounts of L172A/K173A IN viral lysate failed to generate the 27-mer SalI digestion product. The failure to observe uracil processing upon incubation of the DNA substrate with UNG2-deficient viruses serves as an internal control to certify that cellular proteins in micrococcal nuclease did not significantly contaminate our purified virion preparations (28) and to eliminate the possibility that the G:U versus G:C correction was the result of a virion-independent endonucleolytic processing of the substrate. As a control, we incubated a primer-template DNA substrate containing a G:U mismatch instead of a G:U mismatch with increased amounts (0.3, 0.6, and 1.2 µg of CA p24 antigen) of wild-type viral lysate, and we subjected it to SalI digestion. As shown in Fig. 4B, right panel, no SalI digest product was observed, indicating the specificity of the repair process. Altogether, our data indicate that uracil residues are corrected from G:U mismatches to G:C pairs in the presence of HIV-1 viral lysate and that virion-associated UNG2 plays a key role in initiating the repair process.

**Physical Association of UNG2 with the Viral Reverse Transcriptase Enzyme—**In a previous study, we reported that a G:U mispair buried in the central part of a double-stranded DNA substrate was not corrected to a G:C pair by HIV-1 viral lysate (20). In contrast, we observed here the correction of G:U mispairs located near the free 3’-OH end of the primer in the primer-template DNA substrate. The presence of a free 3’-OH end in close proximity to the G:U mispair to anchor the viral RT enzyme on the DNA primer seems to be important for uracil processing. We hypothesized that UNG2 and RT might be spatially close to act in a concerted manner to process uracil.

Therefore, we examined whether UNG2 and RT have the ability to associate. GST affinity matrix containing HIV-1 IN, HIV-1 Pr55<sup>Gag</sup>, or UNG2 was incubated with recombinant purified p66/p51 RT heterodimer, and matrix-bound RT was analyzed by SDS-PAGE followed by Western blotting (Fig. 5, left panel). RT binds to HIV-1 integrase as reported previously (29) as well as to UNG2 but not to GST or GST-Pr55<sup>Gag</sup>. The possibility that nucleic acids interfered in the interaction RT-UNG2 was ruled out by pretreating the mixture of RT-GST derivatives with micrococcal nuclease and RNase A, which did not decrease amounts of the RT pull-down assay (data not shown). We also tested the ability of p66/p66 RT homodimer (Fig. 5, middle panel) or p51/p51 RT homodimer (Fig. 5, right panel) to associate with UNG2. The results indicated that each of the homodimers still retained UNG2 association, suggesting that the RNase H domain of RT was probably not involved in the interaction with UNG2. These data indicate that UNG2 and RT physically associate.

**DISCUSSION**

We have previously reported that HIV-1 viral particles have the unique property of packaging the host uracil DNA glycosylase DNA repair enzyme UNG2 into viral particles (20). In this paper, we investigated the functional role of virion-associated UNG2 in uracil processing by studying the ability of HIV-1 viral lysate to correct G:U mismatched pairs incorporated into a primer-template DNA substrate. The repair of G:U mismatches to G:C pairs was revealed by the restoration of the cytidine residue instead of the uridine residue in the nucleotidic se-
We showed that HIV-1 wild-type viral lysate, but not UNG2-deficient viral lysate, can process uracil residues when they are present at the free 3'-OH end of the primer in the primer-template DNA substrate. In our early study (20), we showed that HIV-1 viral lysate failed to process uracil residues present in a G:U mispair located in the middle of a blunt double-stranded DNA substrate, probably because of the absence of a free 3'-OH end to anchor RT on its primer. In contrast, recombinant UNG2 alone efficiently processed uracil residues in this

![Diagram](image)

**Fig. 4. Processing of uracil-containing DNA substrate by HIV-1 viral lysate.** A, the 32/70-mer uracil-containing substrate used is depicted. Asterisk indicates the 5' end-labeling. The *SalI* restriction site is boxed. B, the DNA substrate containing G:U mispairs was incubated with increasing amounts (0.3, 0.6, and 1.2 μg of CA p24 antigen) of wild-type or 172/173 IN viral lysate and subjected to *SalI* restriction enzyme digestion. Resulting DNA products were resolved on a 15% denaturing gel and revealed by autoradiography. As a control (right panel), viral lysate was incubated with a DNA substrate containing G:T mispairs instead of G:U pairs. Arrow indicates the position of the *SalI* digest product.

![Diagram](image)

**Fig. 5. Physical association of UNG2 with RT.** Left panels, recombinant purified p66/p51 RT heterodimer incubated with equivalent amounts of GST, GST-HIV-1 IN, GST-UNG2, or GST-Pr55^Gag^ fusion proteins affinity-purified on glutathione-agarose beads. After washes, bound proteins were analyzed by Western blot with anti-RT antibody. Middle panels, GST pull-down assays performed with GST derivatives and the p66/p66 RT homodimer. Right panels, GST pull-down assays performed with GST derivatives and the p51/p51 RT homodimer. Lane marked input contains one-fifth of RT before binding to GST derivatives. Similar amounts of GST derivatives were revealed by Coomassie Blue staining of the gel. Molecular mass markers are shown in kilodaltons.
case. These findings suggested that UNG2, in the context of HIV-1 viral particles, might act in a manner distinct from that of recombinant UNG2. Indeed, we demonstrated that (i) UNG2 is associated and packaged with IN, (ii) UNG2 can associate with RT, and (iii) RT and IN are associated. This latter association has been previously reported to be critical for the initiation of the retrotranscription of viral genome (29). We hypothesized that RT, IN, and UNG2 are physically associated and are part of the viral retrotranscription complex. As a consequence of such a multi-enzymatic complex, UNG2 would probably not be free in the viral particle and could act only on uracil residues in close proximity of a free 3′-OH anchor for the retrotranscription complex. On the basis of this hypothesis, we proposed that DNA polymerization by RT and DNA repair initiated by virion-associated UNG2 are coupled events.

The viral retrotranscription process is a two-step process consisting of the synthesis of the minus-strand DNA using viral RNA as a template followed by the synthesis of the plus-strand DNA using the minus-strand DNA as a template to obtain the proviral double-stranded DNA. One mispairing mutation occurring in the minus-strand DNA of the heteroduplex is mutagenic and is fixed in the plus-strand DNA of the DNA/DNA homoduplex constituting the proviral genome. For example, dUMP residues in the minus-strand DNA will be paired with dAMP residues during the synthesis of the plus-strand DNA, contributing to the accumulation of G-to-A substitutions. Moreover, we have not looked at the repair process for RNA/DNA heteroduplexes, which are intermediate products formed during the retrotranscription process, but a previous study (30) have shown that the repair process of a RNA/DNA duplex was effective, albeit with a reduced efficiency, compared with that on a DNA/DNA duplex. In conclusion, we propose that HIV-1 viruses with an enhanced efficiency, compared with that on a DNA/DNA duplex, might act in a manner distinct from that of cellular BER and thus contribute to the accumulation of G-to-A substitutions. We have not looked at the repair process for RNA/DNA heteroduplexes, which are intermediate products formed during the retrotranscription process, but a previous study (30) have shown that the repair process of a RNA/DNA duplex was effective, albeit with a reduced efficiency, compared with that on a DNA/DNA duplex. Therefore, we speculate that HIV-1 viruses did not contain a AP-endonuclease activity. An alternative hypothesis would be that the creation of an abasic site by the virally associated UNG2 is followed by the elimination of the baseless nucleotide by the pyrophosphorylolytic activity of RT, leaving a free 3′-OH end to be used by the polymerase activity of RT. Further studies are in progress to understand the molecular mechanism(s) involved in viral BER.

The fact that the HIV-1 reverse transcription process is mainly accomplished in the cytoplasm of infected cells and the fact that cellular enzymes participating in the DNA repair process are nuclear proteins might explain why HIV-1 particles have to be equipped with their own enzymatic DNA repair activities. In conclusion, we propose that HIV-1 viruses in the manner of non-primate lentiviruses, pox viruses, or herpes viruses which replicate in non-dividing cells, have developed an original strategy to bypass the deleterious consequence of uracil in viral DNA by using IN as a carrier to package host UNG2 into viral particles and by using RT to target UNG2 at the site of nascent DNA synthesis.

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