Vpr.A3A Chimera Inhibits HIV Replication*

Received for publication, August 3, 2007, and in revised form, December 5, 2007 Published, JBC Papers in Press, December 5, 2007, DOI 10.1074/jbc.M706436200

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Several APOBEC3 proteins (A3F and A3G), that are cytidine deaminases restrict human immunodeficiency virus (HIV) replication in the absence of the viral infectivity factor (Vif) protein. However, Vif leads to their degradation and counteracts their effects. Another member, A3A, restricts some retrotransposons and another virus but not HIV. We reasoned that this failure was due to the lack of appropriate targeting. Thus, we fused A3A to another viral protein, Vpr, which binds p6 in Gag and is incorporated into viral cores. Indeed, the Vpr.A3A chimera but not A3A was found abundantly in the viral core. It also restricted potently the replication of HIV and simian immunodeficiency virus in the presence and absence of Vif. Because we identified a high frequency of G to A mutations in viral cDNAs, this antiviral activity was mediated by DNA editing. Interestingly, our fusion protein did not restrict murine leukemia virus, which does not incorporate Vpr. Thus, by targeting appropriately a potent single domain cytidine deaminase, we rendered HIV and simian immunodeficiency virus restriction resistant to Vif.

Innate immune defenses, which are poised to neutralize extracellular pathogens, are integral components of eukaryotic systems (1). Although some of these factors can interfere with the replication of HIV,2 viral proteins can counteract them. The ability of members of the APOBEC3 (A3) family of cytidine deaminases (CDAs) to confer intrinsic immunity to retroviral infection was first recognized with the human A3G protein (2). A3G is packaged into newly synthesized viral particles from producer cells and acts in target cells to inhibit viral replication during reverse transcription. In humans, the APOBEC family includes AID, A1, A2, A3A–A3H, and A4 (3). All proteins in this family have one or two CDA motifs that contain one histidine, two cysteines, and one glutamic acid that are involved in the hydrolytic deamination of cytosines, thereby converting cytidine (C) to uridine (U) in a process called editing (for review in Ref. 3). Indeed, G to A changes observed in viral sequences from HIV-infected patients can be explained by direct C to U transitions in the minus-strand cDNA by CDAs (4). However, because mutant A3G proteins lacking the CDA activity retain substantial antiviral effects, the mechanism of this intrinsic restriction is not fully understood (5, 6).

Although A3F and A3G can restrict the replication of HIV, this inhibition is overcome by the viral infectivity factor (Vif). Thus, primate lentiviruses have developed an escape strategy to prevent the incorporation of A3 proteins into progeny virions (7). Vif acts as an adaptor protein, which connects A3F and A3G to an E3 ubiquitin ligase, thereby inducing the polyubiquitilation and proteasomal degradation of these CDAs (8). As a consequence, A3F and A3G are unavailable for subsequent incorporation into viral particles, and their antiviral effects are absent in target cells. Anti-viral effects of A3 proteins are not limited to Vif-deficient forms of HIV but also extend to other lentiviruses, including SIV, the equine infectious anemia virus (EIAV), and more distant retroviruses such as the murine leukemia virus (MLV), foamy viruses, and the mouse mammary tumor virus as well as endogenous retroviruses and retrotransposons (9–13).

In this study we wanted to create an A3 protein that can be targeted into the core of viral particles and is not degraded by Vif. To this end, we chose A3A, which contains a single copy of the CDA motif and is not active against HIV (13). However, it is catalytically active and inhibits endogenous long terminal repeat (LTR)-containing retroelements (MusDs and 1Aps), non-LTR retroelements (LINEs and SINEs), and Alus (14–19). Indeed, A3A inhibits the retrotransposition of 1Aps more potently than do A3B or A3G (18). This CDA also inhibits the replication of the adeno-associated virus (parvovirus AAV) (19). To target A3A, we chose the viral protein R (Vpr), which binds p6 in Gag and is incorporated into the core of HIV particles (20–23). Vpr is a small basic protein (14 kDa), whose functions include the import of preintegrated complexes into the nucleus of cells and the delay of the cell cycle (for review in Ref. 24). Recently, several studies demonstrated that Vpr fusion proteins could be used as intravirin inactivating agents (22, 23). In this study we constructed a Vpr.A3A chimera that was incorporated abundantly into the viral core where it restricted the replication of HIV and SIV in the presence and absence of Vif.

**EXPERIMENTAL PROCEDURES**

**Tissue Cultures**—The human embryonic kidney cell line 293T and GHOST R3/X4/R5 cells were cultured in Dulbec-
co’s modified Eagle’s medium, 10% fetal bovine serum, 100 μg/ml penicillin and streptomycin, and 2 mM glutamate at 37 °C with 5% CO₂. GHOST cells obtained from the AIDS Reference and Reagent Program have been engineered to stably express CD4 and the chemokine receptors CCR3, CXCR4, and CCR5 (25). The expression of these chemokine receptors was maintained by adding to the culture medium hygromycin (100 μg/ml), Geneticin (500 μg/ml), and puromycin (1 μg/ml).

Plasmids—HIV proviral plasmids pNL43-Luc and pNL43-LucΔVif were described before (26). The full-length, envelope-defective SIVmac reporter virus (pSIVmac-Luc E R and pSIVmac-Luc E R Δvif) were kindly provided by Dr. Nathaniel Landau (27). HIV and SIVmac clones encoded luciferase in place of the nef gene. Expression vectors encoding the B-tropic MLV Gag-Pol proteins have been described previously (28). They were used to package MLV-based vectors encoding GFP in place of env but lacking Gag and Gag-Pol genes (pCNCG, Oxford Biomedica). The B-tropic MLV vectors were obtained from Dr. Paul D. Bieniasz. Reporter viruses lacking env were generated as vesicular stomatitis virus glycoprotein (VSV-G) pseudotypes. To construct the Vpr.A3A fusion protein, the human A3A was fused to the C terminus of Vpr in the context of the mammalian expression vector pcDNA3.1/V5-His TOPO (Invitrogen) (Fig. 1A). First, the vpr gene was amplified by PCR using a 5′-primer located before the initiation codon (ATG) of Vpr and 3′-primer at the end of the vpr gene of pNL43 proviral clone (Vpr forward primer, 5′-CAC CAT GGA ACA AGC CCC AGA AGA CCA A-3′; VprGlyClaI reverse primer, 5′-CCC ATC GAT GGG ATG GAA GCC AGC CCA GCA ACC CCC AGA ATT CTG-3′). At the 3′ site of Vpr gene, a linker of four glycines and the ClaI restriction site were introduced, and the VprGly fragment was cloned into pcDNA3.1-V5 TOPO. Next, a PCR-amplified A3A DNA fragment flanked by ClaI and XbaI restriction sites at 5′ and 3′ termini, respectively (ClaIA3Af, 5′-ATC GAT GGG ATG GAA GCC AGC CCA GCA TCC GGG-3′; A3AXbaIrev, 5′-GCT CTA GAG TTT CCC TGA TGG CCC GCA GCC TCC CAC TCA-3′) was amplified from pK3A vector previously described (18), digested with ClaI and XbaI enzymes, and ligated into pcDNA3.1-VprGly vector digested with the same enzymes. In addition, other plasmid constructions were used as controls. The Vpr.Nef fusion protein (pEF.Myc.Vpr.Nef) was described before (29). The expressing vector was constructed as described above, and the Vpr gene was inserted in the pcDNA3.1-V5 TOPO (pcDNA3.1 Vpr). Mammalian expression vectors for human A3C, A3F, and A3G (26) were subcloned into pcDNA3.1D/V5-His-TOPO. The pΔA3 vector expressing hA3A HA-tagged protein was a gift of Dr. Brian Cullen. All constructions were checked by restriction enzyme digestion and DNA sequencing with cytomegalovirus and bovine growth hormone primers.

Viral Production and Infectivity Assay—HIV viruses were produced by transfection of pNL43 Luc vectors (8 μg) with 4 μg of each vector expressing APOBEC, Vpr, or Vpr.Nef fusion proteins. Reporter viruses (SIVmac and B-Tropic MLV) were generated as VSV-G pseudotypes by co-transfection of 293T cells in 10-cm dishes with 8 μg of proviral plasmid DNA, 4 μg of VSV-G, and 4 μg of APOBEC expression vector or the controls plasmids (pcDNA3.1 Vpr or pEF.Myc.Vpr.Nef) using FuGENE 6 transfection reagent (Roche Applied Science). B-tropic MLV viruses were generated using an MLV-based vector encoding GFP in place of env, an expression vector B-tropic MLV Gag-Pol protein (28), the pVSVE-G vector and the A3 and control vectors in the proportion 1:1:0.5:0.5, respectively. Viruses in the supernatant were collected after 48 h of transfection and filtered through a 0.45-μm filter, and the viral titers were measured with an enzyme-linked immunosorbent assay kit against p24 (Roche Applied Science) or p27 (RETRO-TEK; ZeptoMetrix). Equal amounts of HIV-1 and SIVmac were used to infect GHOST-R3/X4/R5 cells in six-well plates. 24 h later cells were lysed in CAT lysis buffer (Promega). After removing the nuclei, the cytosolic fraction was used to determine the luciferase activity using a luciferase assay kit (Promega). MLV infectivity assays were done in six-well plates seeded with GHOST-R3/X4/R5 cells per well the day before infection. 48 h post-infection, the target cells were assayed for GFP expression by fluorescence microscopy, then harvested by trypsinization and resuspended in 1× phosphate-buffered saline. An aliquot was used for flow cytometry (FACSCalibur, BD Biosciences), and the data were analyzed using CellQuest software (BD Biosciences Immunocytometry Systems). Uninfected control cells were also processed as described above. Infectivity assays were done in triplicate; each assay was performed three times with similar results. Values are presented as the percentage of infectivity relative to the virus infectivity in the absence of A3 vectors.

A3 Encapsulation and Isolation of HIV Cores—Normalized amounts of virus were pelleted by ultracentrifugation through 20% sucrose cushion at 45,000 rpm for 1 h (Beckman), 4 °C. Cell lysates and pelleted virions were solubilized in 10 μl of HEPES lysing buffer (8C4). Virion cores were obtained using previously published methods (30). Briefly, viral pellets from the cushion of 20% sucrose ultracentrifugation were resuspended in phosphate-buffered saline (3.5 μl/ml of initial culture volume). Subsequently, 40 μl of fresh virus suspension was mixed with an equal volume of 200 mM NaCl, 100 mM MOPS (pH 7.0), and virions were lysed for 2 min at room temperature by adding Triton X-100 to a final concentration of 0.5%. HIV cores were recovered by centrifugation in a microcentrifuge at full speed (14,000 rpm) for 8 min at 4 °C. Pellets were washed twice with 100 mM NaCl, 50 mM MOPS (pH 7.0) and resuspended in 8 μl of the same buffer. Cores and supernatants were processed immediately for further analysis. Cores and supernatants were then analyzed by immunoblotting to evaluate the efficiency of core isolation. The HIV reverse transcriptase (RT) (8C4), HIV gp41 (no. 50–69), and HIV p24 monoclonal antibodies (183-H12-5C) were obtained from National Institutes of Health AIDS Research and Reference Reagent Program. To examine A3 and Vpr packaging, purified virions were subjected to SDS-PAGE and Western blotting using α-5-vorherdsheridic peroxidase (HRP; Invitrogen) for Vpr.A3A, Vpr, A3G, and A3F proteins and α-HA monoclonal antibody (Sigma-Aldrich) for A3A protein and the appropriate HRP-conjugated secondary antibodies followed by detection with ECL reagents (Amersham Biosciences).
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**Figure 1. Schematic representation of the A3A plasmid effectors and HIV proviral targets.** A, plasmid effectors. Depicted are plasmids that directed the expression of the Vpr.A3A and Vpr.Nef chimeras, Vpr and A3A from the human cytomegalovirus (CMV) IE promoter. V5 (black rectangles) and HA (gray rectangles) epitope tags were placed at the C terminus of these effectors for Western blotting. B, proviral targets. The WT NL4.3, NL4.3Luc (HIV), and NL4.3VifLuc (HIV Vif) proviral targets are presented. Open reading frames are presented above and below the central line. The infectivity of these viruses was evaluated from the luciferase reporter gene, which was inserted in the nef open reading frame. LTR, long terminal repeat; Pol, polymerase.

**Figure 2. Vpr.A3A chimera blocks the replication of HIV and is incorporated into viral particles.** A, Vpr.A3A chimera inhibits the replication of HIV (black bars) and HIV Vif (gray bars). Reporter viruses were co-expressed with the indicated A3 proteins (+ signs) in 293T cells. Equal amounts of virus were used to infect GHOST cells. Luciferase activities were determined 2 days later. Results are presented as percentages of the virus controls without A3 proteins. Error bars reveal S.D. of three independent experiments. B, A3A and the Vpr.A3A chimera are incorporated into viral particles. Virions were generated by co-expressing A3A proteins and proviral targets in 293T cells. The expression of A3A and our chimera was detected by Western blotting with α-HA and α-V5 antibodies, respectively. A3 proteins in virions were normalized to p24 with α-p24 antibodies (middle panel).

Detection of Hypermutation in Viral DNA—35-mm subconfluent monolayers of GHOST R3/X4/R5 cells were infected with 4 ng of p24 wild-type HIV virus produced in the presence or absence of the Vpr.A3A fusion protein in 12 h. Total cellular DNA was purified using the DNeasy kit (Qiagen), treated with DpnI for 2 h at 37 °C to digest any residual plasmid DNA, and subjected to high fidelity PCR (Expand High Fidelity PCR system, Roche Applied Science) using HIV-specific oligonucleotides U3 R (forward) 5′-CAG ATA TCC ACT GAC CTT TGG-3′ and U3 R (reverse) 5′-GAG GCT TAA GCA GTG GGT TC-3′. The PCR products were cloned into pCR4-TOPO (TOPO TA cloning kit for sequencing, Invitrogen) and used to transform Escherichia coli (DH5α). Plasmids were purified from at least 10 individual colonies, and the insert (417-bp) was sequenced using T3 and T7 primers. The nucleotide sequences of independent clones were aligned and analyzed using Hypermut software (31).

**RESULTS**

Vpr.A3A Chimera Blocks the Replication of HIV and Is Incorporated into Viral Particles—To target A3A into the cores of HIV, we constructed a number of plasmid effectors that are depicted in Fig. 1A (effectors). For the expression of the Vpr.A3A chimera, A3A was subcloned from pKA3A expression vector, and Vpr was amplified by PCR from the HIV-1NL4.3 provirus (Fig. 1B, targets). They were ligated in-frame and introduced into the pCDNA3.1-V5 expression vector. Thus, our fusion protein also contained the V5 epitope tag at its C terminus. Control plasmid effectors, e.g. those expressing the Vpr.Nef chimera, or just Vpr or A3A alone are also presented in Fig. 1A. To determine whether the Vpr.A3A chimera possessed any antiviral activity, we used the wild type (WT) (NL4.3Luc, HIV) and mutant HIV-1NL4.3 lacking Vif (NL4.3ΔVifLuc, HIVΔVif) luciferase proviral targets (Fig. 1B, targets). These targets were co-expressed with different A3 proteins or Vpr in 293T cells. Supernatants were harvested and purified, and amounts of viral particles were determined by p24 capture enzyme-linked immunosorbent assay. Equal amounts of different HIVs were then used to infect GHOST R3/X4/R5 (GHOST) cells. The infectivity of our viruses was determined by measurements of intracellular luciferase activities 2 days later. As presented in Fig. 2A, lane 2, the Vpr.A3A chimera blocked the WT HIV and mutant HIVΔVif to background levels (greater than 10-fold reduction compared with the control; lane 1). In contrast, A3A had no effect on the WT HIV or mutant HIVΔVif (Fig. 2A, lane 3). The reduced infectivity of viruses which were produced in the presence of A3G and A3C was consistent with previous reports (13). Briefly, A3G was sufficient to reduce the infectivity of only HIVΔVif to close to background levels (Fig. 2A, lane 5). A3C weakly inhibited only the mutant HIVΔVif (1.6-fold) (Fig. 2A, lane 4). Moreover, the presence of Vpr alone did not influence viral infectivity (Fig. 2A, lane 6). To exclude the possibility that the reduction of viral infectivity was due to a Vpr-based fusion protein, we also co-expressed the virus and the Vpr.Nef chimera (Fig. 2A, lane 7). Again this fusion protein did not interfere with viral replication, validating our results with the Vpr.A3A chimera. We conclude that the
Vpr.A3A chimera blocks the replication of HIV and is not inactivated by Vif.

To evaluate the incorporation efficiencies of A3A and the Vpr.A3A chimera, the same aliquots of viruses and producer cells were subjected to Western blotting. Virions were pelleted through a 20% sucrose cushion and normalized to levels of p24, and their protein profiles were examined in parallel (Fig. 2B). A3A and the Vpr.A3A chimera were identified using α-HA and α-V5 antibodies, respectively. Our fusion protein had an apparent molecular mass of 40 kDa, consistent with the combined sizes of Vpr and A3A (Fig. 2B, lanes 3 and 4). Moreover, Western blotting confirmed that our chimera was packaged efficiently into WT HIV and mutant HIVΔVif particles (Fig. 2B, upper panel, lanes 3 and 4). Interestingly, A3A was also incorporated into progeny virions (Fig. 2B, upper panel, lanes 5 and 6). However, it did not affect the infectivity of HIV (Fig. 2A, lane 3). Most likely, this incorporation was outside the ribonucleoprotein complex or the core (30).

Equal loading of virions on these blots was confirmed with outside the ribonucleoprotein complex or the core (30).

Viral Genome responsible for the antiviral effects of our chimera.

Effect, this finding suggests that the targeting via Vpr was incorporated efficiently into viral particles where it restricts HIV (Fig. 2A, lane 3). Most likely, this incorporation was outside the ribonucleoprotein complex or the core (30).

Vpr.A3A Chimera Induces Extensive G to A Changes in the Viral Genome—Because the Vpr.A3A chimera blocked viral replication, we wanted to know if DNA editing was involved.

To this end, we analyzed sequences of viral reverse transcripts from newly infected cells. WT HIV was co-expressed with the Vpr.A3A chimera in 293T cells, normalized to levels of p24, treated with DpnI to remove contaminating plasmid DNA, and then used to infect GHOST cells. 12 h later a fragment of U3 R was amplified and cloned. Nucleotide sequences were determined for at least 10 independent clonings from each infection.

In cells infected with the WT HIV in the presence of the Vpr.A3A chimera, we found a total of 62 G to A transitions distributed over 125 dG residues in 10 sequences (4170 bp) yielding a mutation frequency of 1.5 per 100 bases (Fig. 3A, left panel, black bars). Only a single non-G-to-A change was found in the same 10 sequences (Fig. 3A, left panel, lollipop). Levels of mutations were high in comparison to those with the WT HIV in the absence of the Vpr.A3A chimera and were similar to those described previously (32). Indeed, we found just one change (T to C) in 10 sequences analyzed (4170 bp) for a mutation frequency of 0.02 per 100 bases (Fig. 3A, right panel, lollipop).

To reveal the base preference for this DNA editing, we analyzed an additional 62 G to A mutations generated in the presence of the Vpr.A3A chimera. The spectrum of mutations revealed that our chimera targets dC residues that are preceded by dC or dT (Fig. 3B, rows C and T). This target specificity agrees with previous reports, where A3A mutated dCs preceded by dC or dT (19). We conclude that the Vpr.A3A chimera has the ability to promote editing of cytidines in the minus strand of viral reverse transcripts and, consequently, to inhibit the replication of HIV.

Vpr.A3A Chimera Inhibits SIV Infection—These findings raised the possibility that the Vpr.A3A chimera could restrict the replication of other retroviruses. To investigate this notion, we evaluated the effects of our chimera on the replication of SIV. In these experiments we produced WT SIVmac-Luc-E−R− (SIV) and mutant pSIVmac-Luc-E−R−Δvif (SIVΔvif) viruses in the presence of the Vpr.A3A chimera and other proteins as in Fig. 2A. Luciferase reporter viruses were pseudotyped with the VSV-G Env in the presence of different A3 proteins in 293T cells. As with HIV, the infectivity of these viruses was determined by infecting GHOST cells. Equivalent amounts of viruses, as determined by p27 capture enzyme-linked immunosorbent assay, were used, and luciferase activities were measured 2 days later. In addition to inhibiting the infectivity of HIV, the Vpr.A3A chimera also inhibited potently the infection by SIV (Fig. 4A). As presented in lane 2 of Fig. 4A, viral infectivity was inhibited 6-fold by our chimera in the presence and absence of Vif. In contrast, the
co-expression of A3A had no effect (Fig. 4A, lane 3). A3C exhibited more potent antiviral effects on the mutant SIVΔVif than on the mutant HIVΔVif, as reported previously (33). Again, the presence of Vpr and the Vpr.Nef chimera had no effect, validating the antiviral restriction of the Vpr.A3A chimera on SIV.

To extend our analyses, we also investigated the incorporation of A3 proteins into SIV particles. In Fig. 4B, Western blotting of producer cells and virions from supernatants revealed the presence of A3A and the Vpr.A3A chimera in producer cells and viral particles (Fig. 4B, top and bottom panel, lanes 3–6). Input viruses are presented in the middle panel and were identified by antibodies against p27. We conclude that the incorporation of our chimera into viral particles restricts the infectivity of SIV irrespective of Vif, suggesting that the Vpr.A3A fusion protein also possesses broad antiviral activity against other primate lentiviruses.

**Vpr.A3A Chimera Does Not Restrict the Replication of MLV**—Results of antiviral restriction of HIV and SIV by the Vpr.A3A chimera were somewhat surprising in light of effects of A3A, which had no effect on the infectivity of these viruses. Thus, we wanted to examine our chimera with MLV, which does not incorporate Vpr (34). To determine whether MLV is sensitive to the Vpr.A3A fusion protein, VSV-G-pseudotyped B-tropic MLV-enhanced GFP reporter viruses were co-expressed with different A3 proteins in 293T cells (28). Virions were harvested, normalized to p30, and used to infect GHOST cells as with HIV and SIV (Figs. 2 and 4). To follow their infectivity, the number of GFP-positive cells was determined by FACS. As presented in Fig. 5A, lanes 2 and 3, neither A3A nor our chimera inhibited the infection by MLV. Controls with other A3 proteins (Fig. 5A, lanes 4 and 5) demonstrated a potent suppression of MLV infection by A3G but no A3F (13). Moreover, Vpr alone had no effect (Fig. 5A, lane 6). Thus, neither the Vpr.A3A chimera nor A3A restricted the replication of MLV.

Next, we wanted to determine whether MLV could package A3A proteins into progeny virions. For this purpose we performed high speed centrifugation through a sucrose cushion to collect viral particles. Western blotting of producer cells and lysed virions identified A3G, A3F, Vpr, and the Vpr.A3A chimera with α-V5 and A3A with α-HA antibodies. As presented in Fig. 5B, bottom panel, lanes 2–5 and 7), all proteins were expressed at high levels in producer cells. As previously reported, MLV virions also packaged A3G, consistent with their sensitivity to this protein (Fig. 5B, upper panel, lane 4) (35). However, the incorporation of A3F into viral particles was reduced, which explains the relative absence of its antiviral effects (Fig. 5B, upper panel, lane 3). Interestingly, both
A3A and our chimera were incorporated to the same extent as A3G into MLV particles (Fig. 5B, upper panel, lanes 2 and 7). However, because it was not found alone in these progeny virions, Vpr did not mediate this incorporation (Fig. 5B, upper panel, lane 5). By probing for p30, we also confirmed that the same amounts of viral particles were used in these experiments (Fig. 5B, middle panel). We conclude that although neither protein affected the infectivity of MLV, A3A and the Vpr.A3A chimera were incorporated efficiently into MLV particles.

**Vpr.A3A Chimera but Not A3A Is Incorporated into the Core of HIV**—We hypothesized that A3A is loosely associated with viral particles and that Vpr targeting carries the Vpr.A3A chimera into the viral core. There, the A3A subunit can act as a CDA and restrict viral infectivity. To prove this notion experimentally, virions were produced in the presence of increasing concentrations of our A3A proteins and pelleted through a sucrose cushion (30). Thus, we co-expressed increasing amounts of A3A and Vpr.A3A fusion proteins with a constant amount of WT HIV. Equivalent amounts of viruses were used to infect GHOST cells. In Fig. 6A, we observed that the Vpr.A3A chimera already restricted potently viral infectivity at a ratio of input plasmids of 1 (Vpr.A3A) to 12 (HIV) (lane 3). In contrast, A3A had no effect even at much higher concentrations (Fig. 6A, lane 6). Viruses from these experiments were isolated by pelleting through a sucrose cushion, resuspended, and solubilized with Triton X-100. This treatment released Env (gp41) into the supernatant and retained p24 and RT in the core (Fig. 6B, lanes 6–15). Moreover, the Vpr.A3A chimera was packaged into the viral core (Fig. 6B, lanes 12–15). Importantly, it was found in the supernatant only in viruses that were produced in the presence of high concentrations of our chimera (Fig. 6B, lanes 9 and 10). In sharp contrast, A3A was absent from the viral core (Fig. 6C, lanes 12–15). These findings indicate that whereas A3A is loosely associated with viral particles, the Vpr.A3A chimera is incorporated efficiently into the viral core, where it blocks the infection by HIV.

**DISCUSSION**

In this study we wanted to simplify HIV restriction by A3 proteins. By using Vpr to target a potent CDA into HIV, we were able to convert an innocent bystander into a powerful inhibitor of viral replication. Indeed, the Vpr.A3A chimera restricted the replication of all primate lentiviruses and was not sensitive to Vif. Our chimera also edited viral cDNAs in target cells. Moreover, the Vpr.A3A chimera had no effect against MLV, whose Gag does not bind Vpr. Finally, whereas A3A was found to be loosely associated with viral particles, the targeting by Vpr led to the incorporation of the Vpr.A3A chimera into the viral core. Thus, we targeted a single CDA domain A3 protein to restrict the replication of primate lentiviruses even in the presence of Vif.

These results were somewhat surprising in light of the effects of A3A alone. Moreover, because A3A and our chimera were found abundantly in HIV, SIV, and MLV particles, their differences were not caused by virion exclusion. More likely, whereas Vpr targeted A3A into the core and possibly into the ribonucleoprotein, A3A alone was only loosely associated with viral particles (36). In support of this notion, recent work suggested different distributions of high and low molecular mass A3G complexes in HIV particles, where only the latter inhibited viral replication in target cells (30). Indeed, several studies already described the presence of A3A in viral particles (19). Our results are also in good agreement with a very recent study by Goila-Gaur et al. (37). Using a different strategy, they fused A3A to the N terminus of A3G (A3G.A3A chimera). However, although also targeted into viral cores, this fusion protein restricted the replication of HIV only in the absence of Vif. Thus, not only did
this chimera behave more like WT A3G and A3F proteins in terms of trafficking, incorporating into viral cores, but it remained sensitive to Vif (37). However, it did extend the notion that single domain CDAs are portable and can modify nucleic acids when targeted appropriately.

Thus, precise targeting of A3 into viral particles was critical for its antiviral effects. Moreover, we observed abundant editing of viral cDNAs, which means that A3A was enzymatically active in our chimera. However, we cannot exclude the possibility that the Vpr.A3A fusion protein also affected other parameters during reverse transcription, some of which have been highlighted recently with enzymatically inactive A3F and A3G proteins. They include interference with primer tRNA processing and removal as well as decreased synthesis of viral nucleic acids when targeted appropriately.

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Because the Vpr.A3A chimera inhibited SIV, such therapeutic strategies could be tried first in the monkey model of AIDS in rhesus macaques.

Acknowledgments—We thank Dr. Nathaniel Landau for SIVmac proviral clones and Dr. Paul D. Bieniasz for the B-tropic MLV provirus.

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