Editing of HIV-1 RNA by the double-stranded RNA deaminase ADAR1 stimulates viral infection

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ABSTRACT

Adenosine deaminases that act on dsRNA (ADARs) are enzymes that target double-stranded regions of RNA converting adenosines into inosines (A-to-I editing) thus contributing to genome complexity and fine regulation of gene expression. It has been described that a member of the ADAR family, ADAR1, can target viruses and affect their replication process. Here we report evidence showing that ADAR1 stimulates human immuno deficiency virus type 1 (HIV-1) replication by using both editing-dependent and editing-independent mechanisms. We show that over-expression of ADAR1 in HIV-1 producer cells increases viral protein accumulation in an editing-independent manner. Moreover, HIV-1 virions generated in the presence of over-expressed ADAR1 but not an editing-inactive ADAR1 mutant are released more efficiently and display enhanced infectivity, as demonstrated by challenge assays performed with T cell lines and primary CD4+ T lymphocytes. Finally, we report that ADAR1 associates with HIV-1 RNAs and edits adenosines in the 5′ untranslated region (UTR) and the Rev and Tat coding sequence. Overall these results suggest that HIV-1 has evolved mechanisms to take advantage of specific RNA editing activity of the host cell and disclose a stimulatory function of ADAR1 in the spread of HIV-1.

INTRODUCTION

One of the best-characterized mechanisms of RNA editing is the conversion of adenosine to inosine (A-to-I) mediated by the Adenosine DeAminase enzymes that act on double-stranded RNA or ADARs. In mammals, three different ADAR enzymes have been identified: ADAR1, ADAR2 and ADAR3 (1–3). ADAR1 and ADAR2 are expressed in many different tissues (4,5), while ADAR3 is expressed exclusively in the brain and is inactive on all the RNA substrates tested *in vitro* (6,7). The common structural features shared by ADARs include the N-terminal double-stranded RNA-binding domains (dsRBDs) and the catalytic domain at the C-terminus. Human cells express two different ADAR1 isoforms: a constitutive 110-kDa protein (ADAR1 p110) and an interferon inducible 150-kDa protein (ADAR1 p150) (8). ADAR1 exhibits some features that make this enzyme different from the other two: the presence of two Z-DNA-binding domains and an extra dsRBD at the amino terminus.

Inosine acts as guanosine during both splicing and translation events (9,10), therefore A-to-I editing within pre-mRNA can alter both splicing patterns and amino acid sequence with important consequences for the final function of the coded protein. Indeed, it has been shown that RNA editing can profoundly affect the bio-chemistry of receptors expressed in the brain such as the glutamate receptor GluR-B, a subunit of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), and the serotonin receptor 2C (5-HT$_{2C}$) (2,3). Recent evidence demonstrated that most of the A-to-I substitutions occur within non-coding sequences of pre-mRNAs enriched in inverted repeated Alu elements, such as introns and untranslated regions (UTRs) (11–13). RNA editing of non-coding sequence can alter the fate of pre-mRNAs by affecting their splicing, localization, stability or translation (1,3).

ADARs can target viruses, as suggested by numerous reports showing A-to-I changes identified in viral genomes or transcripts that are consistent with editing mediated by

these enzymes (1,14). This is the case in multiple editing events described for several negative-stranded RNA viruses such as measles virus, human parainfluenza virus 3 and respiratory syncytial virus (15), although their functional consequences are poorly understood. A direct effect of RNA editing mediated by ADAR1 has been clearly demonstrated for hepatitis C virus (HCV). The A-to-I editing of multiple sites within the HCV RNA replicon impairs viral replication and leads to its clearance from infected cells (16). There is also evidence of highly selective editing of viral RNA mediated by ADAR1, for example the A-to-I editing of the amber/w site in the antigenomic RNA of hepatitis delta virus (HDV), a change that is essential for viral replication (17,18).

Despite an increasing attention on the role of A-to-I RNA editing in the biology of viruses, so far little effort has been dedicated to testing the involvement of ADARs in the life cycle of the human immunodeficiency virus type 1 (HIV-1). HIV-1 gene expression is tightly regulated at the level of transcription and maturation of an unspliced primary transcript (9-kb RNA) in distinct classes of partially and completely spliced RNA molecules (4-kb and 2-kb RNAs). This is accomplished by a coordinated interaction between viral and cellular factors (19,20). In addition, HIV-1 RNAs contain several double-stranded regions, some of them critical for the different steps of the viral life cycle such as the Rev responsive element (RRE), *trans*-activation responsive element (TAR) and dimerization domain (DIS) (19,21,22) that could be possible ADARs substrates. So far, a report showed A-to-I editing of TAR sequence in *Xenopus laevis* (23) and, in a more recent study, ADAR1 was shown to edit HIV-1 RNA and enhance the expression of p24 Gag protein (24).

The aim of this study was to further investigate the role of ADAR1 in the regulation of HIV-1 replication. Here we report for the first time that the RNA editing activity mediated by ADAR1 stimulates the release and the infectious potential of HIV-1 progeny viruses.

MATERIALS AND METHODS

DNA constructs

The full-length hADAR1 was inserted into the XbaI restriction site of the pEGFP-C3 (Clontech) with in-frame EGFP (enhanced green fluorescent protein) at the N-terminus in order to generate an EGFP-ADAR1 construct. A single point mutation in the catalytic domain of ADAR1 was introduced directly into the EGFP-ADAR1 construct using a site directed mutagenesis kit (Stratagene) in order to change the amino acid E955 into A955 within the catalytic domain (changing the sequence from GAA to GCA) giving rise to the inactive mutant EGFP-ADAR1 E/A. The PCR reaction was performed following the manufacturer's instructions. The EGFP-ADAR1 and the inactive EGFP-ADAR1 E/A were tested for the editing activity assay in a cell system (293T) using the miniB13 (encoding a portion of the editing-competent murine glutamate receptor GluR-B gene) as substrate. All the DNA constructs were confirmed by sequencing. The pEGFP-CTNS (cystinosin-expressing construct) was kindly provided by Dr A. Taranta and Dr F. Emma, Bambino Gesù Children Hospital, Rome, Italy.

Cells and antibodies

293T cells were maintained in Dulbecco's modified Eagle's medium. Jurkat E6-1, U937 and CEM-GFP cells (25) were maintained in RPMI 1640 medium. Both media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin–streptomycin. The medium for CEM-GFP was also supplemented with 100 μg/ml G-418. Tissue culture reagents were from GIBCO-BRL. Western blotting analysis was performed with the following antibodies: polyclonal rabbit anti-ADAR1 developed 'in house' (26), polyclonal rabbit anti-p24 (kindly provided by Dr O. T. Fackler, Heidelberg, Germany), anti-pThr451PKR (Santa Cruz), anti-PKR (BD Biosceces), and anti-pS51 eIF-2α and eIF-2α (Cell Signalling), polyclonal goat anti-gp160/gp120 (HT3, kindly provided by Dr M. Federico, Istituto Superiore di Sanità, Rome), polyclonal sheep anti-Nef (clone 444, kindly provided by Dr M. Harris, Leeds, UK), monoclonal anti-tubulin (Sigma) and anti-GFP (Clontech), and human serum from HIV-1-infected individual (kindly provided by Dr M. Federico). The PE-conjugated anti-HIV p24 monoclonal antibody (KC57-RD1, Coulter Immunology) was used for flow cytometry.

HIV-1 expression and detection

293T cells were transfected with the indicated amounts of pNL4-3 proviral plasmid (NIH reagent program) alone or with pEGFP-derived vectors by using the standard calcium-phosphate method. Forty-eight hours post-transfection, cells were analyzed by western blotting and culture supernatants were collected, clarified by low-speed centrifugation, and stored in aliquots at –80°C. Viral stocks were titrated by anti-p24 ELISA (Immunogenetics NV) according to the manufacturer's instructions.

HIV-1 infection

To evaluate viral infectivity, CEM-GFP indicator cells were infected in triplicate for 4 h at 37°C with 400 ng of p24/5×10^4 cells of virus and 2 μg/ml of polybrene. This virus dose resulted in a percentage of infected cells that matched the linear range of infectivity, as determined in pilot experiments with serial dilutions of viral preparations. After viral exposure, cells were washed twice and resuspended at 5×10^5 cells/ml. After 40 h, cells were collected and the number of GFP+ cells was determined by flow cytometric analysis. For HIV-1 infection of primary human CD4+ T lymphocytes, purified cells were derived from healthy donors as described (27). To evaluate the efficiency of single-cycle infection, CD4+ T cells were infected by incubation for 4 h at 37°C with 100 ng of p24/10^6 cells of virus. Then, cells were washed twice, resuspended at 1.5×10^6/ml in complete RPMI medium supplemented with 100 IU/ml of human rIL-2, and stimulated by the addition of PHA (SIGMA) at a final concentration of 3 μg/ml and irradiated allogeneic PBMCs at a 1:1 ratio. Finally, infected CD4+ T cells were harvested

after 3 days for FACS analysis. The efficiency of viral infectivity was calculated as percentage of p24+ cells with the control virus produced by EGFP-expressing 293T set at a 100%.

Flow cytometry

For detection of intracellular p24 in HIV-1-infected $CD4^+$ T cells, 5×10^5 infected or uninfected cells were fixed and permeabilized with reagents from BD Biosciences and incubated with the PE-conjugated anti-HIV p24 mAb. Finally, cells were washed, resuspended in 1% paraformaldehyde and analyzed by flow cytometry on a FACSCalibur with CellQuest software (Becton Dickinson).

Western blotting analysis

Cells were lysed in buffer containing 1% Triton X-100 as described elsewhere (28). Equal amounts of total cellular lysates (40 μg) were separated by 10% SDS–PAGE, transferred on nitrocellulose and analyzed by immunoblotting with the appropriate antibodies and the ECL system (Amersham Pharmacia Biotech) as previously described (28). The proteins' specific signals were quantified by densitometric analysis.

Determination of HIV-1 RNA editing sites

Total RNA was isolated with TRIzol reagent (Invitrogen) from transfected 293T cells according to the manufacturer's instructions. The cDNA pools generated by SuperScript III reverse transcriptase (Invitrogen) using 1 μg of total RNA and random hexamer primers were amplified by PCR using Expand High Fidelity PCR System (Roche) and specific primers for the analysis of different HIV-1 RNA regions. TAR5′ (5′-GGGTCTCTC TGGTTAGACCAG-3′) and TAR2R (5′-CTGCTAGAG ATTTTCCACACTG-3′) were used to amplify a fragment of 181 bp containing a region of the 5′ UTR shared by all the viral transcripts. Rev-forward (5′-ATGGCAGGAAG AAGCGGAGAC-3′) and Rev-reverse (5′-CTATTCTTT AGTTCCTGACTCC-3′) were used to amplify a fragment of 351 bp containing the Rev mRNA coding sequence. Tat-forward (5′-ATGGAGCCAGTAGATCC TAGAC-3′) and Tat-reverse (5′-CTAATCGAATGGAT CTGTCTCTG-3′) were used to amplify a fragment of 306 bp containing the Tat mRNA coding sequence. For the sequence analysis of the 5′ UTRs harbored in the three different HIV-1 RNA species (2 kb, 4 kb and 9 kb), the cDNA pools were first subjected to PCR reaction using the TAR5′ forward primer and three different reverse primers as previously described (29) to amplify specific fragments of the three viral RNA species. The resulting PCR products were subjected to a second PCR reaction with TAR5′ and TAR2R primers to amplify a fragment of 181 bp containing part of the 5′ UTR. The resulting RT–PCR products were analyzed by direct sequencing. The extent of editing at each site was determined by using the electropherograms from the sequencing reactions to estimate the relative amounts of A and G (30). Total RNA isolated from three independent

co-transfection experiments was used for RT–PCR and sequencing analysis.

Immunoprecipitation experiments and RT–PCR analysis

Transfected 293T cells were homogenized and immuno-precipitated with anti-ADAR1 antibody as previously described (26). The resulting beads were washed five times with PBS 1× and one-fourth of them were resuspended in SDS sample buffer for western blotting analysis. The remaining beads were first incubated with lysis buffer in the presence of 60 units RQ1 DNase (RNase free) for 30 min at 37°C, washed three times with PBS 1× and then incubated with lysis buffer with 5 mM EDTA, 0.5% SDS and 50 μg proteinase K (Promega) for 20 min at 50°C. Co-precipitated RNA was isolated by phenol and chloroform extraction method followed by ethanol precipitation. Each RNA sample was DNase treated (Promega, RQ1 DNase) and used for RT–PCR experiments using random hexamer and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The resulting cDNA was amplified by PCR using the TAR5′ and TAR2R primers (see above) to detect the TAR hairpin located at the extreme 5′ termini of all HIV transcripts, the C primer (5′-CTTCTAAGCATCTT AAATTTGC-3′) and D primer (5′-TTTAGCCTATGAG ATCTGGATGTGC-3′) to detect the miniB13 transcript, and the GAPDH5′ (5′-ACCACAGTCCATGCCA TCAC-3′) and the GAPDH3′ (5′-TCCACCACCCTGTT GCTGTA-3′) primers to detect GAPDH mRNA. To determine the HIV-1 RNA species that are specifically co-immunoprecipitated with anti-ADAR1 antibody, three different reverse primers were used as previously described (29) while TAR5′ was used as the forward primer.

RESULTS

T lymphocytes express ADAR1

To establish a model system for studying the interaction between ADAR1 and HIV-1, we first evaluated ADAR1 expression in viral cell targets (primary $CD4^+$ T lymphocytes, Jurkat leukaemic T cell line, and U937 monocyte lymphoma cell line) as well as in 293T, an embryonic kidney cell line that is widely used for *in vitro* HIV-1 production. Western blotting analysis of total cell lysates showed that ADAR1 is expressed in both $CD4^+$ T and Jurkat cells (Figure 1), in agreement with previous
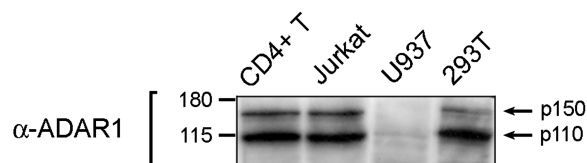

Figure 1. ADAR1 expression in HIV-1 target cells. An amount of 40 μg of total cell lysates from freshly purified $CD4^+$ T lymphocytes, Jurkat, U937 and 293T cells were analyzed by western blotting analysis with anti-ADAR1 rabbit serum. Both the long (p150) and short (p110) isoforms of ADAR1 are indicated.

studies describing A-to-I RNA editing in human T lymphocytes (31–33). 293T cells express comparable levels of ADAR1 (Figure 1), although these cells display very low/undetectable editing activity on endogenous or plasmid-derived RNA substrates of ADAR enzymes (26,34,35). In contrast, U937 cells express only barely detectable levels of ADAR1 p110. Thus, U937 cell line was excluded from further analysis and this study focused on a T-tropic HIV-1 strain, NL4-3.

HIV-1 protein expression is regulated by ADAR1 independently of its editing activity

Recent evidence suggested that ADAR1 enhances the expression of the p24 Gag protein of HIV-1 (24). To further investigate this phenomenon, 293T cells were co-transfected with the NL4-3 proviral DNA and a vector expressing EGFP-ADAR1 or EGFP-ADAR1 E/A mutant that lacks editing activity. For controls, a vector expressing an unrelated EGFP-tagged protein (cystinosin or CTNS) or, alternatively, pEGFP vector alone was used. Comparable transfection efficiency was confirmed by determining the percentage of EGFP-positive cells by flow cytometric analysis (data not shown). Forty-eight hours post-transfection, total cell extracts were prepared and analyzed by western blotting with antibodies specific for EGFP, HIV-1 Nef protein, tubulin, and with a total serum of an HIV-infected patient. Strikingly, the entire set of viral proteins detected with the anti-HIV-1 human serum was strongly enhanced over-expressing ADAR1 if compared to the control EGFP-CTNS- or EGFP-expressing cells (Figure 2A and B). In addition, a similar increase in HIV-1 proteins was observed upon over-expression of the ADAR1 E/A mutant (Figure 2A). The levels of three HIV-1 proteins, gp120 envelope protein, p24 Gag capsid antigen, and the regulatory Nef protein, were analyzed with specific antibodies (Figure 2A and data not shown) and calculated in four independent experiments by considering as 100% the relative expression in control cells (Figure 2C). Results showed that over-expression of ADAR1 increases the levels of gp120, p24 and Nef by about 3-, 7- and 2-fold, respectively. Importantly, since a similar effect was observed also with the ADAR1 E/A mutant, the function of ADAR1 on HIV protein expression is editing-independent. Indeed, ADAR1 was previously shown to stimulate the replication of vesicular stomatitis virus (VSV) in an editing-independent manner through the inhibition of the RNA-activated protein kinase PKR that would otherwise shut down protein synthesis by phosphorylating the α subunit of eukaryotic initiation factor 2, eIF-2α (36). Therefore, we tested the status of PKR and its substrate eIF-2α in our experimental cell system by performing western blotting analysis with specific antibodies against total PKR and eIF-2α proteins and against their phosphorylated forms (pThr451PKR and pS51 eIF-2α). As shown in Figure 2D, upon expression of EGFP-ADAR1 with or without HIV-1 expression, the low basal level of phosphorylated PKR became undetectable and the phosphorylated eIF-2α was reduced by about 30%. Down-regulation of pThr451PKR and pS51eIF-2α was

also observed in cells expressing EGFP-ADAR1 E/A. These data suggest that the editing-independent capacity of ADAR1 to stimulate HIV-1 protein expression may be contributed by the enzyme's inhibitory activity on PKR function.

ADAR1 increases the release of progeny virions

To further investigate the role of ADAR1 in the biology of HIV-1, we analyzed the release of viral particles produced by cells over-expressing either EGFP-ADAR1 or EGFP-ADAR1 E/A mutant. 293T cells were transfected as described above and 48 h later p24 expression was measured in both cellular lysates and in cell culture media by means of western blotting analysis and ELISA, respectively (Figure 3A and B show a representative experiment). The relative HIV-1 release was calculated in four independent experiments by setting at a 100% the release of viral particles in the absence of ADAR1 over-expression (Figure 3C). We found that both EGFP-ADAR1 and EGFP-ADAR1 E/A over-expressed in producer cells increased the intracellular accumulation and the concentration in the cell supernatant of p24 (Figure 3A and B). However, over-expressed EGFP-ADAR1 but not EGFP-ADAR1 E/A resulted in a 2-fold higher release of viral particles (Figure 3C). These data show that the productive assembling and/or release of HIV-1 progeny virions can be stimulated by ADAR1 through its editing activity.

ADAR1 stimulates HIV-1 infectivity

Next, the impact of ADAR1 in the infectivity of viral particles was analyzed. The viruses produced by cells expressing EGFP, EGFP-ADAR1 or EGFP-ADAR1 E/A were quantified measuring by ELISA the p24 protein concentration in the culture supernatant and used for single-round infection of CEM-GFP indicator cells (400 ng of p24/5×10^4 cells). Figure 4A and B show that the virus produced by EGFP-ADAR1 + cells displayed an infectious capacity about 2.5-fold higher than that of virus released by control EGFP + cells. A smaller increase in viral infectivity was also observed when EGFP-ADAR1 E/A was expressed in producer cells, although it did not differ significantly from control virus. Moreover, the stimulatory effect of EGFP-ADAR1 on viral infectivity was dose-dependent (Figure 4C) and maintained when different cell targets were used such as Jurkat cells (data not shown) and, more importantly, primary CD4$^+$ T lymphocytes (Figure 4D and E). Taken together, these results indicated that ADAR1 increased the infectious potential of HIV-1 through an editing-dependent mechanism.

HIV-1 RNAs associate with ADAR1

Given that ADARs exert their editing activity upon binding to dsRNA, the physical interaction between ADAR1 and HIV-1 RNAs was tested by co-immunoprecipitation experiments. 293T cells were transiently co-transfected with miniB13 plasmid (encoding a natural substrate of ADAR1), and pEGFP-ADAR1 expression vector in the presence or absence of NL4-3 proviral DNA. 48 h post-transfection total cell extracts were prepared and immunoprecipitated with either anti-ADAR1 antibody
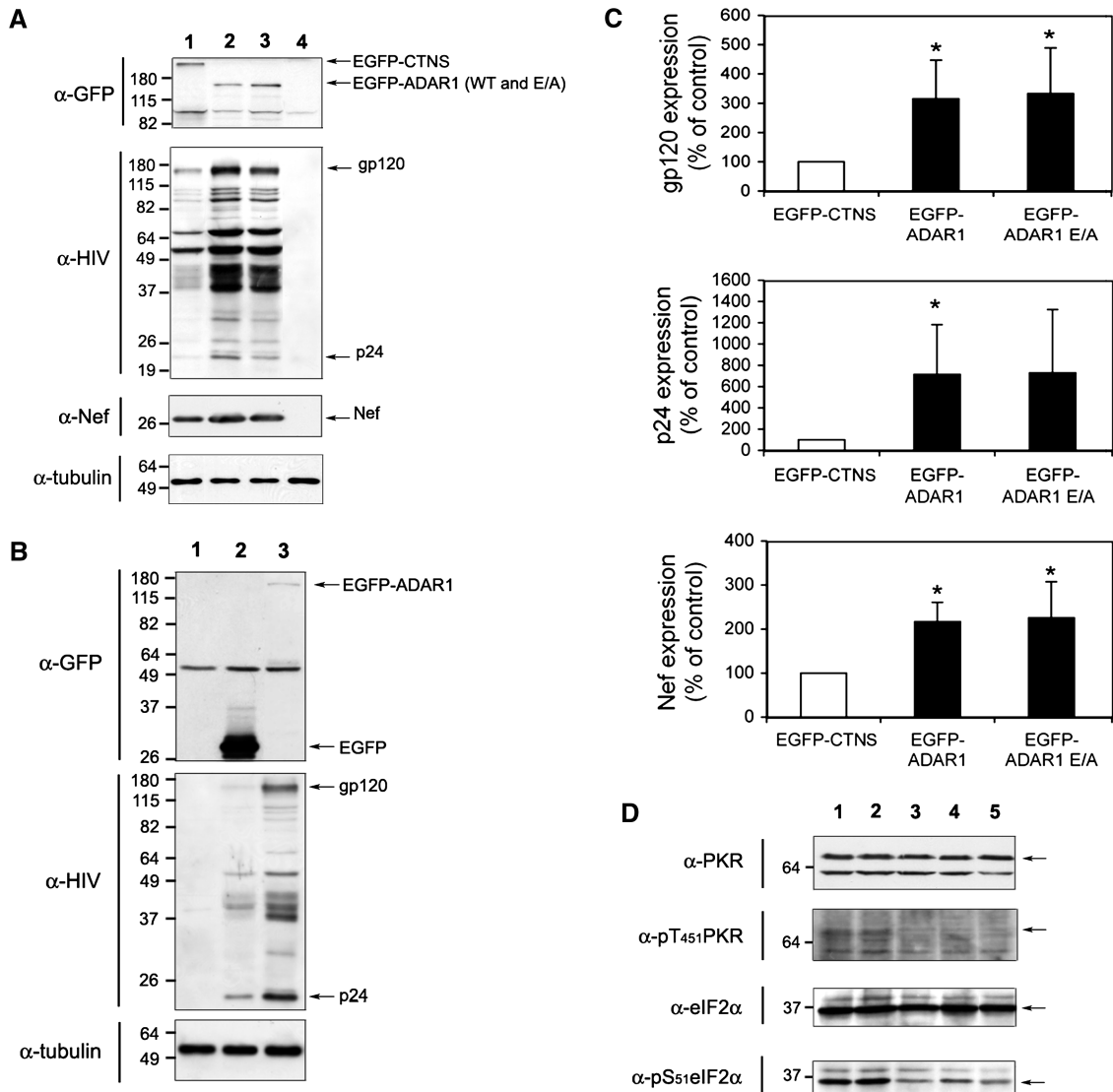
Figure 2. ADAR1 enhances HIV-1 protein expression. (**A**) Total cell lysates (40 µg) prepared from 293T cells transfected with 5 µg of pNL4-3 proviral plasmid together with 3 µg of pEGFP-CTNS, pEGFP-ADAR1 or pEGFP-ADAR1 E/A (lanes 1, 2 and 3) or mock transfected (lane 4) were analyzed by western blotting with antibody specific for GFP, HIV total proteins, Nef and tubulin. (**B**) Western blotting was performed as described in panel A with lysates of 293T cells mock transfected (lane 1), transfected with 5 µg of pNL4-3 together with 3 µg of pEGFP or pEGFP-ADAR1 (lanes 2 and 3). (**C**) The average levels of gp120, p24 and Nef HIV-1 proteins were calculated by densitometric analysis of the corresponding bands normalized by tubulin (data not shown) in four independent experiments like the one shown in panel A and by setting at a 100% the value obtained in EGFP-CTNS+ control cells. As calculated by paired *t*-test, significant differences between control and other samples are indicated (*$P < 0.05$). The error bars represent standard deviations (SD). (**D**) The expression of total or phosphorylated PKR and eIF-2α was evaluated by western blotting analysis of lysates from 293T mock transfected (lane 1), co-transfected with pNL4-3 and pEGFP-CTNS, pEGFP-ADAR1 or pEGFP-ADAR1 E/A (lanes 2, 3 and 4, respectively) or transfected with pEGFP-ADAR1 alone (lane 5).

or control rabbit IgGs. Western blotting analysis confirmed that ADAR1 was specifically immunoprecipitated with anti-ADAR1 antibody in the presence and absence of HIV-1 (Figure 5A and data not shown). An RT–PCR analysis performed on RNA extracted from the immunoprecipitates demonstrated that ADAR1 specifically binds both HIV RNAs and miniB13 transcript but not GAPDH mRNA (Figure 5B). Moreover, by performing RT–PCR analysis specific for the three different HIV-1 RNA classes (9-kb primary transcript, 4-kb singly spliced and 2-kb completely spliced RNAs), we demonstrated that all viral transcripts are specifically co-immunoprecipitated with ADAR1 (Figure 5C).

ADAR1 edits HIV-1 RNAs

Finally, we investigated whether ADAR1 binding to HIV-1 transcripts resulted in editing of viral sequences. Co-transfection experiments with NL4-3 proviral DNA and either pEGFP-ADAR1 or pEGFP-ADAR1 E/A were carried out in 293T cells. Total RNA isolated from the transfected cells was subjected to reverse transcription generating cDNA pools that were amplified by PCR using specific primers for the analysis of different HIV-1 regions and then sequenced. Inosine is read as guanosine during reverse transcription; therefore the A-to-I changes in the RNA appear as A-to-G changes in the
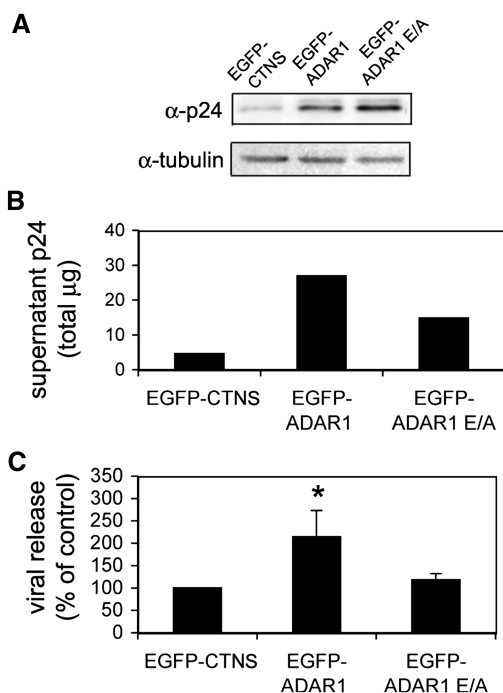
Figure 3. The release of HIV-1 virions is stimulated by ADAR1. 293T cells were co-transfected as described in Figure 1A with pNL4-3 and the indicated pEGFP-based vectors. (**A**) After 48 h, cell lysates were prepared and analyzed by western blotting with antibodies specific for p24 and tubulin to evaluate the steady-state expression of intracellular p24. (**B**) In addition, virion production from transfected 293T was measured by p24 capture ELISA of culture supernatant. (**C**) The efficiency of viral release was calculated by dividing the total amount of p24 in the culture supernatant by the amount of intracellular p24 normalized by tubulin (densitometric units) and by setting at a 100% the release of viral particles in the presence of EGFP-CTNS. Results shown are the mean ± SD of four independent experiments including the one shown in panels A–B ($^*P < 0.05$).

resulting PCR products. Since ADARs can mediate editing events in both non-coding and coding RNA, a region of the 5′ UTR shared by all HIV RNAs (rich in double-stranded hairpins that are crucial for viral replication) and Rev and Tat mRNA coding sequences were analyzed. This analysis revealed the presence of editing events in viral RNAs (Figures 6 and 7 and Supplementary Figures 1 and 2) isolated from 293T cells over-expressing EGFP-ADAR1 but not EGFP-ADAR1 E/A mutant or EGFP alone (Figure 6 and Supplementary Figure 1). In the 5′ UTR, five editing events were identified (Figure 6A and B), four of which (edited sites 520, 521, 551 and 552) occurred at sites located within the poly(A) hairpin that encompasses the AAUAAA polyadenylation signal (Figure 6C). The poly(A) hairpin is a functional element of the repeat (R) region of the 5′ UTR sequence and is reiterated at the extreme 3′ end of viral RNA where it is required for 3′ end formation. Notably, the analysis of the processed poly(A) hairpin located at 3′ terminus of the polyadenylated viral transcripts did not reveal any A-to-I modifications (data not shown).

Since we analyzed a region of the 5′ UTR shared by all HIV-1 RNAs, the A-to-I changes identified represent the

sum of all the editing events occurred in the various viral transcripts. Therefore, we further extended our analysis to distinguish the 5′ UTR harbored in each HIV-1 RNA class (9 kb, 4 kb or 2 kb) and determined that editing occurred in all viral transcripts, albeit with different efficiencies, with the highest level of editing found in the completely spliced 2-kb transcripts and the lowest in the 9-kb primary transcript (Supplementary Figure 2).

Direct sequence analysis of the RT–PCR fragments corresponding to viral coding regions allowed identification of six major editing events in Rev and one in Tat sequence. While five out of six editing events occurring within the Rev coding sequence (Figure 7 and Supplementary Figure 1A) lead to codon changes that altered the protein primary sequence, the single editing event detected in the Tat sequence does not (Figure 7 and Supplementary Figure 1B). Since Tat and Rev exons partially overlap within the HIV-1 genome, their corresponding transcripts share some coding sequences. Interestingly, a common adenosine (A_{6036}) is edited by ADAR1 in both viral transcripts although with different efficiencies (32% in Tat mRNA, Supplementary Figure 1B and 67% Rev mRNA, Supplementary Figure 1A), suggesting that this residue corresponds to a hot spot for ADAR1.

Overall these results prove that HIV-1 RNAs can be edited by ADAR1. Moreover, the identification of ADAR1-specific A-to-I changes in the viral sequences supports and correlates with the evidence of an ADAR1 editing-dependent stimulatory activity on virus release and infectivity.

DISCUSSION

Here we provided evidence of a possible role for ADAR1 in the regulation of some critical steps of the HIV-1 life cycle. We showed that over-expressed ADAR1 strongly increases the overall accumulation of HIV-1 proteins in producer cells independently of its editing activity (Figure 2). This mechanism may rely on the previously described capacity of ADAR1 to bind and inhibit the PKR kinase that, once phosphorylated/activated, can suppress protein synthesis by phosphorylating eIF-2α (36). Indeed, in cells over-expressing wt or inactive ADAR1 we observed reduced levels of phosphorylated PKR and eIF-2α that could account for enhanced synthesis of HIV-1 proteins (Figure 2C). However, further studies are needed to test whether ADAR1 can stimulate HIV-1 expression by additional editing-independent mechanisms, for instance through protein-protein interactions with cellular factors that regulate RNA transcription and/or translation, as previously reported with nuclear factor 90 (NF90) (37). Moreover, results here presented demonstrated that HIV-1 virions produced in ADAR1 over-expressing cells are released more efficiently and, importantly, displayed a higher infectious potential (Figures 3 and 4). These effects of ADAR1 on HIV-1 viral particles apparently depend on its editing activity. It is possible that ADAR1-mediated editing in mRNAs coding for encapsidated viral proteins and/or cellular co-factors that participate in virion assembly and release
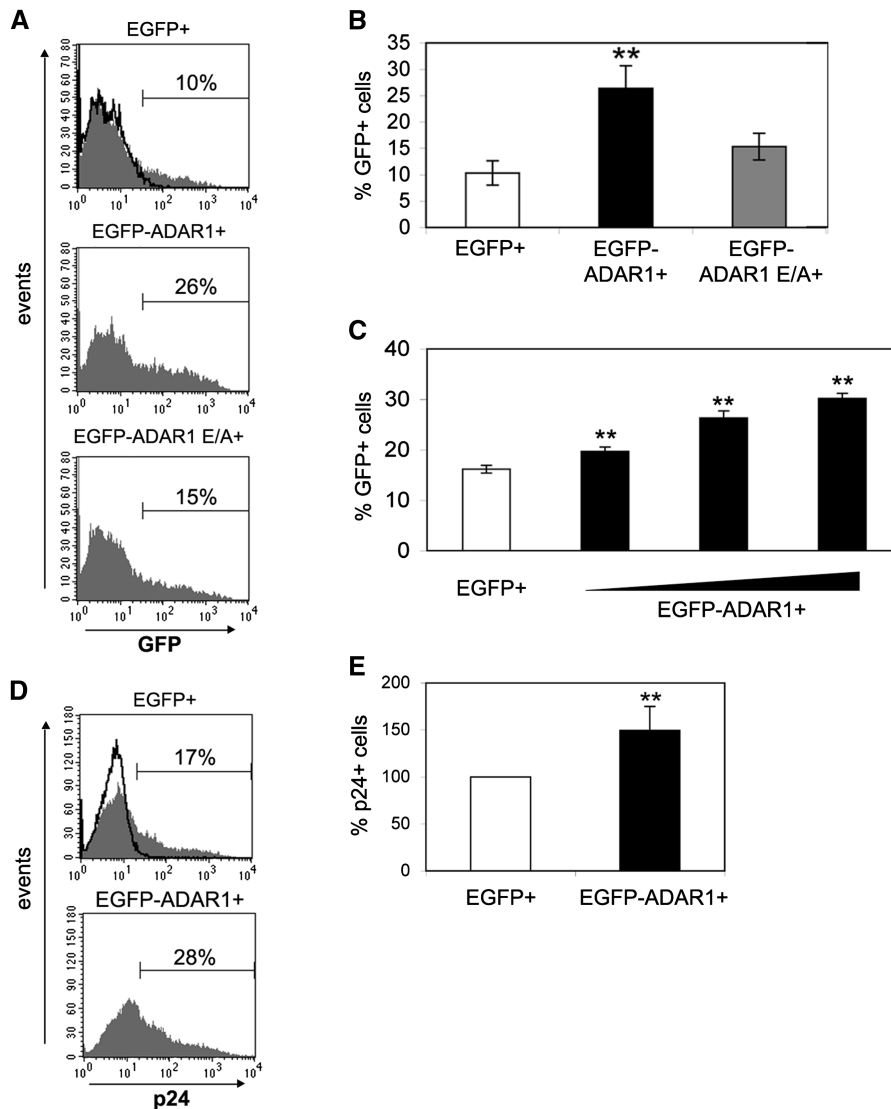
Figure 4. ADAR1 increases the infectivity of progeny virions. EGFP +, EGFP-ADAR1+ and EGFP-ADAR1 E/A + viruses were produced by 293T cells transfected with pNL4-3 (15 μg) together with pEGFP (8 μg), pEGFP-ADAR1 (8 μg and 1, 4 and 8 μg in panel C), or pEGFP-ADAR1 E/A (8 μg), respectively. (**A**) Equal amounts of virus were used for single-round infections of CEM-GFP target cells as described under 'Materials and Methods' section. Forty-eight hours post-infection, the relative percentage of GFP+ cells was analyzed by flow cytometry. The unshaded histogram show uninfected cells. Results shown in (**B**) and (**C**) are the mean ± SD determination from at least three independent experiments (**$P < 0.01$). (**D**) Freshly isolated CD4+ T lymphocytes have been infected, activated and analyzed after 3 days by flow cytometry to evaluate the percentage of p24 + cells as soon as they appeared. The unshaded histogram show uninfected cells. (**E**) The efficiency of viral infectivity was calculated in three independent experiments as percentage of p24 + cells with the control virus produced by pEGFP-transfected cells (EGFP +) set at a 100%.

could result in the production of higher levels of well fit viral particles.

Indeed, in this study we demonstrated that ADAR1 binds to the three different classes of HIV-1 RNA (Figure 5) and identified editing events in both coding and non-coding regions of viral transcripts (Figures 6 and 7 and Supplementary Figure 1).

The sequence analysis of viral RNAs isolated from ADAR1 wt over-expressing cells showed specific A-to-I changes in a region of the 5′ UTR shared by all viral transcripts as well as in the Rev and Tat mRNA coding sequences, providing the proof that ADAR1 can target and modify HIV-1 RNAs.

The adenosines that are targeted by ADAR1 in the 5′ UTR of the viral transcripts are mostly clustered in the poly(A) hairpin (Figure 5C), and their editing can alter the thermodynamic stability of the dsRNA structure (data not shown). The poly(A) hairpin motif in the 5′ UTR is highly conserved and essential for viral replication (38,39). The sequence of the 5′ UTR downstream of the common region analyzed (the first 181 nt) differs in various HIV-1 RNAs due to alternative ligation with distal exons (40). Interestingly, the analysis of the common region of 5′ UTR in the three HIV-1 RNA classes showed that the 2-kb transcripts are more efficiently edited by ADAR1 than the other species
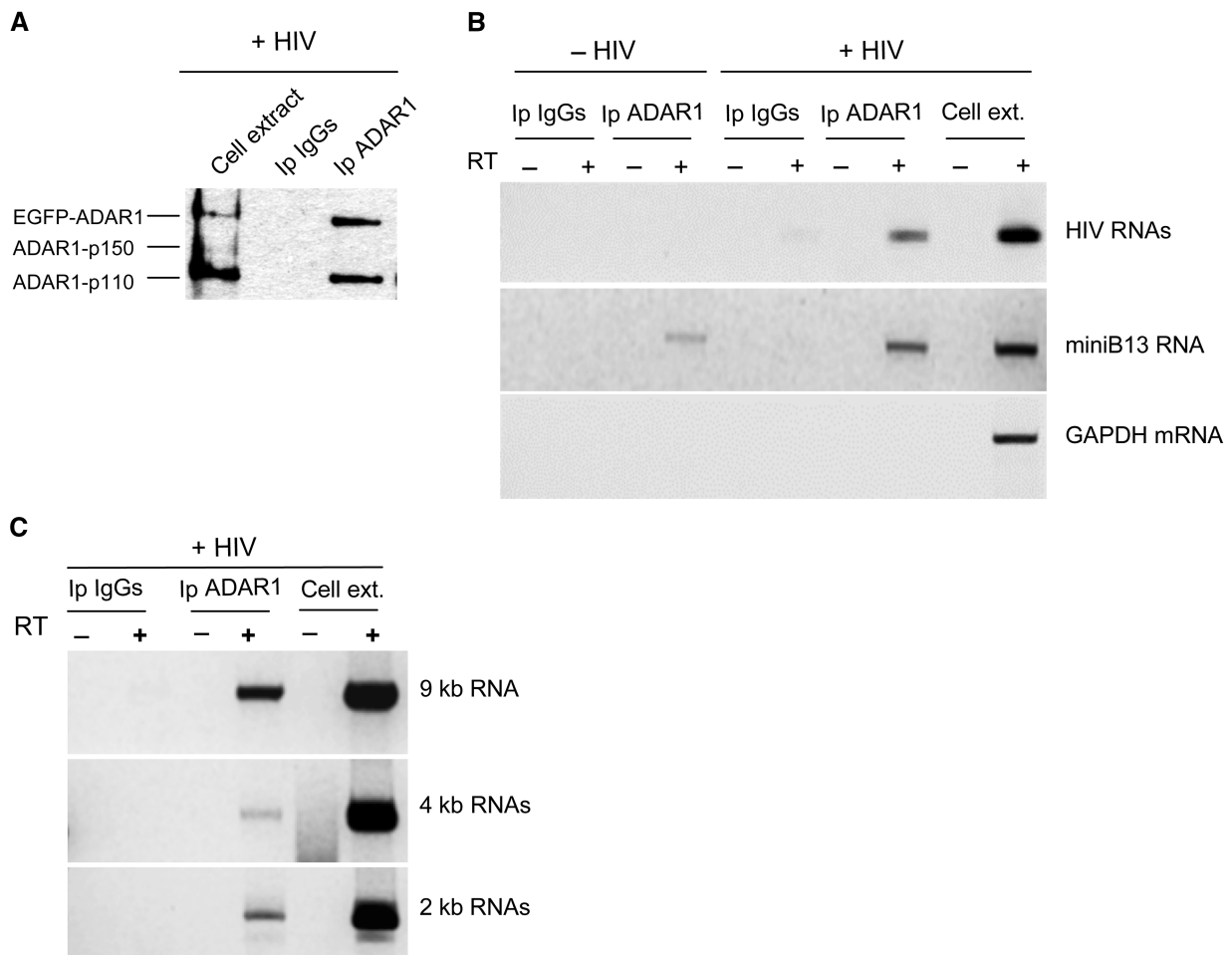
Figure 5. ADAR1 associates with HIV-1 RNAs. 293T cells were transiently co-transfected with miniB13 plasmid (2 μg) and pEGFP-ADAR1 (8 μg) in the presence or absence of NL4-3 proviral DNA (15 μg). Forty-eight hours post-transfection total cell extracts were prepared and immunopre-cipitated with anti-ADAR1 antibody or with control rabbit IgGs. (**A**) Western blotting analysis of an aliquot of the immunoprecipitated proteins and total cell extract probed with anti-ADAR1 antibody. (**B**) RNA isolated from the immunoprecipitates was retrotranscribed and amplified by PCR using specific primers to detect the TAR sequence harbored at the extreme 5′ end of all HIV-1 transcripts, the miniB13 transcript and the endogenous GAPDH mRNA. As a positive control, an RT–PCR experiment was performed with total RNA isolated from 293T co-transfected with miniB13 plasmid along with pEGFP-ADAR1, and NL4-3 proviral DNA (Cell ext.). Exclusion of RT from the RT–PCR reaction served as a specific negative control. (**C**) An RT–PCR analysis was carried using specific primers designed to distinguish the different HIV-1 RNA species (unspliced 9-kb RNA, spliced 4-kb and 2-kb RNAs) associated with ADAR1.

(Supplementary Figure 2). This result could be explained by the different ADAR1-binding affinity for the diverse 5′ UTR of viral transcripts. In addition, a common adenosine residue (A_{6036}) is edited with higher frequency in the Rev RNA (67%) if compared to the Tat RNA (32%), suggesting a higher binding affinity of ADAR1 for the Rev-specific 2-kb transcript. An alternative explanation that does not exclude the former is that editing of the A_{6036} residue in the 9-kb primary transcript may lead to its preferential processing into the Rev RNA. Of note, A_{6036} resides in a regulatory region that contributes to the splicing site selection necessary for the generation of the Rev RNA as well as other viral transcripts (41).

While the only editing identified in the Tat sequence causes a codon change that doesn't alter primary protein sequence (Figure 7 and Supplementary Figure 1), five out of six editing events found in the Rev coding sequence lead

to amino acid changes in motifs that are crucial for the regulative function of Rev (Figure 7 and Supplementary Figure 1) (42), such as the oligomerization motif, Nuclear localization signal and RNA-binding (RRE) motif, and Nuclear export signal. How the editing events identified mediate the effects we have observed on HIV-1 virions remains to be investigated. We believe that an extensive sequencing analysis to gain a complete mapping of the A-to-I changes occurring in HIV-1 RNA sequence will help to identify the specific adenosines critical for the biological effects we have observed.

Preliminary analysis of the HIV-1 RNA isolated from $CD4^+$ T infected *in vitro* indicate that A-to-G is the most frequent change occurring in the Rev coding sequence (data not shown). It is unlikely that the observed A-to-G changes resulted from errors of the HIV-1 RT enzyme since this would imply an insertion of dCTP opposite
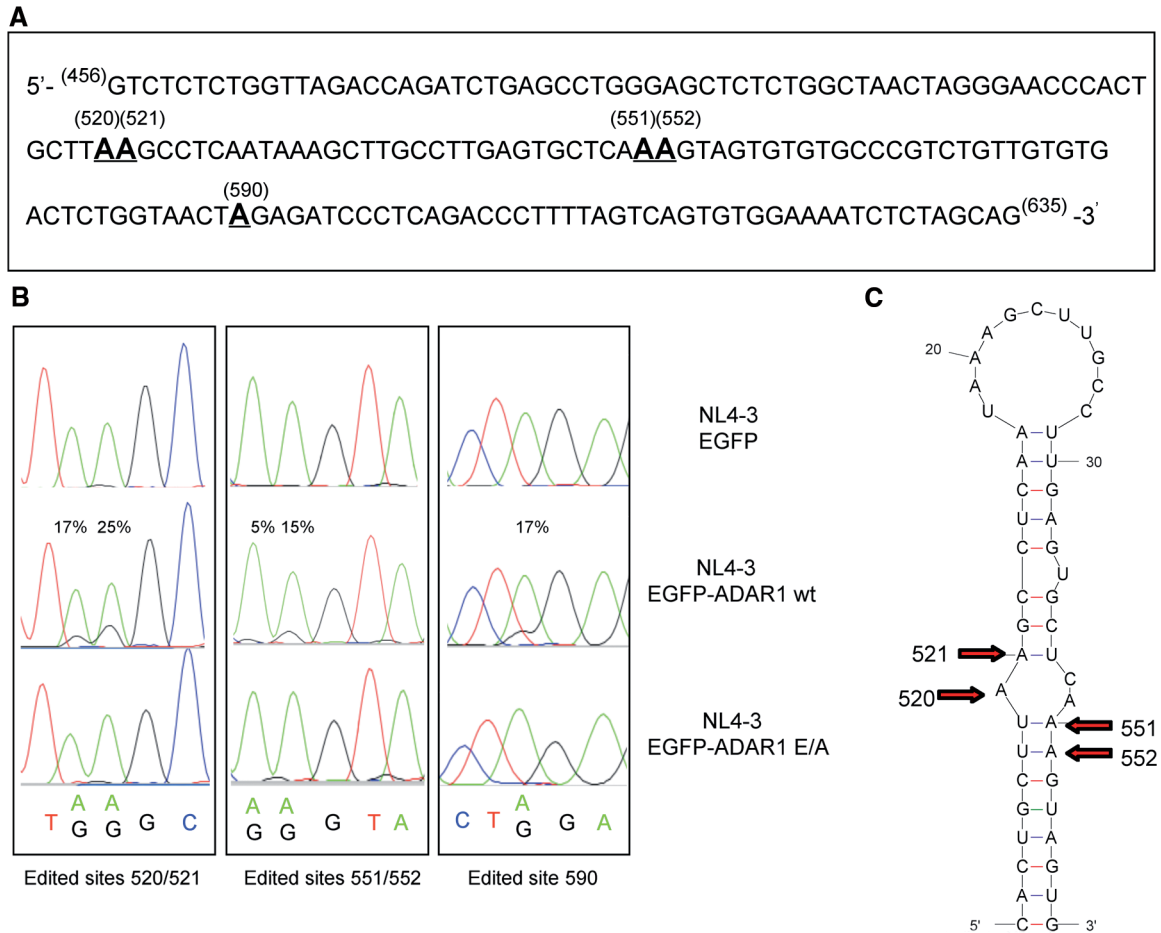
A

5'- (456)GTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACT

(520)(521)   (551)(552)

GCTT**AA**GCCTCAATAAAGCTTGCCTTGAGTGCTCA**AA**GTAGTGTGTGCCCGTCTGTTGTGTG

(590)

ACTCTGGTAACT**A**GAGATCCCTCAGACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAG(635) -3'

B   **C**

Figure 6. A-to-I RNA editing at specific sites in the 5′ UTR of HIV-1 RNAs. The 5′ UTR sequence of HIV-1 was analyzed by direct sequencing of RT–PCR products generated using as substrate the total RNA isolated from 293T cells co-transfected with NL4-3 proviral DNA (15 μg) together with either pEGFP (8 μg) or pEGFP-ADAR1 (8 μg) or pEGFP-ADAR1 E/A (8 μg) plasmids. (**A**) Edited adenosines in the 5′ UTR sequence analyzed are shown in bold and underlined and their nucleotide position in the HIV-1 genome is indicated (NL4-3 numbering). (**B**) Representative DNA sequencing chromatograms of the RT–PCR products. Edited adenosines appear as mixture of A and G, and the estimated percentage of editing efficiency is indicated (30). Results shown are representative of three independent co-transfection experiments. (**C**) Schematic representation of the poly(A) hairpin (generated by using mfold server: http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi) harbored in the 5′ UTR of HIV-1 RNA. Red arrows show adenosines that undergo ADAR1 editing and their relative nucleotide position in the HIV-1 genome.
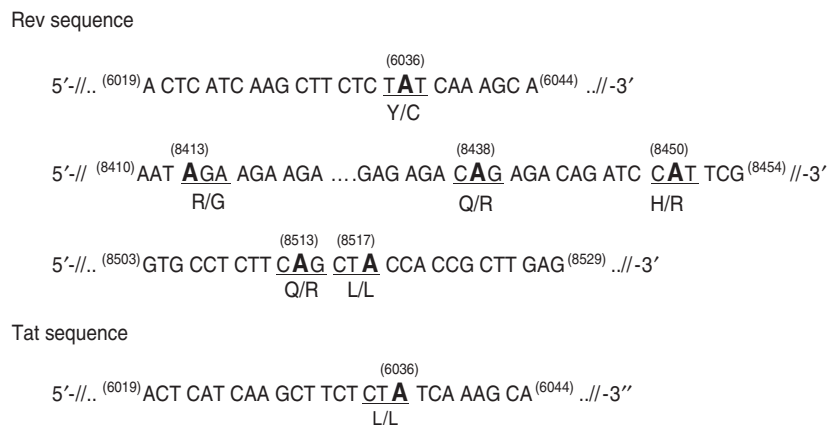
Rev sequence

5′-//.. (6019)A CTC ATC AAG CTT CTC T**A**T CAA AGC A(6044) ..//-3′
                                          (6036)
                                          Y/C

5′-// (8410)AAT **A**GA AGA AGA ….GAG AGA C**A**G AGA CAG ATC C**A**T TCG (8454) //-3′
            (8413)   (8438)   (8450)
            R/G   Q/R   H/R

5′-//.. (8503)GTG CCT CTT C**A**G CT**A** CCA CCG CTT GAG (8529) ..//-3′
                          (8513) (8517)
                          Q/R   L/L

Tat sequence

5′-//.. (6019)ACT CAT CAA GCT TCT CT**A** TCA AAG CA(6044) ..//-3″
                                  (6036)
                                  L/L

Figure 7. A-to-I RNA editing at specific sites in the Rev and Tat coding sequence. The Rev and Tat coding sequence were analyzed by direct sequencing of RT–PCR products generated using as substrate the total RNA isolated as described in Figure 6. Three independent co-transfection experiments with relative RT–PCR analysis were carried out. The edited adenosines found in the Rev and Tat coding sequence are numbered (relative to NL4-3) and shown in bold. The codons encompassing modified adenosines are underlined and the corresponding amino acids (before/after editing) are indicated.

to A in viral RNA, an event that occurs very rarely compared to a normal Watson–Crick base pair formation (A + dTTP) (43). The most plausible explanation for these changes is that the Rev RNA is edited by cellular ADAR enzymes during HIV-1 replication in $CD4^+$ T cells. Interestingly, adenosines that are modified in $CD4^+$ T cells do not coincide with those targeted by ADAR1 in 293T cells although some are located in close proximity. Several factors may influence the specificity of ADAR1 including cell-specific proteins that modulate ADAR1 activity and/or the local structure of viral RNA and the ratio between the enzyme and its target RNA substrate. Indeed, Phuphuakrat and colleagues (24) showed that in the monkey COS7 cell line HIV-1 RNA is edited by ADAR1 at sites different from those found in the present study. In addition, both the frequency and specificity of editing events in the HIV-1 RNA varied upon ADAR1 over-expression in COS7 cells (24). Variation of RNA editing specificity seems to be a feature shared by other viruses (44,45). Of note, during the course of HIV-1 infection the levels and/or the function of ADAR1 may be modulated, as suggested by the fact that in T lymphocytes proinflammatory stimuli may increase both the expression and the activity of the ADAR1 enzyme (32). Future studies are needed to understand the regulation of viral RNA editing as well as the impact of ADAR1 in the viral life cycle.

Our study confirms and extends a recent report showing that ADAR1 over-expression in HIV-1 producer cells increases extra-cellular levels of the p24 protein in an editing-dependent manner (24). Here, we disclose a novel function of ADAR1 in the stimulation of virion release and infectivity (Figure 3 and 4). On the other hand, Phuphuakrat and colleagues (24) found that the virus produced from ADAR1-overexpressing COS7 cells exhibits normal infectivity if tested on GHOST-CXCR4 cells. Differences in the experimental systems employed may possibly account for this discrepancy. In particular, we used 293T cells to produce the virus and natural HIV-1 T cell targets (CEM-GFP and Jurkat cell lines as well as primary $CD4^+$ T lymphocytes) to evaluate viral infectivity. Different cell systems employed may also account for divergent editing events found in the non-coding TAR region of HIV-1 transcripts that was analyzed in both studies.

It is well known that editing enzymes that belong to the APOBEC family of cytidine deaminases can edit HIV-1 DNA (46). In particular, human APOBEC3G mediates extensive cytidine deamination in the viral-minus strand DNA and induces multiple G-to-A changes in viral plus strand during reverse transcription. These hypermutations lead to incomplete reverse transcription or production of non-functional viral proteins (46). It's intriguing that editing of HIV-1 at the DNA and RNA level mediated by two different host enzymes APOBEC3G and ADAR1 could exert opposing effects on viral replication. The interaction of APOBEC3G and ADAR1 with HIV-1 and their possible coordinated interplay may shed light on a novel regulatory network that could take place in the infected host cells.

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