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Enzymatically Active APOBEC3G Is Required for Efficient Inhibition of Human Immunodeficiency Virus Type 1[▽]

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APOBEC3G (APO3G) is a cellular cytidine deaminase with potent antiviral activity. Initial studies of the function of APO3G demonstrated extensive mutation of the viral genome, suggesting a model in which APO3G's antiviral activity is due to hypermutation of the viral genome. Recent studies, however, found that deaminase-defective APO3G mutants transiently expressed in virus-producing cells exhibited significant antiviral activity, suggesting that the antiviral activity of APO3G could be dissociated from its deaminase activity. To directly compare the antiviral activities of wild-type (wt) and deaminase-defective APO3G, we used two approaches: (i) we titrated wt and deaminase-defective APO3G in transient-transfection studies to achieve similar levels of virus-associated APO3G and (ii) we constructed stable cell lines and selected clones expressing comparable amounts of wt and deaminase-defective APO3G. Viruses produced under these conditions were tested for viral infectivity. The results from the two approaches were consistent and suggested that the antiviral activity of deaminase-defective APO3G was significantly lower than that of wt APO3G. We conclude that efficient inhibition of *vif*-defective human immunodeficiency virus type 1 requires catalytically active APO3G.

The human immunodeficiency virus type 1 (HIV-1) accessory protein Vif plays an important role in regulating virus infectivity (8, 38). It is now well established that HIV-1 Vif can counteract the human cytidine deaminase APOBEC3G (APO3G). The inhibition of APO3G's antiviral effects has been attributed to a reduction in cellular expression of APO3G protein, which is due to Vif-mediated degradation of APO3G by cytoplasmic proteasomes (6, 20, 25, 27, 34, 37, 43). On the other hand, we recently found that Vif could prevent encapsidation of a degradation-resistant APO3G variant, suggesting that Vif can inhibit the APO3G antiviral activity through multiple independent mechanisms (29). In the absence of Vif, APO3G is efficiently packaged into HIV virions and inhibits virus replication. A number of studies reported that the presence of APO3G in the virus can result in hypermutation of the viral minus-strand cDNA during reverse transcription (11, 18, 23, 24, 42, 45), inhibition of reverse transcription (9), tRNA annealing or tRNA processing (10, 26), DNA strand transfer (19, 26), or integration (22, 26).

Some of these effects do not require catalytically active APO3G (19, 22), and several reports suggested that deaminase-defective APO3G and APO3F have antiviral activity when transiently coexpressed with HIV-1 in 293T cells (3, 12, 28, 35). Our own data concerning the antiviral properties of the deaminase-defective APO3G C288S/C291A mutant supported these conclusions (30). However, in our previous study we found that comparable inhibition of viral infectivity required higher levels of deaminase-defective APO3G protein than that of wild type (wt) (30). The purpose of the current study was to characterize in more detail the antiviral properties of deaminase-defective APO3G. We performed side-by-side analyses of wt and deaminase-defective APO3G using transient-transfection protocols as well as stable cell lines expressing wt and deaminase-defective APO3G. Our results demonstrate that wt APO3G has significantly higher antiviral activity than deaminase-defective APO3G. In particular, deaminase-defective APO3G in stably transfected HeLa cells had negligible antiviral activity compared to the wt protein even though the two proteins were encapsidated with very similar efficiencies and were equally sensitive to Vif. Deaminase-defective APO3G, like wt APO3G, copurified with the viral nucleoprotein complexes. Our finding that the deaminase-defective APO3G C288S/C291A variant had antiviral activity only when expressed at high levels raises questions about the physiological relevance of deaminase-independent activities of APO3G.

MATERIALS AND METHODS

Cell culture, transfections, and construction of HeLa-APO3G cell lines. HeLa cells were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. HeLa cell lines stably expressing wt APO3G or APO3G C288S/C291A or containing empty vector (HeLa-Ctrl) were constructed by transfecting HeLa cells in a 100-mm dish with pcDNA3.1-APO3G, pcDNA3.1-APO3G C288S/C291A, or empty vector DNA [pcDNA3.1(−), Ctrl]. Two days following transfection, cells were treated with G418 (800 μg/ml) and cultured until G418-resistant colonies were apparent. Individual colonies were transferred to 24-well plates and maintained in DMEM containing 10% fetal bovine serum and 400 μg/ml of G418. Cells were passaged three times and then screened by immunoblot analysis for APO3G expression. Clones expressing equivalent levels of wt APO3G (HeLa APO3G wt) and APO3G C288S/C291A (HeLa APO3G mut) were selected for functional studies. LuSIV cells are derived from CEM-X174 cells and contain a luciferase indicator gene under the control of the SIVmac239 long terminal repeat. These cells were obtained through the NIH AIDS Research and Reference Reagent Program (catalog no. 5460) and were maintained in complete RPMI 1640 medium supplemented with 10% fetal bovine serum and hygromycin B (300 μg/ml). For transfection of HeLa

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cells, cells were grown in 25-cm^2 flasks to about 80% confluence. Cells were transfected using TransIT-LT1 (Mirus Corp., Madison, WI) following the manufacturer's recommendations. A total of 5 to 5.5 μg of plasmid DNA per 25-cm^2 flask (~5 × 10^6 cells) was generally used. Cells were harvested 24 to 48 h posttransfection.

Antibodies. A peptide antibody to human APO3G was prepared by immunizing rabbits with keyhole limpet hemocyanin-coupled peptides corresponding to residues 367 to 384 of human APO3G. A monoclonal antibody to Vif (monoclonal antibody no. 319) was used for all immunoblot analyses and was obtained from Michael Malim through the NIH AIDS Research and Reference Reagent Program (catalog no. 6459). A monoclonal antibody to alpha-tubulin (T-9026; Sigma-Aldrich, Inc., St. Louis, MO) was used as a loading control. An HIV-positive patient serum was used for the identification of HIV-1 capsid (CA) protein.

Plasmids. The full-length molecular clone of HIV-1 (pNL4-3) was used for the production of wt infectious virus (1). Construction of its *vif*-defective variant pNL4-3 vif(−) was described previously (15). Vectors for the expression of untagged wt APO3G and APO3G C288S/C291A in the backbone of pcDNA3.1(−) (Invitrogen Corp., Carlsbad, CA) have been previously described (30).

Immunoblotting. For immunoblot analysis of intracellular proteins, whole-cell lysates were prepared as follows. Cells were washed once with phosphate-buffered saline (PBS), suspended in PBS, and mixed with an equal volume of sample buffer (4% sodium dodecyl sulfate [SDS], 125 mM Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue). To analyze virus-associated proteins, cell-free filtered supernatants from transfected HeLa cells (3.5 ml) were pelleted (75 min, 35,000 rpm) through a 20% sucrose cushion (5.5 ml) in an SW41 rotor. The concentrated virus pellet was suspended in PBS and mixed with an equal volume of sample buffer. Proteins were solubilized by being heated for 10 to 15 min at 95°C. Cell and virus lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE); proteins were transferred to polyvinylidene difluoride (PVDF) membranes and reacted with appropriate antibodies as described elsewhere in the text. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Piscataway NJ), and proteins were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences).

Northern blot analysis. Total RNA from H9 cells or from HeLa cells harvested 24 h after transfection was prepared using the RNeasy minikit according to the manufacturer's instructions (Qiagen, Valencia, CA). Total RNA (20 μg) was electrophoresed on a denaturing 1% agarose gel and capillary blotted onto a nylon membrane using a Turbo blotter (Schleicher & Schuell, Inc., Keene, NH). After UV cross-linking, the membrane was stained for rRNA. For that purpose the membrane was soaked in 5% acetic acid for 15 min at ambient temperature and then incubated in a solution of 0.5 M sodium acetate (pH 5.2) and 0.04% methylene blue for 5 min at ambient temperature. The membrane was rinsed in water for 10 min, and the stained membrane was photographed. Following staining, the membrane was prehybridized with 10 ml of QuickHyb hybridization solution (Stratagene, La Jolla, CA) for 1 h at 68°C. The membrane was then hybridized with probes for 5 h at 68°C. The probe for APO3G mRNA was a 998-bp BglII and HindIII restriction fragment isolated from pcDNA-APO3G (nucleotides 1119 to 2116 of the APO3G gene [13]). Probes were labeled with deoxy-[^{32}P]TTP and random primer using a Ladderman labeling kit (PanVera, Madison, WI), and 1 × 10^7 cpm of the probe was added after premixing with 100 μl sonicated salmon sperm DNA (Stratagene, La Jolla, CA), heating at 94°C for 5 min, and chilling on ice. Following hybridization, membranes were washed twice with wash buffer {2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), 0.1% SDS } for 15 min at ambient temperature, followed by a 15-min wash in 0.2× SSPE-0.1% SDS at 60°C. RNA bands were visualized by autoradiography.

Immunofluorescence and confocal microscopy. HeLa cells stably expressing wt APO3G or APO3G C288S/C291A were trypsinized, and single-cell suspensions were distributed into 12-well plates containing 0.13-mm coverslips. Cells were grown for 15 h at 37°C in DMEM containing 10% fetal bovine serum. Cells were fixed at −20°C in precooled methanol (−20°C) for 10 min followed by two washes in PBS. For antibody staining, coverslips were incubated in a humid chamber at 37°C for 1 h with an APO3G-specific peptide antibody in 1% bovine serum albumin in PBS. Coverslips were washed once in PBS (5 min, room temperature) and incubated with Texas red-conjugated secondary antibodies (diluted in 1% bovine serum albumin in PBS) for 30 min at 37°C in a humid chamber. Coverslips were then washed twice with PBS and mounted onto microscope slides with glycerol gelatin (Sigma-Aldrich Inc., St. Louis, MO) containing 0.1 M *N*-propyl gallate (Sigma) to prevent photobleaching. For confocal microscopy, a Zeiss LSM410 inverted laser scanning microscope equipped with a krypton-argon mixed-gas laser was employed. Images were acquired with a Plan-Apochromat 63×/1.4 oil-immersion objective (Zeiss). Image quality was enhanced during data acquisition using the LSM line average feature (8×). Postacquisition digital image enhancement was performed using the LSM software.

Virus preparation. Virus stocks were prepared by transfection of HeLa cells with appropriate plasmid DNAs. Virus-containing supernatants were harvested 24 to 48 h after transfection. Cellular debris was removed by centrifugation (5 min, 1,000 rpm), and clarified supernatants were filtered (0.45 μm) to remove residual cellular contaminants. For immunoblot analyses, filtered virus stocks were concentrated by pelleting through a 20% sucrose cushion (75 min, 4°C at 35,000 rpm in an SW41 rotor).

Viral infectivity assay. To determine viral infectivity, unconcentrated virus stocks were normalized for equal reverse transcriptase activity and used to infect 5 × 10^5 LuSIV cells (31) in a 24-well plate in a total volume of 1.2 to 1.4 ml. Infection was allowed for 24 h at 37°C. Cells were then harvested and lysed in 150 μl of Promega 1× reporter lysis buffer (Promega Corp., Madison, WI). To determine the luciferase activity in the lysates, 20 μl of each lysate was combined with luciferase substrate (Promega Corp., Madison, WI) by automatic injection and light emission was measured for 10 seconds at room temperature in a luminometer (Optocomp II; MGM Instruments, Hamden, CT).

Sucrose step gradient analysis. Sucrose step gradients were prepared as follows: 2.0 ml of a 60% sucrose solution was placed into the bottom of SW55 centrifuge tubes and overlaid with 2.1 ml of a 20% sucrose solution. Immediately prior to adding concentrated virus stocks (500 μl), the step gradients were overlaid with 100 μl of either PBS or 0.5% Triton X-100. Samples were then centrifuged in a SW55Ti rotor for 60 min at 35,000 rpm and 4°C. Four fractions of 1.1 ml each were collected from the top (see diagram in Fig. 5). Aliquots from each fraction were combined with sample buffer, incubated at 95°C for 10 min, and then processed for immunoblot analysis. Reverse transcriptase activity was determined as reported previously (40). Briefly, 10 μl of each fraction was combined with 50 μl of reverse transcriptase cocktail {50 mM Tris, pH 7.8, 75 mM KCl, 5 mM $MgCl_2$, 0.1% NP-40, 1 mM EDTA, 5 μg/ml poly(A), 0.16 μg/ml oligo(dT), α-[^{32}P]dTTP (10 μCi/ml) } and incubated for 1 h at 37°C. Samples (10 μl each) were spotted on DE81 anion-exchange paper (Whatman Inc., Clifton, NJ) and air dried. Filter papers were then washed three times for 5 min ea. with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), rinsed with ethanol, and dried. Reverse transcription products were visualized by autoradiography.

RESULTS

Transiently expressed deaminase-defective APO3G has antiviral activity. In our previous study we found that APO3G C288S/C291A transiently overexpressed in HeLa cells was efficiently packaged into *vif*-deficient HIV-1 virions and had antiviral activity (30). In that study untagged and epitope-tagged APO3G proteins were compared. For a more careful analysis of the antiviral activity of APO3G C288S/C291A relative to that of wt APO3G, we used untagged APO3G proteins throughout this study.

In our first experiment, pNL4-3vif(−) DNA was cotransfected into HeLa cells with increasing amounts of either APO3G C288S/C291A (Fig. 1A, lanes 1 to 4) or wt APO3G (Fig. 1A, lanes 6 to 9). Lane 5 is a control of *vif*-deficient NL4-3 produced in the absence of APO3G. Transfected cells and virus-containing supernatants were harvested 24 h later and subjected to immunoblot analysis using a polyclonal antibody to APO3G (Fig. 1A, α-APO3G) or an HIV-positive patient serum (APS) for the detection of viral capsid protein (Fig. 1A, CA). As in our previous study (30), APO3G C288S/C291A was expressed better (based on transfected plasmid DNA) and packaged at higher levels into HIV-1 virions than wt APO3G.

The infectivity of the viruses produced in Fig. 1A was assessed by infecting LuSIV indicator cells as described in Materials and Methods. Virus input was normalized by p24 enzyme-linked immunosorbent assay (ELISA). Virus-induced luciferase activity was measured 24 h after infection and cal-

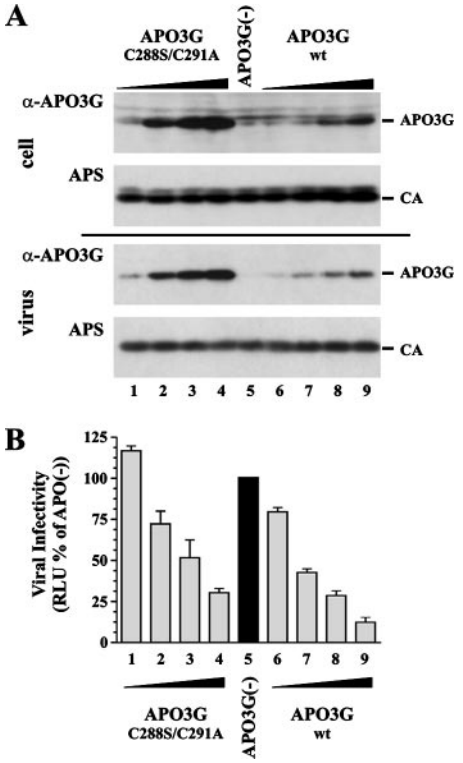

FIG. 1. Transiently expressed deaminase-defective APO3G has antiviral activity. (A) HeLa cells (5×10^6) were cotransfected with 4 μg of pNL4-3vif(−) and increasing amounts of either APO3G C288S/C291A (lanes 1 to 4) or wt APO3G (lanes 6 to 9). Amounts of APO3G DNA transfected were 0.125 μg (lanes 1 and 6), 0.25 μg (lanes 2 and 7), 0.5 μg (lanes 3 and 8), and 1.0 μg (lanes 4 and 9). Lane 5 is a control of HeLa cells transfected with 4 μg of pNL4-3vif(−) in the absence of APO3G. Total DNA amounts were adjusted to 5 μg with empty pcDNA3.1(−) vector DNA. Transfected cells and virus-containing supernatants were harvested 24 h following transfection. Whole-cell lysates and concentrated viral extracts were prepared as described in Materials and Methods and subjected to immunoblot analysis by using a polyclonal antibody to APO3G (α-APO3G) or an HIV-positive patient serum (APS) for the detection of viral capsid proteins (CA). (B) The infectivity of viruses produced in panel A was determined by infecting LuSIV indicator cells. Virus input was normalized by p24 ELISA. Virus-induced luciferase activity was determined in a standard luciferase assay as described in Materials and Methods. The infectivity of virus produced in the absence of APO3G was defined as 100% (black bar, lane 5). The infectivity of the other viruses was expressed as a percentage of that of the control virus. Error bars reflect standard deviations calculated from three independent infections.

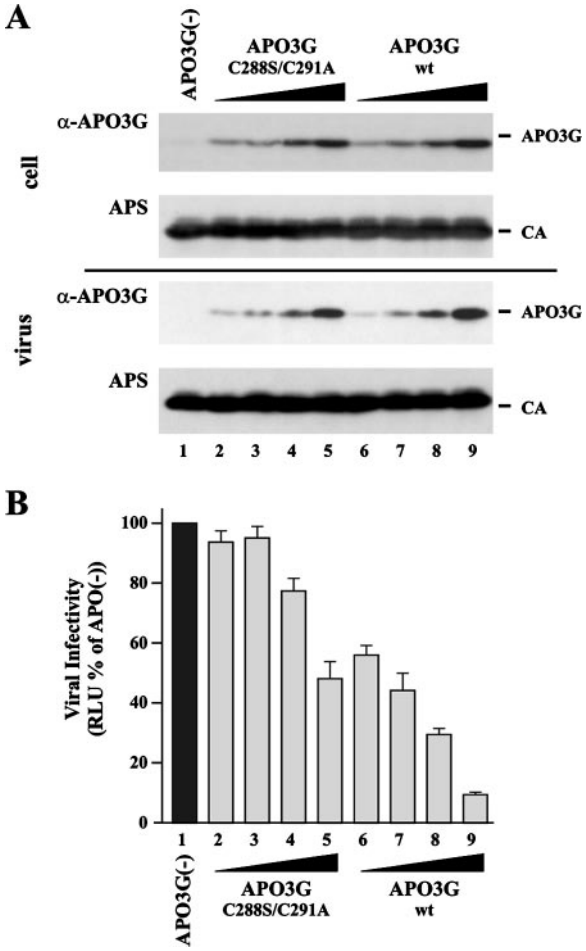

FIG. 2. Antiviral activity of wt APO3G is more potent than that of APO3G C288S/C291A. (A) HeLa cells (5×10^6) were transfected with 4 μg of pNL4-3vif(−) together with increasing amounts of vector encoding APO3G C288S/C291A (lane 2, 0.0625 μg; lane 3, 0.125 μg; lane 4, 0.25 μg; lane 5, 0.5 μg) or wt APO3G (lane 6, 0.187 μg; lane 7, 0.375 μg; lane 8, 0.75 μg; lane 9, 1.5 μg). Total DNA amounts were adjusted to 5.5 μg using empty vector DNA. Lane 1 is a control representing HeLa cells transfected with 4 μg of pNL4-3vif(−) and 1.5 μg of empty vector DNA in the absence of APO3G. Cells and viruses were harvested and processed for immunoblotting as described for Fig. 1A. (B) The infectivity of the viruses produced in panel A was determined as described in the legend to Fig. 1B. The infectivity of the viruses was expressed as a percentage of that of the control virus, which was defined as 100% (lane 1, black bar). Error bars reflect standard deviations calculated from three independent infections.

culated relative to the APO3G-negative control virus, which was defined as 100% (Fig. 1B, lane 5). Consistent with our previous report, we found that deaminase-defective APO3G C288S/C291A had antiviral activity although higher amounts of APO3G C288S/C291A protein than wt APO3G were required for comparable inhibition (Fig. 1A and B, compare lanes 4 and 8).

The antiviral activity of wt APO3G is more potent than that of APO3G C288S/C291A. To better compare the relative antiviral effects of wt and mutant APO3G, we modified the experimental conditions from Fig. 1 such that equivalent amounts of wt and deaminase-defective proteins were expressed (Fig. 2A). HeLa cells were transfected with DNA encoding *vif*-defective pNL4-3 together with increasing amounts of vector encoding APO3G C288S/C291A (Fig. 2A, lanes 2 to 5) or wt APO3G (Fig. 2A, lanes 6 to 9) as detailed in the legend to Fig. 2. Lane 1 is an APO3G-negative control. Cells and viruses were harvested and processed for immunoblotting as described for Fig. 1A. As can be seen in Fig. 2A, intracellular expression of wt and mutant APO3G and encapsidation of the proteins into *vif*-deficient HIV-1 virions were very similar over the range of protein levels tested.

To compare the antiviral activities of wt and mutant APO3G, virus from the experiment in Fig. 2A normalized by p24 ELISA was used to infect LuSIV indicator cells and virus infectivity was determined as described for Fig. 1B. As before,

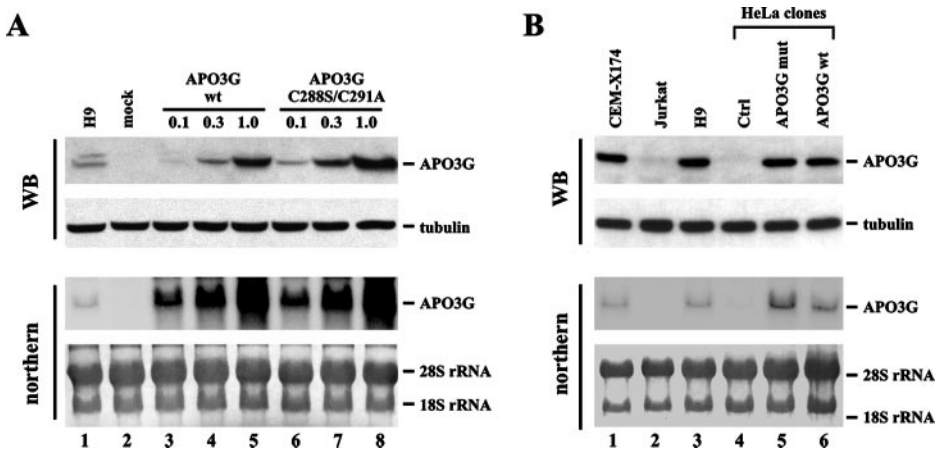

FIG. 3. Transient and stable expression of APO3G in HeLa cells. (A) APO3G mRNA levels and protein expression from transiently transfected HeLa cells were compared to those in H9 cells. HeLa cells were transfected with 0.1 μg (lane 3), 0.3 μg (lane 4), or 1 μg (lane 5) of pcDNA-APO3G wt or 0.1 μg (lane 6), 0.3 μg (lane 7), or 1 μg (lane 8) of pcDNA-APO3G C288S/C291A. Total DNA was adjusted to 5 μg with empty vector DNA. Untransfected HeLa cells were included as a control (lane 2). Total cell lysates from normal H9 cells (lane 1) and transfected HeLa cells were normalized for total protein levels and subjected to immunoblot analysis using an APO3G-specific polyclonal antibody (APO3G). The same blot was then reprobed with a tubulin-specific monoclonal antibody (tubulin) to control for loading errors. WB, Western blot. In parallel, total RNA was isolated from H9 and HeLa cells. Equal amounts (20 μg) of total RNA were separated by denaturing 1% agarose gel electrophoresis and transferred to a nylon membrane. Membranes were stained with methylene blue to identify rRNA species (Northern, lower panel). The membranes were then probed with an APO3G-specific ^{32}P-labeled probe (Northern, upper panel). APO3G mRNA was visualized by autoradiography (APO3G). (B) Expression of APO3G mRNA and protein from G418-resistant stable HeLa clones (lanes 5 and 6) was compared to that of APO3G mRNA and protein in CEM-X174 cells (lane 1), Jurkat cells (lane 2), and H9 cells (lane 3). HeLa-Ctrl cells stably transfected with empty vector DNA and selected for G418 resistance were included as a negative control (lane 4). Total cell lysates were analyzed for APO3G expression by immunoblotting as described for panel A. Total cellular RNA was extracted from the three T-cell lines and the stable HeLa clones and subjected to Northern blot analysis as described for panel A.

the infectivity of virus produced in the absence of APO3G was defined as 100% (Fig. 2B, lane 1). As expected wt APO3G inhibited viral infectivity in a dose-dependent manner (Fig. 2B, lanes 6 to 9). Deaminase-defective APO3G also inhibited viral infectivity (Fig. 2B, lanes 2 to 5). However, four- to eight-times-higher amounts of virus-associated APO3G C288S/C291A protein than of wt APO3G were required to achieve comparable inhibition of viral infectivity (compare Fig. 2A and B, lane 5 to lanes 6 and 7). These results suggest that deaminase-defective APO3G retains only partial antiviral activity compared to wt APO3G and requires higher levels of virus-associated protein to achieve inhibitory effects comparable to those of wt APO3G.

Construction and characterization of HeLa cell lines stably expressing wt APO3G and APO3G C288S/C291A. A recent study reported that virus-associated APO3G is recruited mainly from the pool of newly synthesized protein (36). Thus, the rate of de novo synthesis of APO3G rather than the total intracellular APO3G pool may determine the efficiency of encapsidation into HIV-1 virions. Since the rate of APO3G de novo synthesis from cytomegalovirus promoter-driven vectors is very high in transiently transfected cells compared to de novo synthesis of endogenous APO3G in nonpermissive cells, we were concerned that the antiviral activity of APO3G C288S/C291A was at least in part due to protein overexpression. To illustrate this problem, we compared relative APO3G protein and mRNA levels in HeLa cells transiently transfected with increasing amounts of pcDNA-APO3G (Fig. 3A, lanes 3 to 5) and pcDNA-APO3G C288S/C291A (Fig. 3A, lanes 6 to 8) to endogenous APO3G protein and mRNA levels in H9 cells (Fig. 3A, lane 1). APO3G in the cell lysates was identified by immunoblotting using an APO3G-specific peptide antibody (Fig. 3A, WB, APO3G). We found that transfection of about 0.3 μg of pcDNA-APO3G produced levels of APO3G protein comparable to those in H9 cells (Fig. 3A, compare WB, APO3G, lanes 1 and 4). Deaminase-defective APO3G was expressed more efficiently than wt APO3G, and less than 0.3 μg of DNA was sufficient to produce protein levels equivalent to those in H9 cells (compare lanes 1 to lanes 6 and 7). To compare APO3G transcription levels, equal amounts of total RNA from transfected HeLa cells isolated 24 h after transfection and from H9 cells were subjected to Northern blot analysis as described in Materials and Methods. The size of the mRNA expressed from pcDNA-APO3G was predicted to be approximately 1.5 kb, which is similar to the size observed for endogenous APO3G mRNA (33). As expected, no APO3G-specific mRNA was detected in untransfected HeLa cells (Fig. 3A, Northern blot, lane 2). In contrast, transfection of pcDNA-APO3G (lanes 3 to 5) or pcDNA-APO3G C288S/C291A (lanes 6 to 8) resulted in a dose-dependent increase in APO3G mRNA levels in transfected HeLa cells. Importantly, APO3G mRNA levels in H9 cells were dramatically lower than those observed in transfected HeLa cells even at the lowest level of transfected APO3G (Fig. 3A, compare lanes 1 and 3).

To overcome the potential problems associated with the different rates of APO3G mRNA synthesis, we constructed stable HeLa cell lines expressing wt and deaminase-defective APO3G. Construction of the HeLa-APO3G lines is described in Materials and Methods. Expression of APO3G from G418-resistant stable HeLa clones was compared both on protein and on RNA levels to that of the nonpermissive CEM-X174 and H9 T-cell lines. Two cell lines were selected (HeLa

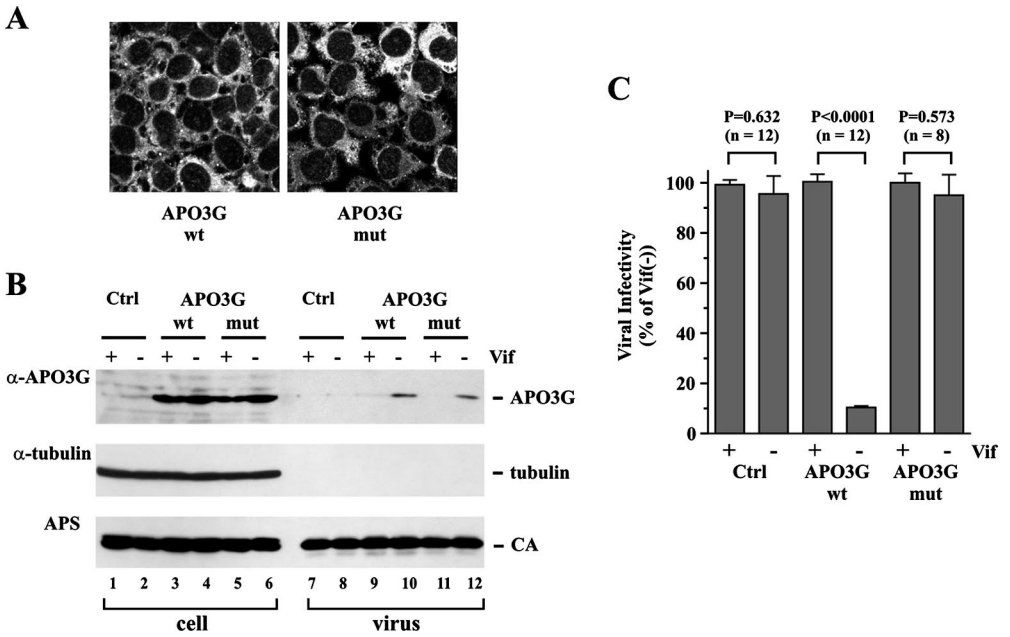

FIG. 4. Deaminase activity is required for efficient antiviral activity of APO3G in stable HeLa cells. (A) APO3G expression in stable HeLa cells was analyzed by indirect immunofluorescence and confocal microscopy as described in Materials and Methods. (B) Stable HeLa cell lines were transfected with pNL4-3 (Vif^+) or pNL4-3vif(−) (Vif^-) DNA. Transfected cells and virus-containing supernatants were harvested 24 h later. Cell-free virus was concentrated by pelleting through 20% sucrose. Proteins from whole-cell lysates and concentrated virus pellets were separated by SDS-PAGE and transferred to a PVDF membrane. The same membrane was sequentially probed in the order shown, i.e., first with a polyclonal APO3G-specific peptide antibody (α-APO3G), followed by probing with a tubulin-specific monoclonal antibody (α-tubulin) and finally an HIV-positive human serum recognizing HIV-1 capsid protein (APS). Proteins are identified on the right. (C) Virus samples from panel B were normalized for equivalent reverse transcriptase activity and used to determine the viral infectivity as described for Fig. 1B. Luciferase activity observed with wt virus was defined as 100% infectivity for each producer cell type. The infectivity of the *vif*-defective viruses was calculated relative to the corresponding wt viruses. Error bars reflect standard deviations from 8 to 12 independent infections of each of the three producer cell types. *P* values were calculated using a two-tailed unpaired *t* test (GraphPad Prism 4).

APO3G wt and HeLa APO3G mut) expressing levels of wt and deaminase-defective APO3G protein similar to those observed in H9 or CEM-X174 cells (Fig. 3B). As can be seen, APO3G mRNA levels in these HeLa cell lines (Fig. 3B, lanes 5 and 6) were also similar to those in H9 and CEM-X174 cells (lanes 1 and 3, respectively) and were significantly lower than mRNA levels in the transiently transfected cells in Fig. 3A. As expected, no APO3G-specific mRNA was found in Jurkat and HeLa-Ctrl cells (Fig. 3B, lanes 2 and 4). Analysis of APO3G protein levels by immunoblot analysis showed that both mutant and wt APO3G (Fig. 3B, WB, lanes 5 and 6, respectively) were expressed at levels similar to those in CEM-X174 (lane 1) and H9 (lane 3) cells. As expected, Jurkat cells (lane 2) and HeLa-Ctrl cells (lane 4) were APO3G negative. We conclude that the expression profiles of wt APO3G and APO3G C288S/C291A in stably transfected HeLa cells were comparable to those in H9 and CEM-X174 cells with respect to both mRNA and protein levels.

Deaminase activity is required for antiviral activity of APO3G from stable HeLa cells. HeLa cell lines stably expressing APO3G were used for further functional analysis. First, expression of wt and deaminase-defective APO3G was assessed by indirect immunofluorescence and confocal imaging as described in Materials and Methods. All cells in the cultures were APO3G positive with cell-to-cell fluctuation in APO3G expression consistent with a Gaussian distribution (Fig. 4A).

To see if APO3G C288S/C291A in stable HeLa cells retained antiviral activity, the HeLa cell lines were transfected with pNL4-3 (Fig. 4B, Vif^+) or pNL4-3vif(−) DNA (Fig. 4B, Vif^-). Transfected cells and virus-containing supernatants were harvested 24 h later. Cell-free virus was concentrated by pelleting through 20% sucrose to remove soluble viral proteins. Proteins in whole-cell lysates and concentrated virus pellets were analyzed by immunoblotting. The same membrane was sequentially probed with (i) a polyclonal APO3G-specific peptide antibody (Fig. 4B, α-APO3G), (ii) a tubulin-specific monoclonal antibody (Fig. 4B, α-tubulin), and (iii) an HIV-positive human serum recognizing HIV-1 capsid protein (Fig. 4B, APS). The tubulin blot served as a loading control to ascertain similar total protein levels in all cell lysates (lanes 1 to 6). Tubulin was absent from cell-free virus preparations (lanes 7 to 12). All cells produced comparable amounts of viral capsid protein (Fig. 4B, APS, lanes 1 to 6) and released equivalent amounts of cell-free virus (Fig. 4B, APS, lanes 7 to 12). Of note, while similar amounts of wt APO3G (wt) and APO3G C288S/C291A (mut) were packaged into *vif*-deficient HIV-1 (Fig. 4A, lanes 10 and 12), both APO3G variants were efficiently excluded from wt NL4-3 virions (lanes 9 and 11). As in our previous studies (13, 14), the exclusion of APO3G from cell-free virions was paralleled by only a modest reduction in intracellular APO3G in Vif-expressing cells (Fig. 4A, α-APO3G; compare lanes 3 and 4 and lanes 5 and 6).

The infectivity of the viruses produced in Fig. 4B was assessed by infecting LuSIV indicator cells with equivalent

amounts of cell-free viruses. Infection of LuSIV cells was measured by the Tat-induced expression of luciferase. The infectivity of wt NL4-3 viruses from each cell type was defined as 100%, and the infectivity of *vif*-defective NL4-3 was calculated as a percentage of the respective wt virus (Fig. 4C). As expected, the infectivity of *vif*-defective virus produced from APO3G-negative cells (Ctrl) did not significantly differ from that of wt virus ($P = 0.632$) while the presence of wt APO3G significantly ($P < 0.0001$) reduced the infectivity of *vif*-defective NL4-3 particles compared to wt NL4-3. Surprisingly, deaminase-defective APO3G (APO3G mut) caused only an insignificant ($P = 0.573$) reduction in viral infectivity. Thus, deaminase-defective APO3G in stably transfected HeLa cells had minimal, if any, antiviral activity. Mutant APO3G when expressed at three- to four-times-higher levels than those shown in Fig. 5 partially inhibited the infectivity of *vif*-defective virus, consistent with the results from our transient-expression studies shown in Fig. 2 (data not shown). These results suggest that at low expression levels catalytic activity of APO3G is important and relevant to the control of retroviral replication.

APO3G C288S/C291A assembles into viral nucleoprotein complexes. APO3G is packaged into viruses or virus-like particles in a process that involves nucleocapsid protein and viral genomic RNA (4, 5, 7, 17, 21, 32, 39, 44). Moreover, we previously reported that APO3G packaged in the presence of viral genomic RNA stably associates with viral nucleoprotein complexes and is resistant to detergent treatment while APO3G packaged in the absence of viral genomic RNA is detergent sensitive and presumably not core associated (17). It is conceivable that the lack of antiviral activity of APO3G C288S/C291A when expressed from stably transfected HeLa cells is due to aberrant packaging of the protein into HIV-1 particles. To address this issue, we compared the detergent sensitivities of wt APO3G and APO3G C288S/C291A in virions produced from stable HeLa-APO3G cells. Cells (5×10^6) were cotransfected with 4.5 μg of pNL4-3vif(−) and 0.5 μg of pCMV-VSVg. Vesicular stomatitis virus g-protein DNA was added here to increase virus yield by allowing reinfection of HeLa cells. Virus-containing supernatants were harvested 48 h after transfection, and viruses were pelleted by ultracentrifugation through 20% sucrose. Pelleted virus was suspended in 1 ml of DMEM and split into two equal fractions. One fraction was left untreated; the other fraction was adjusted to 0.1% Triton X-100. Untreated and detergent-treated samples were layered on top of a 20% to 60% sucrose step gradient and subjected to ultracentrifugation as described previously (16, 17). Four fractions (1.1 ml each) were collected from the top. Aliquots of each fraction were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were sequentially probed first with an APO3G-specific polyclonal antibody (Fig. 5, APO3G) followed by an HIV-positive human serum to identify viral capsid protein (Fig. 5, CA). In addition, the presence of reverse transcriptase in individual gradient fractions was determined by a standard enzymatic reverse transcriptase assay (40) (Fig. 5, RT). As expected, untreated virions were recovered from the interphase of 20% and 60% sucrose and contained all of the extracellular APO3G (Fig. 5, lane 3). Small amounts of protein recovered from fraction 4 (Fig. 5, lane 4) were presumably due to spillover since samples were collected from the top of the gradient. Detergent treatment of virions resulted in the release of a significant portion of capsid protein from the particles, which was found at the top of the gradient (Fig. 5, lane 5). Residual core-associated capsid protein was identified in fraction 3 (Fig. 5, lane 7). Importantly, both wt and mutant APO3G were similarly resistant to detergent treatment and copurified with viral reverse transcriptase (Fig. 5, RT). Thus, detergent treatment of virus preparations failed to reveal any differences between wt and deaminase-defective APO3G, suggesting that the lack of antiviral activity was not due to aberrant packaging of the deaminase-defective APO3G.

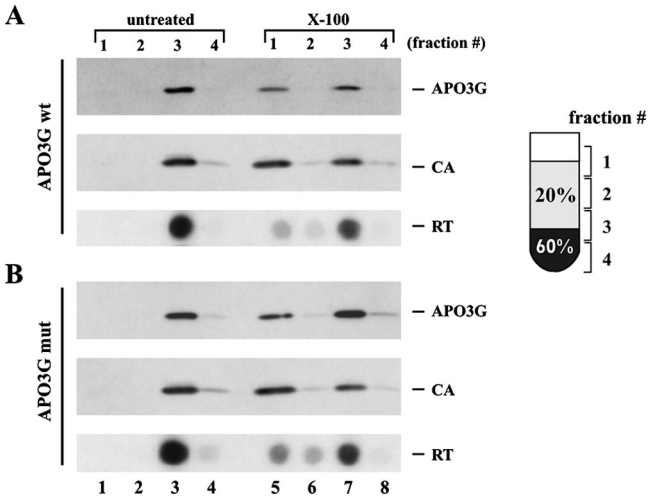

FIG. 5. Deaminase-defective APO3G C288S/C291A is packaged into viral nucleoprotein complexes. HeLa APO3G wt (A) or HeLa-APO3G mut (B) cells were cotransfected with 4.5 μg of pNL4-3vif(−) and 0.5 μg of pCMV-VSVg. Virus-containing supernatants were harvested 48 h after transfection, and viruses were purified and concentrated by ultracentrifugation through 20% sucrose. Pelleted viruses were suspended in 1 ml of DMEM and divided into two equal fractions. One fraction was left untreated; the other fraction was treated with 0.5% Triton X-100 (X-100). Untreated and detergent-treated samples were subjected to step gradient centrifugation as described in Materials and Methods and elsewhere in the text. Four fractions (1.1 ml each) were collected starting from the top of each gradient. Aliquots from each fraction were analyzed by immunoblotting using an APO3G-specific polyclonal antibody (APO3G). The same blot was then reprobed with an HIV-positive human serum to identify viral capsid protein (CA). The presence of reverse transcriptase was tested in an enzymatic in vitro reverse transcription reaction as described in Materials and Methods. An autoradiogram of the reverse transcription products is shown (RT). The step gradient fractionation procedure is illustrated on the right.

DISCUSSION

The identification of APO3G as the principal cellular target of HIV-1 Vif was a milestone for Vif research and provided valuable new insights into the biological function of Vif (33). A series of subsequent studies demonstrated that packaging of APO3G into *vif*-deficient HIV-1 virions caused hypermutation of the viral genome (11, 18, 24, 45). This observation was confirmed by numerous labs including our own, and cytidine deamination of the viral genome was considered to be the major cause of APO3G's antiviral activity.

Other studies, however, also observed deaminase-indepen-

dent inhibitory effects of APO3G on HIV-1 replication (3, 9, 12, 22, 28, 30, 35), leading to discussions about the true mechanism of APO3G-mediated inhibition of HIV-1. Since natural cell lines expressing deaminase-defective APO3G were not available, most studies employed transient expression of APO3G in 293T or HeLa cells to analyze cytidine deaminase-dependent and deaminase-independent effects. In our own study, comparable inhibition of HIV-1 infectivity by wt and deaminase-defective APO3G required higher levels of the enzymatically inactive protein, leading us to conclude that wt APO3G had stronger antiviral potential than its deaminase-defective counterpart (30). Similar conclusions were drawn by Holmes et al. (12). The requirement for high levels of virus-associated deaminase-defective APO3G for virus inhibition is reminiscent of one of our previous studies, in which we noted that the HIV-1 Vif protein, when packaged at supraphysiological levels into HIV-1 virions, had strong antiviral activity (2). It is well accepted, however, that, at physiological levels, Vif positively regulates viral infectivity, and the antiviral effect at high expression levels was therefore attributable to protein overexpression (2). In the case of Vif, we found that packaging of the protein into HIV-1 virions induced an assembly defect, caused by the binding of Vif to Gag at or near the primary Gag processing site (2).

Given the opposing effects of Vif on viral infectivity at physiological versus supraphysiological expression levels, the goal of this study was to compare the antiviral effects of wt and deaminase-defective APO3G under physiological and nonphysiological conditions. Our results confirm previous reports showing that deaminase-defective APO3G exhibited antiviral properties in transient-transfection studies. However, we also found that deaminase-defective APO3G had very little antiviral activity when expressed under conditions that closely mimic those observed in nonpermissive H9 and CEM-X174 cells. wt and deaminase-defective APO3G expressed in stable HeLa cell lines had very similar biophysical and biochemical properties as far as expression, viral encapsidation, and the association with viral nucleoprotein complexes were concerned (Fig. 3 to 5). In addition, wt and deaminase-defective APO3G in the stable HeLa cell lines formed similar RNase-sensitive high-molecular-mass complexes (data not shown). Thus, we were unable to identify any differences between wt and deaminase-defective APO3G—other than the lack of enzymatic activity in APO3G C288S/C291A—that could explain the difference in their antiviral properties. We therefore propose that enzymatic activity of APO3G is crucial for the antiviral effects of APO3G, at least with respect to HIV-1 replication.

It is unclear how deaminase-defective APO3G inhibits viral infectivity when expressed and packaged at high levels. In analogy to our previous study of Vif (2) we investigated possible effects of APO3G on virus assembly. Unlike Vif, which caused a clearly identifiable assembly defect, no changes in Gag maturation or viral protein composition were apparent in the presence of increasing amounts of deaminase-defective APO3G (data not shown). However, considering their differing specific antiviral activities, it seemed likely that inhibition of viral infectivity by wt APO3G and APO3G C288S/C291A was affected by different molecular mechanisms. It is interesting in that regard that *vif*-deficient particles derived from peripheral blood mononuclear cells were found to contain less than 10 copies of APO3G yet were noninfectious (41). In contrast, viruses produced with transfected 293T cells contained up to 30-fold more APO3G (41). Our own data are consistent with these findings. In fact, we found that, depending on transfection conditions, virus produced with transfected HeLa cells contained up to 500 copies of APO3G (K. Strebel, unpublished data). It is conceivable that the packaging of excessive amounts of APO3G into viral cores and APO3G's interaction with viral nucleoprotein complexes impose a physical block to virus replication that is independent of APO3G enzymatic activity but unrelated to natural infections.

It remains to be investigated how cytidine deamination affects viral infectivity. Accumulation of deamination-induced mutations in the viral genome certainly is detrimental to viral replication. On the other hand, the single-round infectivity assay used in our studies is relatively insensitive to such APO3G-induced mutations since it requires only the production of functional Tat protein (31). Thus, the cytidine deaminase-induced loss of viral infectivity is more likely explained by effects on DNA stability or other effects such as the reported inhibition of tRNA-primed reverse transcription (9), inhibition of plus-strand transfer (26), or inhibition of integration (22, 26).

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