APOBEC3 proteins comprise a multigene family of antiviral cytidine deaminases that are active against human immunodeficiency virus, simian immunodeficiency virus, endogenous retroelements. The Vif protein of lentiviruses binds to specific APOBEC3 proteins, notably A3F and A3G, to induce their degradation by proteasomes. APOBEC3 proteins are of two types, those with a single deaminase domain such as human (h)A3A and hA3C and those with two cytidine deaminase domains (CDD) such as hA3G, hA3F, hA3B and the mouse APOBEC3, mA3. In hA3G, both active sites are required for antiviral function but serve separate functions. CDD2 mediates the C to U deamination of the human immunodeficiency virus type 1 genome, whereas CDD1 binds the viral RNA to allow for virion packaging. Here we analyzed the role of the two domains in additional APOBEC3 family members. We analyzed APOBEC3 proteins in which either the critical glutamic acid residue or the Zn^2+ coordination amino acid residues in the active sites were mutated. The separation of function of the domains is maintained in hA3B and hA3F, but in the mouse protein mA3, the roles of the two domains are reversed. Deamination is mediated by CDD1, whereas encapsidation and dimerization are mediated by CDD2. Antiviral function of each of the APOBEC3 proteins was largely attributable to deaminase activity. Deaminase-independent antiviral activity of the active site mutants was minor. These findings suggest that the two active sites have different functions but that these functions can be interchanged in different APOBEC3 family members.

The APOBEC3 (apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3) proteins comprise a family of antiviral cytidine deaminases that are active against viruses such as human immunodeficiency virus, type 1 (HIV-1), simian immunodeficiency virus, adeno-associated virus, and hepatitis B virus (1–5). In human, APOBEC3 has been expanded to include eight members designated human APOBEC3A (hA3A), hA3B, hA3C, hA3D/E, hA3F, hA3G, hA3H, and a pseudogene to hA3G, whereas in mice only one mouse APOBEC3 (mA3) exists (3, 6, 7). hA3F and hA3G are of particular interest because HIV-1 has developed the Vif accessory gene, which is dedicated to inducing their degradation (8–11). APOBEC3 proteins have been reported to possess activity against other retroviruses such as human T cell leukemia virus 1 (12, 13), murine leukemia virus (2, 14), and possibly foamy viruses (15, 16).

In cells infected with HIV-1 deleted for Vif (Δvif), hA3G molecules are packaged into the assembling virion, causing a dramatic reduction in particle infectivity. The ability to be packaged in retroviral virions is a property that is shared among the APOBEC family members, regardless of whether they have antiviral activity or not (17). Virus that lacks an intact nucleocapsid domain in the Gag precursor polyprotein does not efficiently package hA3G, suggesting either a direct interaction with Gag or binding to the viral genomic RNA (13). Upon infection of target cells with Δvif virus, the packaged hA3G deamidates the minus strand reverse transcript as it is synthesized. The specificity for the minus strand results from the requirement of APOBEC3 for single-stranded DNA. The majority of the virus DNA molecules are degraded prior to integration by DNA repair enzymes such as uracil DNA glycosylase, and the few proviruses that form are generally inactivated by the G→A mutations (2, 3, 14, 18–20). hA3F is similar to hA3G regarding the anti-HIV-1 function and the sensitivity to Vif, and those mechanisms seem to resemble those of hA3G except for the target sequence preference by which hA3F favors dC found in the different context as seen for hA3G (14, 18, 21–25). In contrast to hA3G and hA3F, hA3B does not have Vif sensitivity and has a different target preference (17, 21, 22). mA3 also does not possess Vif sensitivity (3).

The cytidine deaminase domains (CDD) of APOBEC proteins contain an active site with a conserved consensus motif His-X-Glu-X23–28-Pro-Cys-X4–4-Cys in which the His-Cys residues coordinate a Zn^2+ ion and the glutamic acid residue serves an essential role in catalysis as a proton shuttle (26). Some of the APOBEC3 proteins contain a single CDD (hA3A and hA3C), whereas others (hA3B, hA3G, hA3F, and mA3) have two tandem CDDs. In the case of hA3G, both domains contain an intact active site consensus sequence motif, but only CDD2 appears to be catalytically active (13). hA3G with a mutation of the critical Glu-67 in active site 1 maintained its antiviral activity, whereas a mutant in which the analogous Glu-259 of active site 2 was changed to Ala lost antiviral activity (13).
The catalytic function of CDD2 was also demonstrated using hA3G/hA3F chimeras where it was shown that the target site preference of the chimeras was specified by CDD2 (27). AS1 of hA3G was required for virion encapsidation and for dimerization but not to induce G→A mutations in the HIV-1 reverse transcripts (13).

Although it is clear that deamination is the primary mechanism by which APOBEC3 proteins inhibit virus replication, an additional deaminase-independent antiviral function was suggested by Newman et al. (28). In that study, hA3G E259Q was found to lack detectable activity in a bacterial mutagenesis assay, failed to induce G→A mutations on HIV-1 DNA, yet was fully active in a single cycle reporter virus assay. Additional support for a deaminase-independent antiviral activity was suggested by the finding that hA3G inhibited the replication of hepatitis virus in culture in the absence of detectable G→A mutations (5) and the report of hA3A activity against retroelement (29).

Here we have analyzed the function of the deaminase domains of the two domain APOBEC3 family members, hA3F, hA3B, and mA3. We found that as for hA3G, CDD2 was the catalytically active domain of hA3B and hA3F. In contrast, in the mouse enzyme, mA3, the situation was reversed. Deamination was mediated by AS1, and packaging and homodimerization were mediated by AS2. In each of the APOBEC3 proteins, the antiviral activity was dependent on deaminase activity.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids**—The C-terminal hemagglutinin (HA)-tagged expression vectors for hA3G, hA3G, AS1(AA), and hA3G, AS2(AA), hA3B, mA3, and Myc-tagged hA3G have been described previously (3, 13, 17). Single cycle HIV-1 luciferase reporter plasmids, pNL-Luc-E RΔvif and pNL-Luc-E Rvif, and vesicular stomatitis virus glycoprotein (VSV-G) expression vector, pVSV-G, have been described previously (30). C-terminal HA-tagged hA3F expression vector pchA3F.HA was constructed by amplifying hA3F cDNA from a hA3F cDNA template with the sense primer 5′-GGGTGATCATGAAGCCTACCTGAGACCTACACTAG-3′ that has a KpnI site and antisense primer 5′-GCTCTAGACTAAGCGTAGATCTGGGACGTCGTATGGGTACTCGAGAATCTCCTGCAG-3′ (31). The amplicon was digested with KpnI and cloned into the KpnI and EcoRV sites of pcDNA3.1(+) (Invitrogen). Myc-tagged mA3 expression vector pcmA3.MyC was constructed by PCR amplification of a cloned mA3 cDNA with the sense primer 5′-GGGTGATCATGAAGCCTACCTGAGACCTACACTAG-3′ and anti-sense 5′-TCCTCTAGATCACAGACATCTCTCTCTGAGATGAGTTTTTGTTCAGACATCGGGGGTCCAAGCTG-3′. The amplicon was ligated to pcDNA3.1(+) cleaved at the KpnI and EcoRV sites. Mutations in APOBEC3 expression vectors were generated using PCR-based site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions and confirmed by nucleotide sequence analysis.

**Cell Lines**—HEK 293T and human osteosarcoma cell line HOS.T4.X4 that expresses CD4 and CXCR4 were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics at 37 °C in 5% CO₂.

**Infectivity Assay**—VSV-G-pseudotyped HIV-1 luciferase reporter viruses were generated as described previously (3, 13, 17). Briefly, 293T cells were seeded at 1 × 10⁵ cells/well in 6-well plates and 1 day later transfected with 1 μg each of pNL-Luc-E RΔvif, pVSV-G, and wild type, or mutant APOBEC3 expression vector or pcDNA3.1(+) empty vector control using Lipofectamine 2000 (Invitrogen). Negative control transfections were included in which the reporter virus plasmid was substituted with pcDNA3.1(+) (+). In APOBEC3 titrations, the total amount of transfected DNA was kept constant by the addition of pcDNA3.1(+) (+). Virus-containing supernatant was harvested 2 days post-transfection and filtered through a 0.45-μm filter, and the p24 concentration was determined by ELISA. APOBEC3 mutants had no effect on p24 release. Supernatant containing 0.25 ng of p24 was added in triplicate to 1.0 × 10⁴ HOS.T4.X4 cells in a 96-well plate. After 3 days, luciferase activity was measured using Steady Lite HTS (PerkinElmer Life Sciences). The data are presented as the average of the triplicates with the standard error.

**APOBEC3 Encapsulation**—Virions were produced by 293T cells that were cotransfected with 1 μg of pNL-Luc-E RΔvif and APOBEC3 expression vector or empty vector control. To control for nonspecific release of APOBEC3 into the supernatant, mock virions were produced by transfection of the cells with APOBEC3 expression vector without the viral plasmid. Two days post-transfection, the culture supernatant was harvested, and cell lysates were prepared. Level of p24 in the supernatant of the sample transfected with pNL-Luc-E RΔvif was determined to be about 50 ng/ml by ELISA. The supernatant (2 ml) was filtered through a 0.45-μm filter and then ultracentrifuged for 1 h through a 20% sucrose cushion at 150,000 × g. Approximately 40% of p24 in the supernatant was pelleted. The pelleted virions were solubilized in 100 μl of lysis buffer containing 1% Triton X-100. Virion lysates were quantitated by p24 ELISA, and the protein concentration of the lysates was measured by Bradford assay. Solubilized virions (1.5 ng of p24) and cell extracts (6 μg) were separated by SDS-PAGE on a 4–12% gradient gel. The proteins were transferred to a polyvinylidene difluoride membrane and probed with anti-HA monoclonal antibody (Amersham Biosciences), and the blot was developed with horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham Biosciences), and the blot was developed with ECL reagents (Amersham Biosciences). To quantitate the signal a Typhoon fluorescence gel reader was used (Amersham Biosciences).

**APOBEC3 Dimerization Assay**—293T cells were seeded at 1 × 10⁵ cells/well in 6-well plates and transfected a day later with 1.0 μg each of Myc-tagged APOBEC3 expression vector or empty pcDNA3.1(+) and HA-tagged APOBEC3. Two days post-transfection, the cells were lysed in 1% Nonidet P-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40) supplemented with protease inhibitor mixture set III (Calbiochem) for 1 h on ice. The lysate was clarified by centrifugation for 15 min at 10,000 × g and precleared with protein-G-Sepharose. Myc-tagged APOBEC3 was immunoprecipitated by addition of 2 μl of mAb 9E10 (Covance) for 1 h at 4 °C followed by 20 μl of protein-G-Sepharose for 1 h at 4 °C. The resin was washed five
times with lysis buffer, and then 50 μl of reducing sample buffer was added (Invitrogen). The immunoprecipitates were analyzed on an immunoblot probed with 16B12 and 9E10 mAbs.

**Sequencing of Viral Reverse Transcripts**—Virions were prepared by transfection of 1 × 10^6 cells in a 10-cm dish with 8.0 μg of APOBEC3 expression vector, 8 μg of proviral plasmid, and 4 μg of pVSV-G. Two days post-transfection, the supernatant was harvested and treated with DNase-I for 1 h at 37 °C. The virus (10 ng) was added to 5.0 × 10^5 HOS.T4.X4 cells in a 6-well plate. The cells were cultured for 8 h at which time total DNA was prepared using a DNeasy kit (Qiagen). A portion of gag was amplified with the sense primer 5'-CACAATCACACTCCATGCAGAAT-3' and antisense primer 5'-GGTAGCT TCATGGAACAGCAGGCT-3' using Expand DNA polymerase (Roche Applied Science). The amplicon was cloned into the TOPO TA-cloning vector (Invitrogen). The nucleotide sequence of 10 independent clones was determined.

**In Vitro Deaminase Assay**—293T cells were seeded at 1 × 10^5 cells/well in a 6-well plate and a day later transfected with 1 μg of APOBEC3 expression vector. Two days post-transfection, the lysates were lysed for 30 min on ice in 0.1% Triton X-100 buffer (50 mM Tris, pH 8.0, 40 mM KCl, 50 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 10 mM DTT). The lysate was clarified by centrifugation at 10,000 × g for 10 min and precleared with 40 μl of protein-G-Sepharose. The precleared lysate was incubated with anti-HA mAb bound to protein-G-Sepharose for 1 h at 4 °C. The resin was washed three times with 0.1% Triton X-100 buffer. 1/5 of the resin was removed for immunoblot analysis, and the remainder was washed once with deaminase reaction buffer (40 mM Tris, pH 8.0, 10% glycerol, 40 mM KCl, 50 mM NaCl, 5 mM EDTA, 1 mM DTT). Biotin-labeled oligonucleotide (biotin-5'-T28CCCGT28-3' for hA3G, hA3B, and mA3 or biotin-5'-T28CAT28-3' for hA3F) in 20 μl of deaminase reaction buffer was added, and the reaction was incubated at 37 °C for 20 h. The reactions were stopped by heating to 90 °C for 5 min, cooled on ice, and then centrifuged to collect the resin at the bottom of tube. The supernatant was incubated with uracil DNA glycosylase (New England Biolabs) in buffer containing 20 mM Tris, pH 8.0, 1 mM DTT for 1 h at 37 °C and treated with 150 mM NaOH for 37 °C. The resin was harvested and treated with DNase-I for 1 h at 37 °C. The samples were separated by 15% TBE/urea-PAGE, transferred to a nylon membrane, and incubated with streptavidin-horseradish peroxidase conjugate. The bound oligonucleotide was detected with chemiluminescent reagents (Pierce), and the bands were quantitated on a Typhoon fluorescence gel reader.

**RESULTS**

**The Antiviral Activity of mA3 Requires the Catalytic Glutamic Acid of AS1**—To extend our analysis of the function of the CDDs of the APOBEC3 proteins (13), point mutants of hA3B, hA3F, and mA3 were generated, in which the catalytic glutamic acid residue of AS1 in CDD1 and AS2 in CDD2 was changed to alanine. For hA3G, AS1 (E67A), AS2 (E259A), and the AS1/AS2 double mutants (E67A/E259A) that were generated and termed hA3GAS1*, hA3GAS2*, and hA3GAS1/AS2*, respectively. Analogous AS1 and AS2 mutations in hA3B and mA3 were also generated and named similarly. Antiviral function of the mutant proteins was evaluated using Δvif single cycle luciferase reporter virus. The virus was produced in 293T cells cotransfected with APOBEC3 expression vector, and its infectivity was measured on HOS target cells. For hA3G, the AS1 mutant, hA3GAS1*, was fully active, whereas the AS2 and AS1/AS2 mutants, hA3GAS2* and hA3GAS1/AS2*, were minimally active, showing only about 40% reduction in infectivity (Fig. 1). For hA3B, the AS1 mutant was as active as wild-type hA3B, whereas the AS2 and the AS1/AS2 double mutants were inactive. HA3B was less inhibitory overall as compared with hA3G, consistent with previous findings regarding its weak activity against HIV-1 (17). For hA3F, the AS1 mutant was fully active, whereas the AS2 and AS1/AS2 mutants, hA3GAS2* and hA3GAS1/AS2*, were minimally active, showing only about 40% reduction in infectivity (Fig. 1). For mA3, the AS1 mutant was as active as wild-type mA3B, whereas the AS2 and the AS1/AS2 double mutants were inactive. mA3B was less inhibitory overall as compared with hA3G, consistent with previous findings regarding its weak activity against HIV-1 (17). For mA3F, the AS1 mutant was fully active, whereas the AS2 and AS1/AS2 double mutants were partially active with 80 and 60% reduction, respectively. This activity, however, was rapidly lost when the APOBEC3 was titrated down in the subsequent experiment shown below, suggesting that the antiviral activity of these proteins is weak. Interestingly, for mA3, the phenotype of the mutants was reversed. The AS1 mutant and the AS1/AS2 double mutant showed only 70 and 60% reduction on the infectivity, whereas the AS2 mutant retained full function.
To more accurately gauge the difference in function of the active sites in mA3, the antiviral activity of the wild-type and mutant proteins was tested over a range of concentrations (Fig. 2). For hA3G, the AS1 mutant was at least as active as wild type over the titration. It was interesting to note that over the titration, the hA3G AS1 mutant was significantly more active than wild type. This suggests that CDD1 has a negative effect on the catalytic function of AS2. The AS2 mutant reduced viral infectivity only about 40% even at the highest dose. For hA3B, the wild-type protein was considerably less active against HIV-1, consistent with our previous findings (17). Over the course of the titration, the AS1 mutant was nearly as active as wild type, whereas the AS2 mutant was inactive. For hA3F, the AS1 mutant was as active as wild type. The AS2 mutant was somewhat active at the highest dose (60% inhibition), but this was lost by the next lower dose. For mA3, the reversed functional organization of the cytidine deaminase domains was most clear with 0.5 μg/H9262 g of plasmid. At the 1.0-μg dose, the AS1 mutant was somewhat active, perhaps reflecting nonspecific effects of the protein.

To test whether the mutant APOBEC3 proteins were similarly expressed and competent for virion encapsidation, lysates of the transfected cells and the virions were analyzed on an immunoblot probed with anti-HA mAb. The results showed that wild-type, AS1, AS2, and AS1/AS2 mutants of hA3G, hA3B, mA3, and hA3F were similarly expressed (Fig. 3, upper panel) and were efficiently loaded into virions (Fig. 3, middle panel). The only exception was the hA3B AS1 mutant, which was not as efficiently packaged as wild-type hA3B (Fig. 3, middle panel). Similar loading of the viruses was confirmed by probing the blot with anti-CA mAb (Fig. 3, lower panel).

Analysis of G→A Mutations in the Viral Genome Confirms Reversed Roles of mA3 CDD1 and CDD2—To determine which of the domains of the different APOBEC3 proteins was required to generate G→A mutations in the viral genome, viral cDNA derived from newly infected cells was PCR-amplified, cloned, and sequenced. Cells were infected with Δvif virions produced by 293T cells cotransfected with the various APOBEC3 active site mutants. After 8 h, the cells were harvested; DNA was prepared, and a 1-kb fragment of the viral genome near the 3' end of env was PCR-amplified. The fragment was then cloned into a plasmid vector, and the nucleotide sequence of 10 independent
clones was determined. The sequences of viral cDNAs derived from infection of CV-1 cells with a virus produced in the absence of APOBEC3 had no mutations (data not shown). Wild-type virus produced in the presence of hA3G had a single $G \rightarrow A$ mutation (mutational frequency = 0.02%; data not shown). As expected, viral cDNAs derived from $Δvif$ virions produced with hA3G were heavily mutated with seven clones containing 10 or more $G \rightarrow A$ mutations (Fig. 4). The other APOBEC3 proteins also induced a significant number of $G \rightarrow A$ mutations (hA3G, 2.28%; hA3B, 0.82%; hA3F, 0.92%; and mA3, 1.52%).

The AS1 mutants for each of the human two-domain APOBEC3 proteins induced mutations with a frequency similar to that of wild type (3.35% for hA3GAS1, 0.45% for hA3BAS1, and 0.23% for hA3FAS1). In contrast, mutation of AS2 reduced the number of $G \rightarrow A$ mutations to near zero (0.02% for hA3GAS2 and 0% for hA3BAS2 and hA3FAS2). The situation was reversed for the mouse protein. The mA3 AS1 mutant was nonfunctional whereas the AS2 mutant retained function. For all APOBEC3 proteins tested, no $G \rightarrow A$ mutation was detected in the AS1/AS2 double mutants. These results suggest that cDNA deamination is mediated by AS2 for the human

APOBEC3 proteins and by AS1 for the mouse protein. In addition, the higher $G \rightarrow A$ mutational frequency of human AS1* and mouse AS2* mutants when compared with human AS2* and mouse AS1* mutants correlated with the higher antiviral function of human AS1* and mouse AS2* mutants than human AS2* and mouse AS1* mutants.

**Catalytic Activity of mA3 Is Mediated by AS1**—To determine the relative contribution of the two active sites to the $G \rightarrow A$ mutational frequency, the catalytic activity of the mutant proteins was measured with an *in vitro* cytidine deaminase assay. For this, we developed an assay to measure the catalytic activity of APOBEC3 proteins immunoprecipitated from transfected cell lysates rather than from solubilized pelleted virions as we have reported previously (13). For the immunoprecipitation deaminase assay, 293T cells were transfected with APOBEC3 expression vector and lysed after 2 days. APOBEC3 was immunoprecipitated from the lysate with anti-HA mAb, and the immunoprecipitate was incubated with a 5'-biotinylated oligonucleotide that contains a target sequence for APOBEC3 deamination. The deaminated product was detected by denaturing PAGE and autoradiography. The assay was validated with control samples, which included an empty vector transfection, which yielded no detectable deamination, and hA3G expression vector transfection, which resulted in a deaminated product with about 23-fold above background band intensity (Fig. 5A).

The catalytic activity of the APOBEC3 active site mutants was tested with the immunoprecipitation assay. For hA3G, the AS1 mutant maintained near wild-type activity (Fig. 5A), whereas the AS2 and the AS1/AS2 double mutants were nearly inactive. For hA3B and hA3F, the AS1 mutants were active and the AS2 and AS1/AS2 double mutants were inactive. The catalytic activities of wild-type hA3B and hA3F were less than that of hA3G. However, because of the difference in target site preferences of the enzymes, the comparison of relative activity of the different APOBEC3 proteins may not be valid. This reduction may not reflect weaker deaminase activity but could result from the different target sequence preferences. For hA3F, an oligonucleotide was used containing the consensus sequence, yet the activity still appeared to be weak, perhaps reflecting decreased activity for this enzyme. For mA3, the situation was reversed. The AS1 mutant was inactive, whereas the activity of

![FIGURE 4. G → A mutations in the viral reverse transcripts are catalyzed by AS2 in the human APOBEC3 proteins but by AS1 in mA3.](image-url)
AS2 mutant was close to that of mA3. The AS1/AS2 double mutant was inactive. Immunoblot analysis of the immunoprecipitated APOBEC3 proteins confirmed that similar amount of APOBEC3 proteins was used for the deaminase assay (Fig. 5B).

These results further confirmed the reversal in the functional roles of the active sites in mA3.

**AS2 Is Required for Mouse APOBEC3 Encapsulation**—Encapsulation of hA3G requires the cysteine residues of AS1 (13). To determine the encapsidation determinants of mA3, active site Cys\(^3\)Ala mutants were generated in which either two Cys residues (designated AA) or a single Cys (AC or CA) was mutated (Fig. 6A). The mutants were tested for encapsidation into Δ\(\psi\)/HIV-1 virions. For hA3G, encapsidation was dependent on AS1. hA3G.AS1(AC) was poorly packaged, whereas hA3G.AS2(AA) was efficiently packaged, consistent with our previous findings (13) (Fig. 6A). For the mouse protein, the situation was not as clear, but the trend was reversed. Each of the AS1 mutants was detectably encapsidated, whereas there was no detectable encapsidation of the AS2 mutants. Two of the AS1 mutants (mA3.AS1(AC) and mA3.AS1(AA)) were noticeably reduced for encapsidation. This could indicate a lesser role for AS1 in packaging or it could be the result of a more global conformational effect caused by the mutations. Similar expression levels of the Cys→Ala mutants were confirmed by immunoblot analysis (Fig. 6B, Cell). Overall, the results showed a much stronger role for AS2 in the encapsidation of mA3.

Analysis of the infectivity of the Cys→Ala mutants showed that all were inactive, regardless of which active site was mutated (Fig. 6C). These results demonstrated the importance of both domains for antiviral function. The requirement for both domains reflects the importance of deaminase activity and encapsidation (26). In vitro deaminase activity analysis showed that mA3.AS1(AA) and mA3.AS2(AA) were both inactive (data not shown). This reflects the role of AS1 in deamination and the role of AS2 in homodimerization.

**Dimerization of mA3 Requires AS2**—Dimerization of hA3G requires the AS1 Zn\(^2+\) coordination residues (13). To determine the role of the two active sites in mA3, dimerization of the AS1 and AS2 mutants was tested. To detect dimers, HA-tagged APOBEC3 was coexpressed with Myc-tagged hA3G.
or Myc-tagged mA3 in transfected 293T cells. The cells were lysed, and Myc-mA3 (lanes 1–10) and myc-hA3G (lanes 11–15) were used as bait. These Myc-tagged APOBEC3 proteins were immunoprecipitated with anti-Myc mAb and analyzed on an immunoblot with anti-Myc mAb (IP; upper panel). Anti-HA mAb was used to detect coimmunoprecipitated APOBEC3 proteins tagged with HA (IP; middle panel). Similar expression of the APOBEC3-HA mutants was confirmed by probing with anti-HA mAb (lower panel). Controls included cells transfected with pcDNA3.1(−) alone (no APOBE–HA).

FIGURE 7. AS2 of mA3 is required for dimerization. HA and Myc-tagged APOBEC3 proteins were expressed in cotransfected 293T cells. The cells were lysed, and myc-mA3 (lanes 1–10) and myc-hA3G (lanes 11–15) were used as bait. These Myc-tagged APOBEC3 proteins were immunoprecipitated with anti-Myc mAb and analyzed on an immunoblot with anti-Myc mAb (IP: upper panel). Anti-HA mAb was used to detect coimmunoprecipitated APOBEC3 proteins tagged with HA (IP: middle panel). Similar expression of the APOBEC3-HA mutants was confirmed by probing with anti-HA mAb (lower panel). Controls included cells transfected with pcDNA3.1(−) alone (no APOBE–HA).

or Myc-tagged mA3 in transfected 293T cells. The cells were lysed, and the Myc-tagged APOBEC3 was immunoprecipitated with anti-Myc mAb. The immunoprecipitates were then analyzed on an immunoblot probed with anti-HA mAb. For hA3G, hA3G.AS1(AA) failed to dimerize, whereas hA3G.AS2(AA) retained this property (Fig. 7, middle panel, lanes 11–15). In contrast, for mA3 the AS1 mutants, mA3.AS1(AC), mA3.AS1(CA), and mA3.AS1(AA), dimerized, and for the AS2 mutants, mA3.AS2(AC), mA3.AS2(CA), and mA3.AS2(AA), did not (Fig. 7, middle panel, lanes 1–10). Equivalent expression levels of the HA-tagged mA3 mutants were confirmed on an immunoblot probed with anti-HA mAb (Fig. 7, lower panel, lanes 1–10), and similar amounts of immunoprecipitated Myc-tagged mA3 were confirmed with anti-Myc mAb (upper panel, lanes 1–10). These results suggested that the Zn\(^{2+}\) coordination residues in AS2 are required for dimer formation of mA3, unlike for hA3G, which requires the AS1 Zn\(^{2+}\) coordination amino acids. These proteins were tested for deaminase activity and found to be inactive in the in vitro deaminase assay (data not shown) despite the presence of the functional cytidine deaminase domain. This suggested that dimerization is important for catalytic function.

DISCUSSION

Analysis of the function of the two-domain human and mouse APOBEC3 proteins (hA3B, hA3F, hA3G, and mA3) showed that for the human proteins, the two domains are organized as in hA3G, where CDD1 mediates virion packaging and dimerization, and CDD2 mediates the C→U catalytic activity. In contrast, the roles of the domains are reversed in the mouse protein mA3 where CDD1 mediates deamination and CDD2 is required for encapsidation and dimerization. There is no obvious difference in the amino acid sequence of the proteins that would indicate why one domain is catalytically active and the other is not. All of these activities are required for antiviral function, and thus both domains are required for antiviral function against HIV-1.

The purpose of the separation of function of the two domains is not clear. It could be a means of increasing the potency of the antiviral effect, as the single domain proteins, hA3A and hA3C, are inactive or only weakly active against HIV-1 (4, 17). However, this explanation does not hold for hA3A, which has only a single domain but is highly active against retroelements and adeno-associated virus (4). It is possible that the mechanism by which hA3A inhibits these differs from its activity against lentiviruses.

The reversed functional roles of the cytidine deaminase domains of the mouse protein was evident in the analysis of the encapsidation determinants. This analysis showed that in hA3G, encapsidation depended on the Zn\(^{2+}\) coordination residues of CDD1, whereas in mA3, CDD2 was required. Analysis of the dimerization requirements showed a reversal in the mouse protein, which was dependent on CDD1 for hA3G and CDD2 for the mA3. In mA3 the distinction between domains was not absolute. The mutant mA3.AS1(AC) was slightly reduced for dimerization, suggesting that CDD1 has a role in dimerization. However, the requirement for CDD2 was much stronger, again consistent with the reversed functional roles of the two domains.

Unlike Newman et al. (28), we did not find evidence of a strong cytidine deaminase-independent antiviral mechanism for hA3G. In addition, we did not detect deaminase-independent antiviral activity for the other two family members hA3B and hA3F. APOBEC3 mutants that lacked cytidine deaminase activity lacked antiviral activity. This differed from the study of Newman et al. (28) in which the hA3G E259Q mutant, where the catalytic Glu of AS2 was mutated, maintained antiviral activity yet lacked deaminase activity in a bacterial mutation assay and did not induce a significant number of G→A mutations in the viral DNA. In our study, the hA3G AS2* mutant contained an E259A mutation (as opposed to E259Q). Tested over a range of APOBEC3 concentrations, the mutant minimally inhibited virus infectivity. We also generated an hA3G E259Q mutant but found that it also had minimal antiviral activity (data not shown). In that study the untagged APOBEC3 expression vectors were used. To determine whether this could be the source of the difference, we generated untagged vectors, but again found that this mutant was minimally active (data not shown). The source of these experimental differences is not clear but could be related to the amount of APOBEC3 expressed by different expression vectors. We found that APOBEC3 proteins with double AS1/AS2 catalytic glutamic acid mutants caused a small but reproducible depression in reporter virus infectivity. If tested at a sufficiently high ratio of APOBEC3 to virus, this effect might become more pronounced. A valuable means of gauging the strength of APOBEC3 mutants is to titrate them. This method has the ability to show that inhibition that appears to be potent with high levels of APOBEC3 expression is actually much weaker. Such an analysis is important given that in vivo, APOBEC3 levels are likely to be much lower than in transfections.

The cause of the weak deaminase-independent inhibition that occurs at high ratios of APOBEC3 to virus is not clear. It
might be caused by physical interference with primer tRNA binding or by steric interference with reverse transcription caused by artificially high amounts of encapsidated APOBEC3. In addition, high amounts of APOBEC3 may have generalized effects on the producer cells. Such effects could occur in viruses generated by transfection but not play a role in vivo where APOBEC3 expression levels are lower. Although hA3GAS2*, hA3FAS2*, and mA3AS1* mutants appear to have weak antiviral activity despite no or little deaminase activity, this may again result from overexpression of those proteins. Overall, our findings support deamination as the major mechanism by which the APOBEC3 proteins inhibit HIV-1 replication.

In the sequence analysis of the HIV-1 reverse transcripts, we observed a few G → A mutations induced by hA3GAS2* and by mA3AS1*. These deaminations could have been caused by residual deaminase activity of the mutants. The residual catalytic activity could be mediated by low level function of the mutated active site or to low level function of the unmutated active site in the protein.

Overall, the two domain APOBEC3 proteins are more active against retroviruses than the one domain proteins. The presence of two domains may provide a means of increasing the potency of the APOBEC3 proteins by providing a means of separating the RNA binding from the catalytic function. One domain could bind the viral RNA genome, keeping the other domain free to catalyze deamination. For AID, a related cellular cytidine deaminase, binding to RNA inhibits catalytic function (32). The presence of a free, unbound domain might ensure the availability of an uninhibited catalytic site.

The conservation of two active sites in the APOBEC3 proteins is surprising considering that only one of the sites appears to be active in catalyzing C → U deamination. It is possible that the noncatalytic active site has not yet had sufficient evolutionary time to diversify. It is also possible that the site plays a role in acting against genetic elements other than HIV-1. As new viral targets for the APOBEC3 proteins are identified, it will be of interest to determine whether the differential domain function is conserved or differs depending on the target.

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