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Functional Domains of APOBEC3G Required for Antiviral Activity

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Abstract The viral protein, Vif, is essential for the production of infectious progeny virions in natural target cells of human immunodeficiency virus type 1 (HIV-1). Several recent reports indicate that Vif acts by antagonizing the activity of an endogenous human antiviral protein, APOBEC3G. To investigate this route to restrict HIV-1 infection, we employed mutagenesis to assess APOBEC3G function during HIV-1 infection including interaction with Vif, localization, and activity in virions. We found that APOBEC3G binds Vif in infected cells and the C′-terminal region is required for this interaction. APOBEC3G was only incorporated into virions in the absence of Vif and deletion of either the N′-terminal or C′-terminal regions of APOBEC3G abrogated virion localization. Assaying endogenous reverse transcription we found that APOBEC3G and its C′-terminal deletion mutant inhibited full-length cDNA synthesis, possibly through binding to viral RNA, a function revealed through gel-shift assays. Taken together, our studies suggest that APOBEC3G inhibits HIV-1 infection through interference with reverse transcription and that Vif counteracts APOBEC3G by impeding its entry into virions. J. Cell. Biochem. 92: 560–572, 2004.

Key words: APOBEC3G; HIV-1; innate immunity

The essential lentiviral protein Vif was discovered in human immunodeficiency virus type 1 (HIV-1) and is widely conserved [Fisher et al., 1987; Oberste and Gonda, 1992]. Despite intensive research, its mechanism of action during the virus life cycle remains controversial. The definition of Vif function has been complicated by the fact that Vif is required for HIV-1 production in its natural target cells, primary human lymphocytes, and macrophages, but can be dispensable for virus replication in transformed cells, which are commonly used to investigate HIV-1 infection in culture [Gabuzda et al., 1992; Sakai et al., 1993; Chowdhury et al., 1996]. Based upon studies in cells that require Vif for HIV-1 infection, it has been postulated to facilitate capsid condensation [Hoglund et al., 1994], endogenous reverse transcription [Goncalves et al., 1994], Gag–Pol protein processing [Kotler et al., 1997], or to counteract the activity of a cellular antiviral protein [Madani and Kabat, 1998; Simon et al., 1998]. Supporting the latter contention, a novel human gene product was identified that blocked the replication of Vif-negative HIV-1 in transformed cells [Sheehy et al., 2002]. This protein, APOBEC3G, belongs to a family of RNA binding proteins [Jarmuz et al., 2002], but its role in the cell, during HIV-1 replication, or affecting Vif function is now being investigated. Several recent reports indicate that one role of Vif is to bind APOBEC3G and prevents its encapsidation [Kao et al., 2003; Mariani et al., 2003; Marin et al., 2003; Sheehy et al., 2003]. We investigated the interaction of Vif and APOBEC3G during HIV-1 infection, focusing upon regions in APOBEC3G required for function. We identify regions in APOBEC3G required for incorporation into virions and inhibition of endogenous reverse transcription in Vif-negative virions a novel function of APOBEC3G that may relate to its antiviral activity. We propose a pathway through which Vif and APOBEC3G function

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during HIV-1 infection and control the infectivity of virions.

MATERIALS AND METHODS

Plasmid Construction

The construction of NL4-3 ΔVif was described [Simm et al., 1995]. RNA was extracted from human fetal astrocytes with Trizol (GibcoBRL Life Technologies, Carlsbard, CA), cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen, Carlsbard, CA) and oligo dT primers and cDNA encoding full length APOBEC3G was amplified by PCR using the sense primer 5′-CCGCCGGAATTCATGAAGCCTCACTTCAGAAAC-3′ and the antisense primer 5′-CCGCCGCTCGAGTCAGTTTTCCTGATTCTGGAG-3′. pHA, a modified expression vector, was constructed from pcDNA 3.1 (Invitrogen) replacing the 90 base pair (bp) fragment containing multiple cloning sites with an 80 bp fragment that was synthesized to contain the HA epitope followed by *Bam*H I, *Eco*R I, *Sac* I, *Sal* I, and *Xho* I restriction sites; expression of HA and its fusion products is driven by the CMV promoter present on pcDNA. The APOBEC3G coding region was inserted in frame into the *Eco*R I and *Xho* I sites of pHA yielding pHA-APOBEC3G. pHA-APOBEC3GΔC was constructed similarly using the APOBEC3G sense primer and the antisense primer 5′-CCGCTCGAGTCAGCAGTGCTTAAATTCACTG-3′ creating a 114 bp deletion at C′-terminal. pHA-APOBEC3GΔ11 and pHA-APOBEC3GΔ67 were constructed by PCR amplification using the sense primers 5′-CCGCCGGAATTCATGTATCGAGACACATTCTCC-3′ and 5′-CCGCCGGAATTCATGAGATTCTTCCACTGGTTC-3′, respectively and the antisense primer listed above. HA-tagged Vpr was similarly constructed by insertion into a pHA fragment generated by PCR amplification using pNL4-3 [Adachi et al., 1986] as a template and the Vpr sense and antisense primers: 5′-CGCGGATCCATGGAACAAGCCCCAGAAGA-3′ and 5′-CCGCTCGAGCTAGGATCTACTGGCTCCATT-3′, respectively. A plasmid containing Vif with a Flag epitope tag at the C′-terminal was generated by PCR using pNL4-3 as a template, the sense primer 5′-GCGCCCAAGCTTATTATGGAAAACAGATGGCAG-3′, and the anti-sense primer containing the FLAG coding sequence 5′-CGGAATTCACTTGTCATCGTCGTCCTTGTAATCGTGTCCATTCGTTGTATG-3′. This fragment was inserted into the *Hin*d III and *Eco*R I sites of unmodified pcDNA3.1 vector yielding pVifFlag. An intermediate vector was generated by ligation of the 4.3 kb *Sph* I to *Eco*R I fragment of pNL4-3 into pUC18 (Invitrogen), the *Nde* I to *Eco*R I fragment was replaced with the corresponding fragment from pVifFlag. The final plasmid encoding intact HIV/VifFlag was created by replacement of the *Age* I to *Eco*R I fragment of pNL4-3 with the corresponding fragment from the intermediate vector. The construction of each plasmid was confirmed by restriction enzyme digest or sequencing.

Cell Culture, Transfection, and Infection

293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics. Transfections were performed using calcium phosphate precipitation at 10–20 μg DNA per 1.5×10^6 cells. 293T cell lines stably expressing APOBEC3G were obtained by transfection of pHA-APOBEC3G, selection in G418 at 25 mg/ml, and cloning by limiting dilution. HeLaCD4-LTR/β-gal cells obtained from Dr. Michael Emerman (through NIH AIDS Research & Reference Reagent Program) were infected with serial dilutions of HIV-1 preparations to determine the infectivity of virus stocks in MAGI assay as described [Kimpton and Emerman, 1992]. At 48 h after infection, cells were fixed and stained for the detection of β-galactosidase activity.

Protein Analysis by Immunoprecipitation and Immunoblot

Virions were collected from supernatants of transfected 293T cells, filtered and concentrated by centrifugation over a 20% sucrose cushion 1 h at 50,000*g*. The viral pellet was lysed in 1× sample buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02% brompherol blue, and 2% 2-mercaptoethanol) and subjected to electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (SDS–PAGE). Cells were lysed in ice-cold lysis buffer (137 mM NaCl, 50 mM Tris, pH 7.2, 3 mM $MgCl_2$, 0.5 mM DTT, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, and 100 μM leupeptin). Insoluble material was pelleted at 14,000*g* for 10 min in a microcentrifuge at 4°C and adjusted to a protein concentration of 1 mg/ml. For immunoprecipitation experiments, 1 ml cell lysate was incubated with 5 μg antibody and 40 μl

Protein A Sepharose beads (Pharmacia Biotech, Piscataway, NJ) on a rotator at 4°C overnight. After washing the beads with lysis buffer five times, the protein complexes were eluted by 20 μl 2× sample buffer for 3 min at 95°C. Western blot was conducted with cell or virion lysates (1–50 μg protein) or immunoprecipitates by SDS–PAGE and analysis by immunoblotting with appropriate primary antibodies, then with horseradish peroxidase-conjugated secondary antibodies before visualization by enhanced chemiluminescence system (Amersham, Piscataway, NJ). Anti-β-actin, anti-Flag, anti-HA monoclonal, and polyclonal antibodies were purchased from Sigma (St. Louis, MO). Anti-Vif serum was kindly provided by Dr. Dana Gabuzda, and hybridomas which produced MAb against HIV-1 p24 (anti-CA) was obtained from Dr. Bruce Chesebro and Dr. Hardy Chen through NIH AIDS Research & Reference Reagent Program. For sequential immunoblots, membranes were stripped of antibodies by incubation in 0.2 M NaOH with vigorous shaking for 10 min.

Purification of HA-Tagged Proteins

293T cells were transfected with pHA-APOBEC3G, pHA-APOBEC3GΔ67, or pHA-Vpr and after 48 h, cells were lysed in ice-cold extraction buffer (137 mM NaCl, 50 mM Tris, pH 7.2, 3 mM $MgCl_2$, 0.5 mM DTT, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, and 100 μM leupeptin). Cell lysates were clarified by sedimentation and loaded onto the column of monoclonal anti-HA agarose conjugate (Sigma). After washing with extraction buffer, the bound HA-tagged fusion proteins were eluted with glycine–HCl buffer (pH 2.5), then HA-tagged fusion proteins were dialyzed with PBS and concentrated by Microcon centrifugal filter devices (Millipore, Bedford, MA). The purified HA-tagged fusion proteins were analyzed by Western blot with anti-HA monoclonal antibody (Sigma). Protein concentration was detected by DC protein assay kit (Bio-Rad, Hercules, CA).

Electrophoretic Mobility Shift Assay (EMSA)

A 110 bp fragment of NL4-3 Tat cDNA was obtained by PCR with the sense primer: 5′-ATGGAGCCAGTAGATCCTAG-3′ and antisense primer: 5-TGCTTTCATTGCCAAGTTTG-3′ using pNL4-3 as a template and was purified after electrophoresis using DNA Gel Extraction Kit (Millipore). This fragment was used as template for in vitro RNA synthesis incorporating ^{32}[P]-CTP using the Lig'nScribe Reaction Kit and MAXIscript In vitro Transcription Kit (Ambion, Austin, TX) following the manufacturer's instruction. Purified HA-tagged fusion proteins were incubated in 10 μl of reaction mixture containing 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 4% glycerol, and 0.05 μg/μl of poly(dI-dC) for 10 min. The radiolabeled RNA probes were added to the reaction mixture and incubated for 20 min at 25°C. The samples were subjected to electrophoresis on a 10% native polyacrylamide gel at 25°C with 0.5× TBE buffer (50 mM Tris, 45 mM boric acid, 0.5 mM EDTA). The gel was dried and analyzed by autoradiography.

Endogenous Reverse Transcription Assay

Virion pellets were concentrated from transfectant supernatants by sedimentation and virions containing 1 μg p24 were first permeabilized with 1 mM *n*-octylglucoside and 0.04% Triton X 100, before addition of purified proteins as indicated in the text, the mixture was incubated on ice for 20 min prior addition of 50 μl reaction buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 2 mM magnesium acetate, 0.1 mM each dNTP, ^{32}[P]-dCTP (10 μCi per reaction mixture) or 50 μM of dATP, dCTP, Dgtp, and 2.5 μM of ^{3}[H]-dTTP (10 μCi per reaction mixture), 1 mM *n*-octylglucoside (Sigma), and 0.04% Triton X 100 for 16 h at 37°C. For ^{3}[H]dTTP incorporation, reactions were stopped by spotting onto DE81 filters for quantitation by liquid scintillation counting. Reactions involving labeling with ^{32}[P]-dCTP were terminated by removing unincorporated radionucleotides with a Sephadex G-50 column (Roche, Indianapolis, IN). The reaction products were subjected to electrophoresis in 1% agarose in Tris-acetic-EDTA (TAE) running buffer. DNA molecular weight markers were run in parallel, and visualized with ethidium bromide. The gels were dried and exposed to film for autoradiography.

Quantitative PCR Amplification

Sedimented virions were treated with 2 μg RNase-free DNase I at 37°C for 30 min, washed twice with PBS, then the endogenous reverse transcription reaction was performed as described above in the presence of unlabeled

dNTPs. The reaction products were diluted 1:100, and 1 μl per reaction was used for quantitative PCR amplification by using designated primers for 25 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min with additional 2 μCi ^{32}[P]-CTP per reaction. The oligonucleotide primer pairs are M667 and AA55 [Zack et al., 1990] R/U5 region, 5′-ATGGAGCCAGTAGATCCT-3′ and 5′-CTGCTTTGATAGAGAAAC-3′ *tat* region, nt 5830 to 6048, and M667 and M661 [Zack et al., 1990], LTR-*gag*. The PCR products were resolved on 6% polyacrylamide gels and visualized by autoradiography. A standard curve was constructed using known copies of NL4-3Δvif plasmid amplified and analyzed in parallel.

RESULTS

We constructed a cell line for high expression of both APOBEC3G and HIV-1 proteins by transfection of 293T cells with an expression vector of HA-epitope-tagged APOBEC3G, selection for G418 resistance, and cloning of resistant cells. The expression of APOBEC3G was assayed by immunoblot using anti-HA with 293T cells transiently transfected with HA-APOBEC3G serving as positive controls (Fig. 1a). Cell extracts were standardized by the expression of β-actin. All four selected stable transfectants expressed APOBEC3G, although at different levels; compare for example lanes 4, 293T/APOBEC3Ghigh and 6, 293T/APOBEC3Glow. A spontaneous mutant in APOBEC3G of the C′-terminal 38 amino acids was expressed by one of the cell lines (lane 5), prompting an investigation of the function of this region. We confirmed the requirement for Vif for virion infectivity in the presence of APOBEC3G by transfection of these cell lines with intact HIV-1/NL4-3 or NL4-3 carrying a deletion in Vif, ΔVif, and titration of the infectivity of their progeny virus by single-cycle MAGI assay [Kimpton and Emerman, 1992] (Fig. 1b–c). The three cell lines expressing intact APOBEC3G produced infectious virus only in the presence of Vif. By contrast, control 293T and 293T/APOBEC3GΔC, were insensitive to Vif activity. It is noteworthy that even in 293T/APOBEC3Glow, where it is expressed at a very low level, APOBEC3G was active in blocking infectivity of ΔVif, (lane 6). These findings confirm the previous study that Vif is specifically opposed by the expression of APOBEC3G, demonstrate that the C′-terminal domain of the protein is required for its activity in HIV-1 infection, and validate the use of 293T expressing APOBEC3G to investigate this interaction.

We next investigated the interaction of APOBEC3G and Vif by evaluating the localization of these proteins in virions (Fig. 1d–f). When over-expressed by transient transfection, both APOBEC3G and Vif were present in virions (lanes 2). By contrast, stable cell lines 293T/APOBEC3Ghigh and low produced virions in which APOBEC3G localized to virions of ΔVif but not wild-type NL4-3 (compare lanes 4 and 5 to 8 and 9, respectively). The previous study that found co-localization of Vif and APOBEC3G in virions employed transient transfection [Sheehy et al., 2002] and our results suggest co-localization is only seen at extremely high levels of expression. Using APOBEC3Ghigh, we demonstrated that expression of Vif abrogated the localization of APOBEC3G in ΔVif virions (Fig. 1g). We adjusted the amount of plasmid DNA transfected to express Vif at either high or low levels. When both highly expressed (lane 1) or moderately expressed (lane 2), Vif prevented encapsidation of APOBEC3G when co-expressed with ΔVif. Vpr, which itself is encapsidated [Accola et al., 2000], had no effect (lane 3).

The ability of Vif to affect APOBEC3G localization confirms previous studies showing that the two proteins interact [Kao et al., 2003; Mariani et al., 2003; Marin et al., 2003; Sheehy et al., 2003]. To investigate this interaction, we probed the regions in APOBEC3G governing the association (Figs. 2 and 3). A APOBEC3G mutant was constructed carrying the HA-epitope tag and a deletion of the C′-terminal 38aa. 293T cells were transiently transfected with HA-APOBEC3G constructs or controls, together with NL4-3 carrying Vif tagged with FLAG. Cell extracts were probed for expression of Vif by immunoblot using anti-FLAG and we found that Vif was similarly expressed in each transfection (Fig. 2a, lanes 2–4). Next, we inquired whether Vif associates with APOBEC3G by sequential immunoprecipatation of APOBEC3G containing complexes using anti-HA and immunoblot using anti-FLAG to detect Vif in complexes (Fig. 2a, lanes 6–8). Vif expressed by HIV-1 transfection was found in complexes with intact APOBEC3G and not APOBEC3GΔC. The specificity of this interaction is indicated in that Vif was absent from immunoprecipitates formed in the absence of

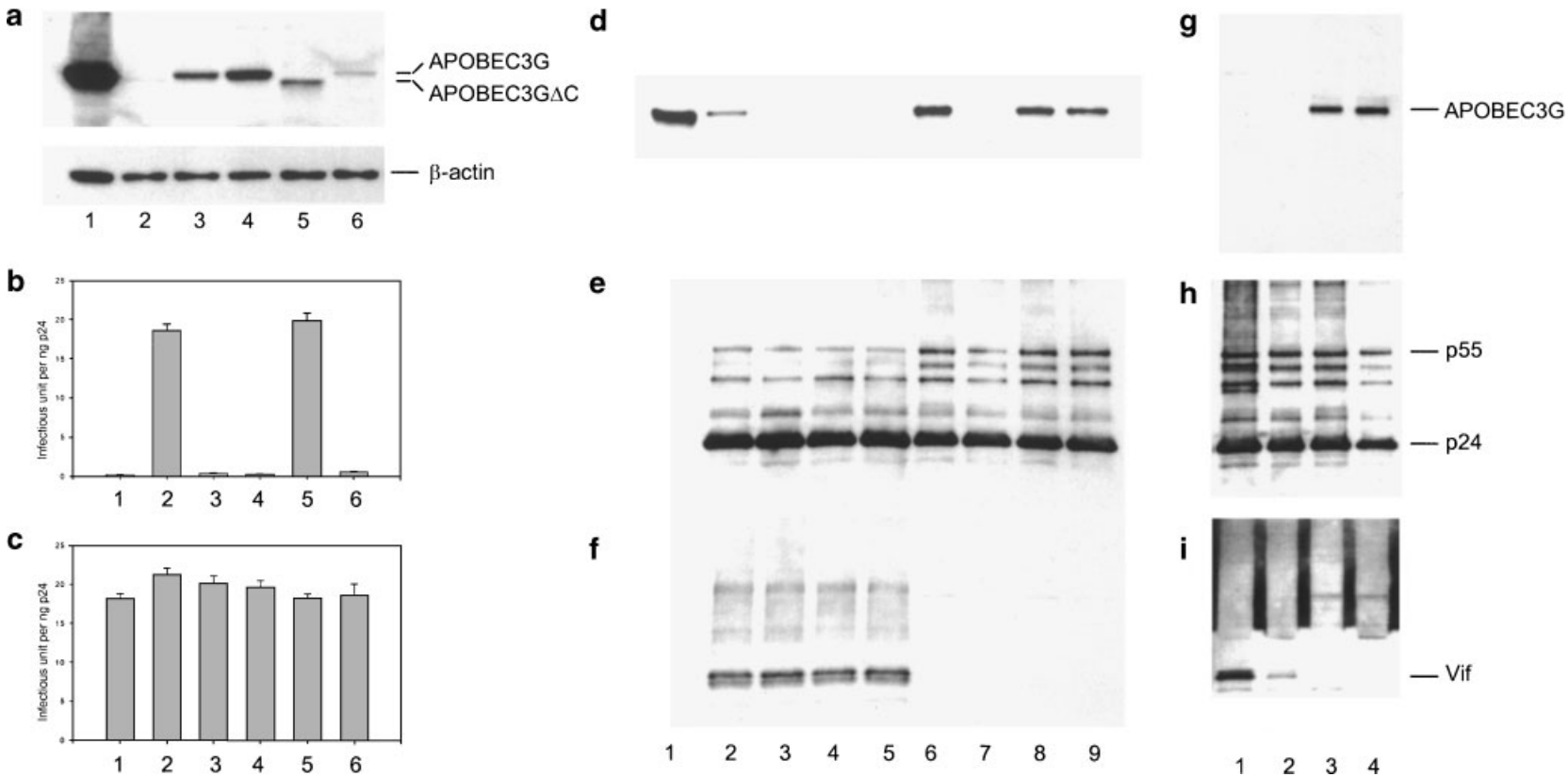

Fig. 1. Vif antagonizes anti-HIV activity and virion localization by APOBEC3G. **a**–**c**: 293T transfected with ΔVif and pHA-APOBEC3G (**lane 1**) or ΔVif and pHA (**lane 2**), or individual 293T/APOBEC3G clones transfected with ΔVif and pHA (**lanes 3**–**6**) (a) were lysed and subjected to Western blot using anti-HA and anti-β-actin or (b) assayed for infectivity of progeny virus normalized by p24 content by MAGI assay (c) or were transfected with wild type NL4-3 and assayed for progeny virus infectivity (**d**) extracts of 293 T cells transfected with pHA-APOBEC3G (lane 1), or virions from cells transfected as follows 293 T with NL4-3 and pHA-APOBEC3G (lane 2), NL4-3 and pHA, (lane 3) ΔVif and pHA-APOBEC3G, (lane 6), ΔVif and pHA (**lane 7**), or 293T/APOBEC3G high with NL4-3 (lane 4) 293T/APOBEC3G low with NL4-3 (lane 5), 293T/APOBEC3Ghigh with ΔVif (**lane 8**) and 293T/APOBEC3Glow with ΔVif. Virions were collected, normalized by p24 content, and subjected to Western blot employing (d) anti-HA, (**e**) anti-CA, (**f**) anti-Vif staining. All transfected plasmids were at a 1:1 ratio. **g, h, i**: 293T/APOBEC3Ghigh were transfected and virions were collected and subjected to immunoblot as follows ΔVif and pVifFlag (1:1 ratio, lanes 1) and (2:1 ratio, lanes 2), ΔVif and pVpr (1:1 ratio, lanes 3) ΔVif and pcDNA (1:1 ratio, lanes 4). The blot were stained with panel (g) anti-HA, panel (h) anti-CA and panel (i) anti-Vif.

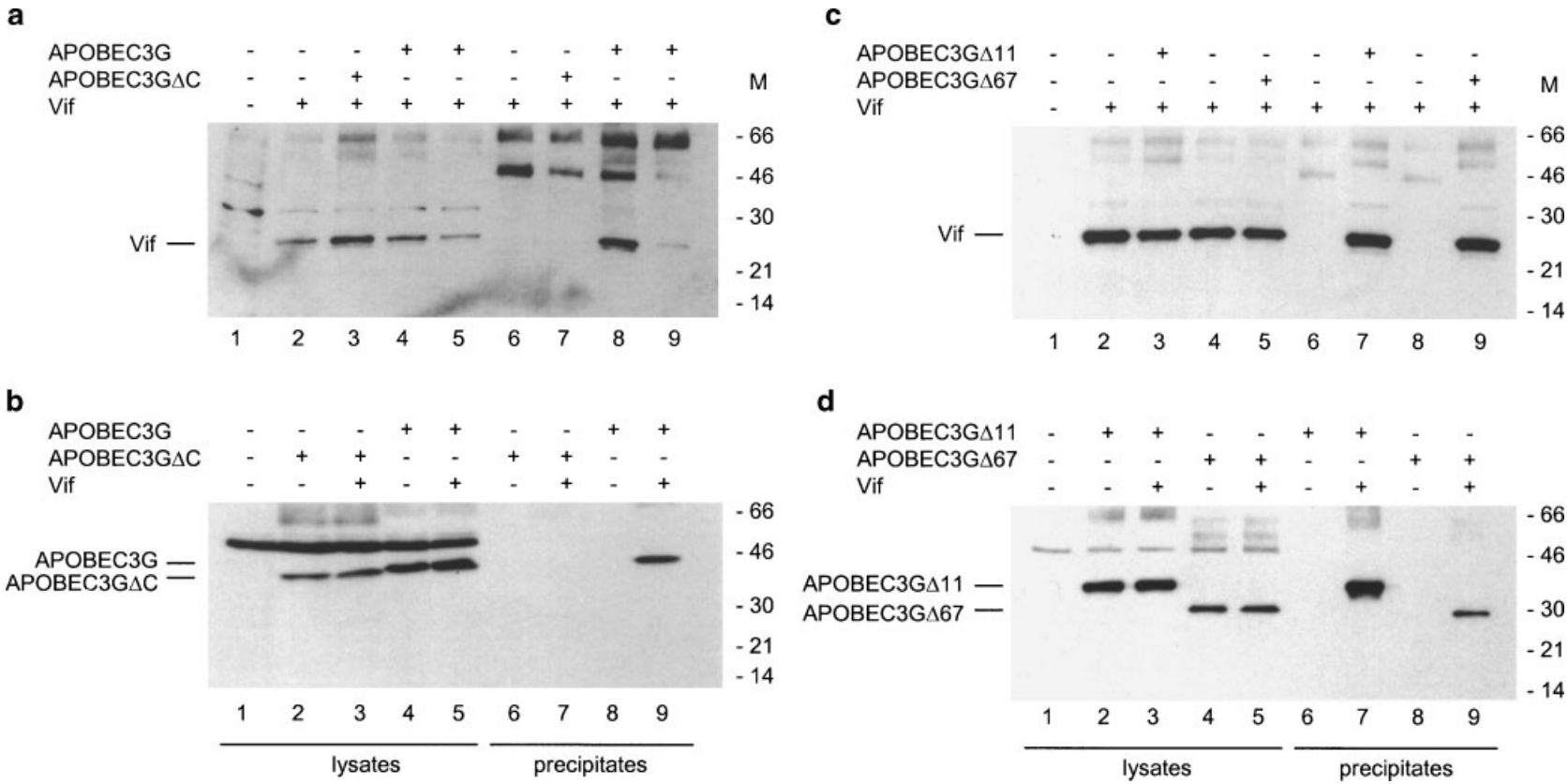

Fig. 2. Regions governing the interaction of APOBEC3G with Vif. Cells were transfected, lysates were prepared and subjected to immunoblot and or immunoprecipitations as follows: (**a**) 293T with HIV/VifFlag and pHA (**lane 2**); 293T with HIV/VifFlag and pHA-APOBEC3GΔC (**lane 3**); HIV/VifFlag and pHA-APOBEC3G (**lane 4**); and 293T/APOBEC3G high with HIV/VifFlag and pHA (**lane 5**). **b**: 293T with pHA-APOBEC3GΔC and pHA (lane 2); pHA-APOBEC3GΔC and HIV/VifFlag (lane 3); pHA-APOBEC3G and pHA (lane 4); pHA-APOBEC3G and HIV/VifFlag (lane 5). **c**: 293T with HIV/VifFlag and pHA (lane 2); HIV/VifFlag and pHA-APOBEC3GΔ11 (lane 3); HIV/VifFlag and pHA-Vpr (lane 4); HIV/VifFlag + pHA-APOBEC3GΔ67 (lane 5). **d**: 293T cells with pHA-APOBEC3GΔ11 and pHA (lane 2); pHA-APOBEC3GΔ11 and HIV/VifFlag (lane 3); APOBEC3GΔ67 and pHA (lane 4); pHA-APOBEC3GΔ67 and HIV/VifFlag (lane 5). a and c: Extracts from lanes 2, 3, 4, and 5 were precipitated with anti-HA and loaded into **lanes 6**, **7**, **8**, and **9**, respectively and the entire blot stained with anti-Flag antibody. b and d: Extracts from lanes 2, 3, 4, and 5 were precipitated with anti-FLAG and loaded into lanes 6, 7, 8, and 9 and the entire blot was stained with anti-HA. All **lanes 1** contained lysates of mock-transfected 293T and all plasmids were transfected at a 1:1 ratio.

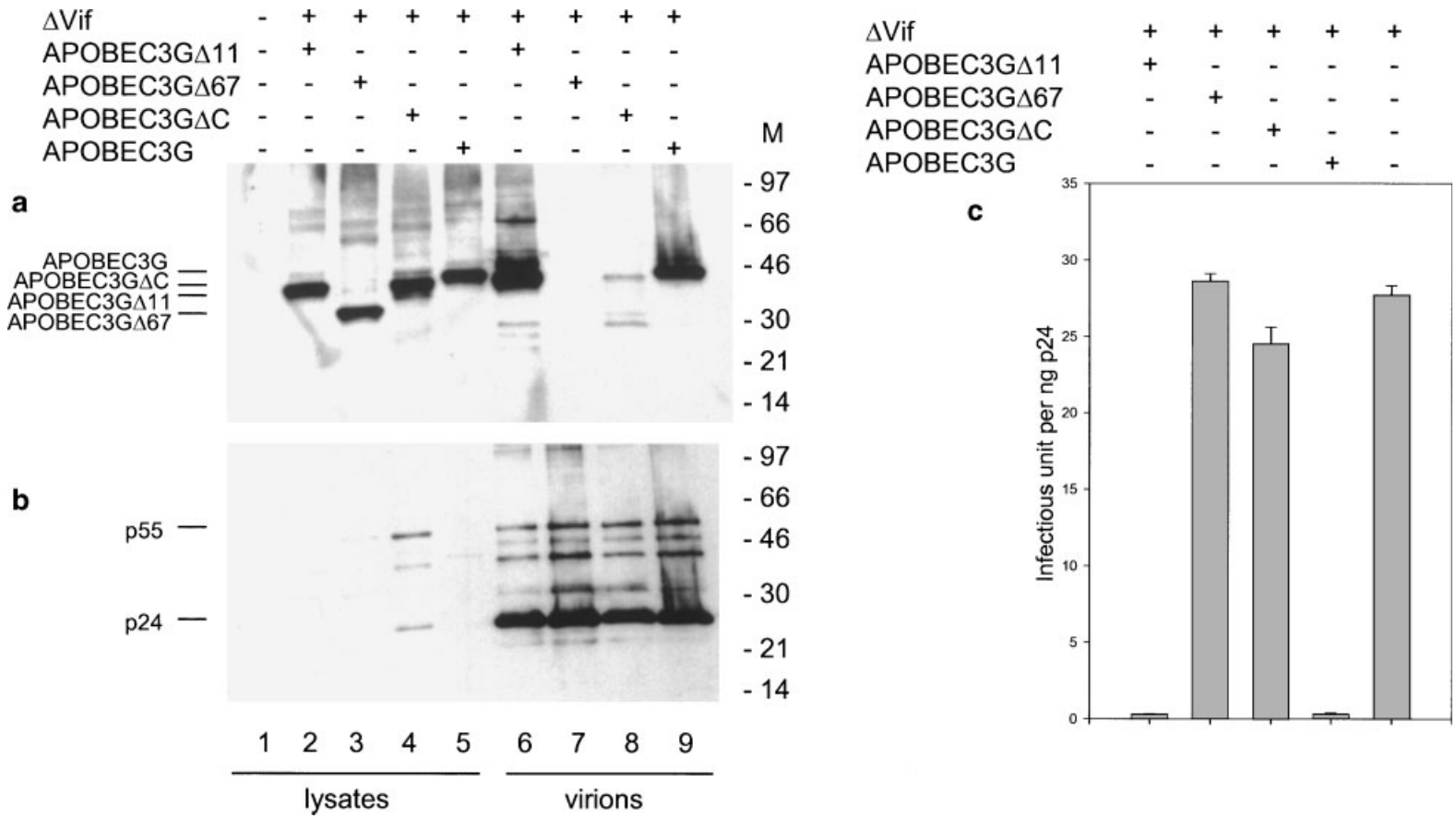

Fig. 3. The same regions of APOBEC3G govern virion localization and anti-HIV-1 activity. Lysates and virions were collected from 293T cells transfected with ΔVif and either pHA-APOBEC3GΔ11, pHA-APOBEC3GΔ67, pHA-APOBEC3GΔC, or pHA-APOBEC3G; respectively. **Lanes 2–5** contain lysates, **lanes 6–9** contain virions standardized by p24 content, respectively. Lane 1 contains extracts of mock transfected 293T cells. **a**: The membrane was stained with anti-HA (**b**) or with anti-CA (**c**) Infectivity in MAGI assay of virions shown in lanes 6–9.

APOBEC3G. This finding indicates that the C′-terminal domain of APOBEC3G is required for its interaction with Vif. We performed the reciprocal experiment using anti-HA in immunoblot, confirming the presence of APOBEC3G in cell extracts and then testing its inclusion in immunoprecipitates of Vif formed by anti-FLAG (Fig. 2b). Both intact APOBEC3G and APOBEC3GΔC were well expressed by transfection, but only the intact protein was detected in complexes with Vif, APOBEC3GΔC failed to immunoprecipitate using an antibody directed against Vif. These findings confirm those shown in panel a indicating that APOBEC3G and Vif stably interact during HIV-1 replication and that the C′-terminal 38aa residues of APOBEC3G is required for this interaction.

Using the same strategy, we investigated the role of the N′-terminal residues of APOBEC3G in the interaction with Vif. Two mutants were constructed, one with an in-frame deletion of the N′-terminal 11 amino acids, one with an N′-terminal deletion of 67 amino acids, both carrying the HA tag. As shown in Figure 2c, in cells co-expressing NL4-3 and these mutants of APOBEC3G, Vif co-precipitated with either deletion mutant. Panel d shows the reciprocal experiment, using antibody to detect APOBEC3G either in cell extracts or in co-precipitates with Vif. In this format, both APOBEC3G deletion mutants stably associated with Vif and could be co-precipitated with antibodies directed against Vif. It is worth noting that the overall expression of APOBEC3GΔ67 is less than APOBEC3GΔ11, but both were sufficiently highly expressed to permit co-precipitation of Vif. These studies indicate that the N′-terminal 67 residues of APOBEC3G are dispensable for the interaction with Vif.

Having defined regions of APOBEC3G required for its interaction with HIV-1 Vif, we investigated the contribution of this interaction to the antiviral function of APOBEC3G in HIV-1 infection. We co-transfected 293T cells with NL4-3ΔVif and one of the HA-tagged APOBEC3G or control vectors and investigated their incorporation into virions and their effects upon the infectivity of virions (Fig. 3). Each APOBEC3G construct was well expressed upon transfection but only intact APOBEC3G and APOBEC3GΔ11 were incorporated into virions (Fig. 3a), standardized by HIV-1 p24 content (Fig. 3b). Virions were tested for infectivity in MAGI assay and the antiviral function of APOBEC3G was conserved in APOBEC3GΔ11, but not in APOBEC3GΔ67 or APOBEC3GΔC, as also shown in stably expressing cells in Figure 1b. These findings indicate that the requirements for virion localization and antiviral function coincide in structural regions of APOBEC3G, raising the possibility that APOBEC3G enacts its function within virions.

Considering the antiviral role that APOBEC3G may play in the virion and its ability to bind RNA [Jarmuz et al., 2002; Sheehy et al., 2002], we tested its binding to HIV-1 RNA in EMSAs. Increasing amounts of affinity purified HA-APOBEC3G or HA-Vpr were incubated with a ^{32}[P]-single-stranded RNA probe from the Tat gene, complexes were permitted to form, and the incubation mixture was electrophoresed in non-denaturing gels (Fig. 4). The addition of HA-Vpr had no effect upon the mobility of the probe, but HA-APOBEC3G bound and retarded the probe in a dose-dependent manner. The ability of APOBEC3G to bind HIV-1 RNA suggests that it may have a role in the utilization of RNA as a template for reverse transcription. To evaluate this proposition, we measured endogenous reverse transcription in virions synthesized in the presence or absence of Vif in which APOBEC3G was co-expressed with HIV-1 or was added exogenously to the reverse transcription reaction. The reaction was monitored by measurement of the incorporation of ^{32}[P]-labeled nucleotides into DNA (Fig. 5). Co-expression of APOBEC3G reduced endogenous reverse transcription by about 40% in Vif-negative but not Vif-positive virions (Fig. 5a). To exclude that reverse transcriptase enzymatic activity was impaired, we also tested these virions in exogenous reverse transcription assays and found that APOBEC3G had no effect upon cDNA synthesis using a synthetic template, indicating that reverse transcriptase is fully active (not shown). We then performed dose-response and specificity studies by adding affinity-purified proteins to endogenous reverse transcription reactions employing virions synthesized in the presence or absence of Vif (Fig. 5b,c). Like the experiment shown in panel a, APOBEC3G reduced the extent of reverse transcription significantly, by about 50%, in ΔVif virions but not in wild-type virions. Vpr had no effect. However APOBEC3GΔC, which is unable to enter virions, also inhibited endogenous reverse transcription when added to the reaction in vitro, indicating that the C′-terminal

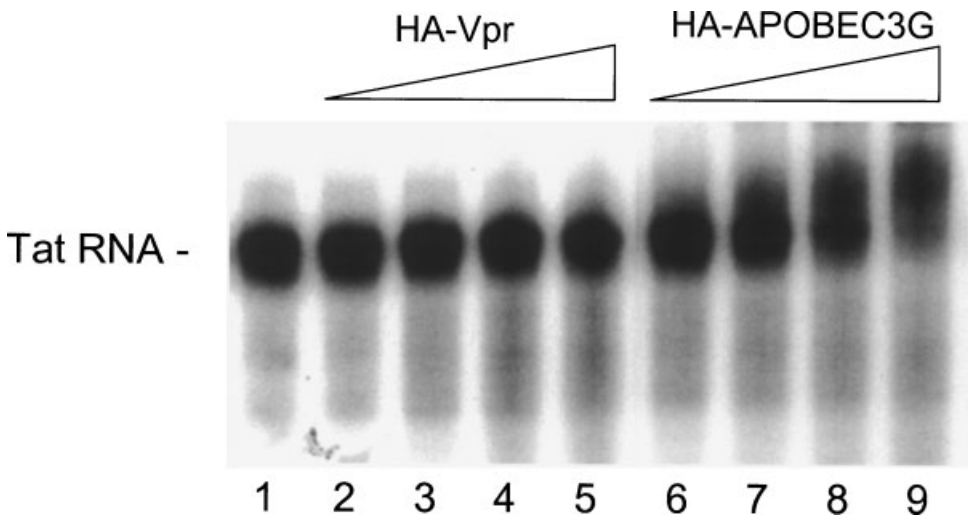

Fig. 4. APOBEC3G binds HIV-1 RNA. A ^{32}[P]-labeled HIV-1 Tat RNA probe was untreated or incubated with increasing amounts of HA-Vpr (**lanes 2–5**) or HA-CEM (**lanes 6–9**). Proteins were added at 1.25 ng, 2,5 ng, 5.0 ng, or 10.0 ng to lanes 2 and 6, 3 and 7, 4 and 8, 5 and 9, respectively to 1 μg p24 virion. After complexes were allowed to form, the reaction mixture was assayed by EMSA.

region is dispensable for this function. We repeated this experiment in a different format and electrophoresed the reaction products for direct visualization (Fig. 5d). APOBEC3G, but neither its deletion mutant Δ67 nor Vpr, blocked the synthesis of the major product of this reverse transcription reaction, the 7 kb intermediate. It is noteworthy that APOBEC3GΔ67 has lost the ability to impair reverse transcription, since its deletion removes an essential histidine in a RNA binding domain [MacGinnitie et al., 1995].

To better resolve the progress of reverse transcription by virions lacking Vif, but exposed to APOBEC3G, we subjected the products of endogenous reverse transcription to polymerase chain reaction using primers that amplify early, intermediate, or late products of cDNA synthesis [Goff, 1990] (Fig. 6). Reverse transcription proceeds through the synthesis of its first product in the presence of APOBEC3G but a product in the middle of the genome requiring two strand transfers is reduced more than 10-fold by the presence of APOBEC3G but not APOBEC3GΔ67. APOBEC3G also inhibits synthesis of the final product of the reaction. This result is in full agreement with an earlier study first defining the defect in endogenous reverse transcription in ΔVif virions [Goncalves et al., 1994] and with a recent study that obtained but discounted the result [Gaddis et al., 2003]. Moreover, our findings explain the cause of impaired infectivity of ΔVif virions. When synthesized in the absence of Vif, virions contain APOBEC3G, which through binding to template RNA, impedes reverse transcription.

DISCUSSION

The results presented here clarify the opposing activities of APOBEC3G and Vif in HIV-1 replication. We report that consistent with the

Fig. 5. APOBEC3G inhibits endogenous reverse transcription in ΔVif, but not wild-type virions. **a**: Virions were isolated from 293T cells transfected either with pNL4-3 ΔVif (bars 1–3) or pNL4-3 (bars 4–6) and pHA (bars 1 and 4), pHA-APOBEC3GΔ67 (bars 2 and 5), or pHA-APOBEC3G (bars 3 and 6) and subjected to assay of endogenous reverse transcription by incorporation of radiolabeled nucleotides. **b**: Virions were isolated from transfectants of pNL4-3ΔVif, left untreated (bar 1) or mixed with increasing amounts of affinity purified HA-APOBEC3G (bars 2–5) or HA-Vpr (bars 6–9), or HA-CEMΔC (10 ng) prior to assay of endogenous reverse transcription. Proteins were added at 1.25, 2.5, 5.0, or 10.0 ng per μg p24 of virion to systems 2 and 6, 3 and 7, 4 and 8, or 5 and 9, respectively. **c**: Virions were isolated from transfectants of pNL4-3 and treated exactly as described in the legend to panel b. **d**: Virions were prepared from transfectants of pNL4-3ΔVif (**lanes 1–5**) or pNL4-3 (**lanes 6–10**) and incubated in the presence of 5 ng affinity purified HA-APOBEC3G (lanes 1 and 6), HA-APOBEC3GΔ67 (lanes 2 and 7), HA-Vpr (lanes 3 and 8), no additional protein (lanes 4 and 9) or no cold dNTP (lanes 5 and 10). Endogenous reverse transcription was conducted in the presence of ^{32}[P]-dCTP and the products were subjected to electrophoresis and autoradiography.

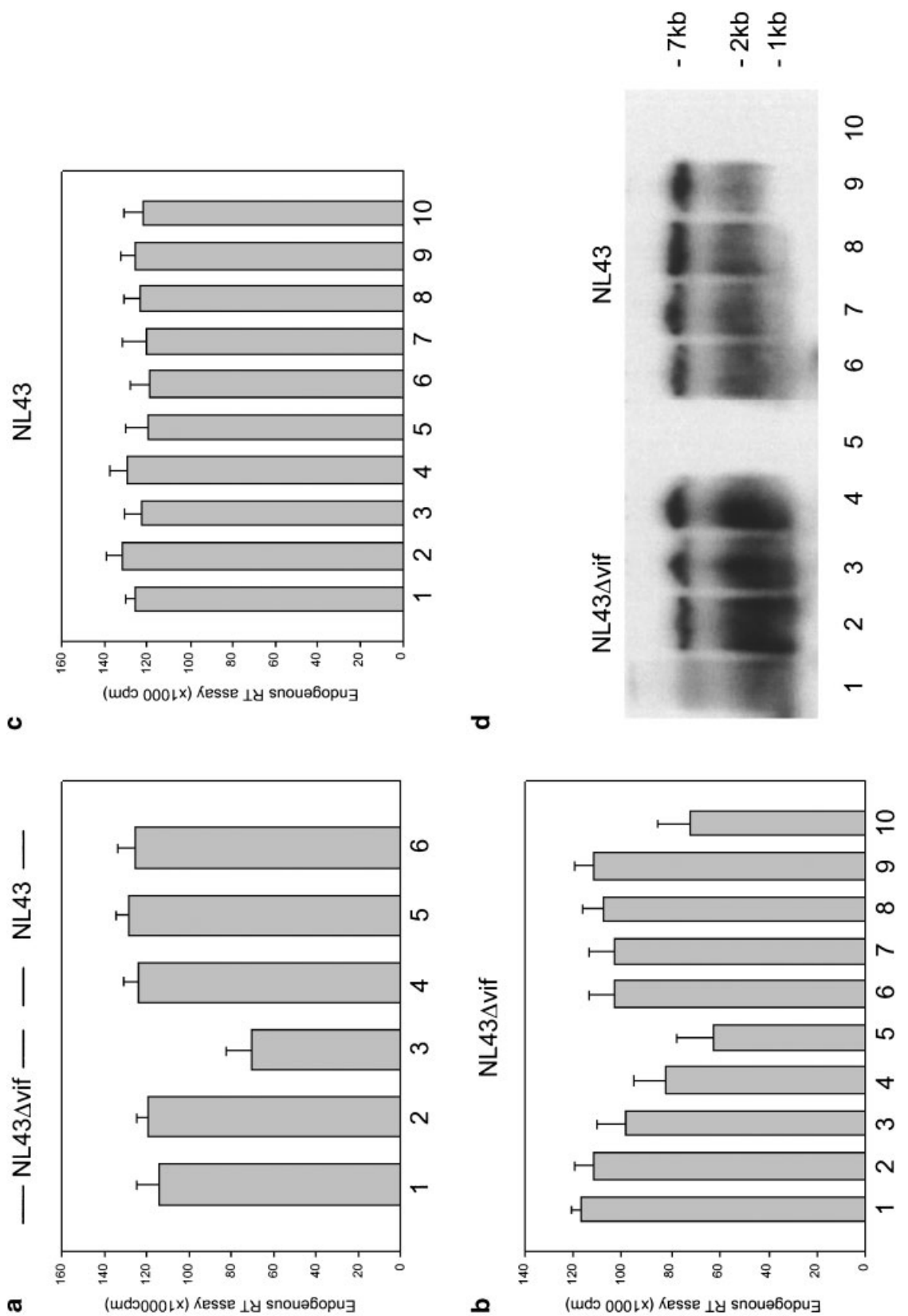
a
— NL43Δvif — — NL43 —
Endogenous RT assay (x1000cpm)
b
NL43Δvif
Endogenous RT assay (x1000 cpm)
c
NL43
Endogenous RT assay (x1000 cpm)
d
NL43Δvif
NL43
- 7kb
- 2kb
- 1kb

Fig. 5.

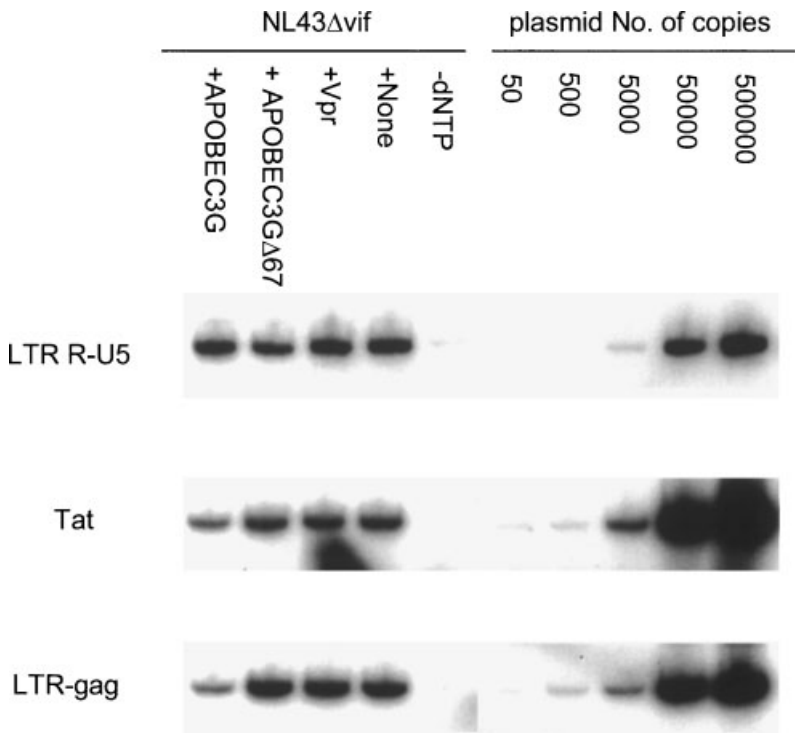

Fig. 6. APOBEC3G blocks HIV-1 the progress of reverse transcription in ΔVif virions. Endogenous reverse transcription in NL4-3ΔVif virions was allowed to proceed as described in the legend to Figure 5d in the absence of radiolabeled nucleotides. Samples of the reaction products was subjected to PCR amplification incorporating radiolabeled nucleotides of regions synthesized at early (R-U5), intermediate (*tat*), or final stages (LTR-*gag*) of reverse transcription. The lanes at the right show the products obtained from amplification of the noted number of copies of the pNL4-3ΔVif plasmid. PCR products were subjected to electrophoresis and autoradiography.

integrity of residues 12–67 APOBEC3G binds HIV-1 RNA and blocks viral cDNA synthesis by reverse transcriptase, providing a mechanism for its impairment of virion infectivity. However, when APOBEC3G is co-expressed with Vif, the two proteins form intracellular complexes involving the C′-terminus of APOBEC3G impeding the entry of APOBEC3G into virions, confirming the mechanism through which Vif antagonizes the effect of APOBEC3G and maintains HIV-1 infectivity in non-permissive cells [Kao et al., 2003; Mariani et al., 2003; Marin et al., 2003; Sheehy et al., 2003].

The behavior of APOBEC3G during HIV-1 replication was partially mapped onto structural determinants using transient or stable transfection of expression vectors and HIV-1 DNA. Two regions, residues 12–67 and residues 346–384, are required both for virion localization and antiviral activity. The N′-terminal 11 amino acids are dispensable for both functions. The region from 12–67 is required for inhibition of reverse transcription and contains a conserved histidine required for RNA binding [MacGinnitie et al., 1995], APOBEC3G interacts with Vif in infected cells as shown by reciprocal co-immunoprecipitation, and the C′-terminal region of APOBEC3G essential for virion localization of APOBEC3G. APOBEC3G localized efficiently in virions only when expressed in cells in the absence of Vif, Vif may act as a decoy binding the C′-terminal region that targets APOBEC3G to the virion. Vif binding of APOBEC3G to inhibit its localization in virions was reported by several investigators during the conduct of this study [Kao et al., 2003; Mariani et al., 2003; Marin et al., 2003; Sheehy et al., 2003].

Like a previous report [Jarmuz et al., 2002], our studies show that APOBEC3G can bind RNA. There is no obvious sequence specificity since the protein bound diverse RNA including an AU polymer, an mRNA of its own family, and in our studies Tat and Gag (not shown) probes. In cells non-permissive to Vif-negative HIV-1 replication, in which APOBEC3G is active,

previous studies reported that endogenous reverse transcription in virions is impaired [Goncalves et al., 1994]. We find that APOBEC3G either co-expressed with HIV-1 or added exogenously to the reverse transcription reaction blocked cDNA synthesis in ΔVif, but not wild-type virions. The block is progressive and late products are selectively reduced versus strong-stop DNA that is fully synthesized immediately after the first strand transfer [Goff, 1990]. Deletion of the N′-terminal 67 residues in APOBEC3G, that contain a histidine residue essential for RNA binding [MacGinnitie et al., 1995; Jarmuz et al., 2002], abolishes the ability to block reverse transcription and abolishes its antiviral function. We suggest that APOBEC3G is a non-specific inhibitor of reverse transcription of the HIV-1 genomic RNA template in virions synthesized in the absence of Vif. The inability of APOBEC3G to affect reverse transcription when added to virions made in cells containing Vif can be explained either by the presence in virions of Vif itself or by structural differences in the virions. Since the presence of Vif in virions is not resolved, further studies will be required. At the quantitative level our results of endogenous reverse transcription fully accord with those recently published [Gaddis et al., 2003] and also confirm the earlier study demonstrating that Vif-negative particles are competent for early but not late phases of reverse transcription in vitro [Goncalves et al., 1994] and during HIV-1 infection [von Schwedler et al., 1993]. In several recent studies, mutations were found in HIV-1 cDNA following cellular infection by ΔVif HIV-1 or by expression of APOBEC3G, leading to the proposal that APOBEC3G enzymatically modifies nascent reverse transcripts to generate frequent mutations [Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Mariani et al., 2003; Zhang et al., 2003]. If the APOBEC3G reduction in completion of reverse transcription reported here and elsewhere [Mariani et al., 2003] is characteristic of native infection then roughly 90% of cDNA will be incomplete. Incomplete molecules may also suffer hypermutation, but in any case they cannot encode virus. It is noteworthy that murine leukemia virus DNA is mutagenized by APOBEC3G without impairment of infectivity [Mangeat et al., 2003] raising the question of the relevance of hypermutation to the defect observed in viral infection. Clearly more studies will be required to fully elucidate the mechanism of action of Vif and APOBEC3G on the control of HIV-1 infectivity in human cells.

Published results and those reported here can be employed to construct a scheme for the antagonistic activities of APOBEC3G and Vif. We suggest that Vif binds APOBEC3G through its C′-terminal region inside infected cells and that this interaction is required for the opposition of Vif and APOBEC3G. In the absence of Vif, APOBEC3G enters virions during assembly and binds viral RNA through its N′-terminal region, impeding its later reverse transcription during target cell infection. Hypermutation may further reduce the integrity of cDNA. Thus, one essential activity of Vif may be prevention of the encapsidation of APOBEC3G. APOBEC3G exerts its antiviral action at the level of reverse transcription.

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