Identification of Two APOBEC3F Splice Variants Displaying HIV-1 Antiviral Activity and Contrasting Sensitivity to Vif

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Approximately half of all human genes undergo alternative mRNA splicing. This process often yields homologous gene products exhibiting diverse functions. Alternative splicing of APOBEC3G (A3G) and APOBEC3F (A3F), the major host resistance factors targeted by the HIV-1 protein Vif, has not been explored. We investigated the effects of alternative splicing on A3G/A3F gene expression and antiviral activity. Three alternatively spliced A3G mRNAs and two alternatively spliced A3F mRNAs were detected in peripheral blood mononuclear cells in each of 10 uninfected, healthy donors. Expression of these splice variants was altered in different cell subsets and in response to cellular stimulation. Alternatively spliced A3G variants were insensitive to degradation by Vif but displayed no antiviral activity against HIV-1. Conversely, alternative splicing of A3F produced a 37-kDa variant lacking exon 2 (A3FΔ2) that was prominently expressed in macrophages and monocytes and was resistant to Vif-mediated degradation. Alternative splicing also produced a 24-kDa variant of A3F lacking exons 2–4 (A3FΔ2–4) that was highly sensitive to Vif. Both A3FΔ2 and A3FΔ2–4 displayed reduced cytidine deaminase activity and moderate antiviral activity. These alternatively spliced A3F gene products, particularly A3FΔ2, were incorporated into HIV virions, albeit at levels less than wild-type A3F. Thus, alternative splicing of A3F mRNA generates truncated antiviral proteins that differ sharply in their sensitivity to Vif.

Human A3G and A3F belong to the family of APOBEC3 cytidine deaminases that exert antiviral activity against diverse exogenous viruses, including HIV-1 (reviewed in Ref. 1). In humans, seven A3 proteins (designated A3A, A3B, A3C, A3D, A3F, A3G, and A3H) are tandemly arrayed on chromosome 22 (2, 3). In contrast, mice have only a single A3 gene (mA3) located on a syntenic region of chromosome 15 (4, 5). Despite extensive evolutionary expansion of this locus, expression of human A3 genes remains tightly regulated and tissue-specific (3). Alternative splicing might represent one mechanism whereby the level and action of the A3 family is modulated.

Alternative splicing of pre-mRNA represents a major mechanism for expanding gene function and diversity (6, 7). Approximately 40–60% of human genes are alternatively spliced (8–13), and about one-quarter of this alternative splicing is conserved across species (11). Read-through alternative splicing of feline A3 genes generates a novel functional isoform (4), whereas alternative splicing and/or diminished expression of mA3 impairs the ability of certain strains of mice to recover from Friend virus complex infection (Rfv3) (14, 15). In humans, gene polymorphisms and alternative splicing of A3H result in diverse effects on antiviral activity (16). However, it is not known whether A3G and A3F, the major anti-HIV A3 enzymes and targets of HIV Vif, are functionally regulated by alternative splicing.

A3G and A3F contain dual conserved catalytic domains located in the N- and C-terminal regions termed CD1 and CD2, respectively (3). The HIV-1 auxiliary protein Vif overcomes the antiviral activity of A3G and A3F by targeting both of these host factors for ubiquitin-dependent proteasomal degradation thereby preventing their incorporation into virions (17–23). Although they share some similar antiviral functions, A3G and A3F differ in their interactions with Vif, their overall antiviral activity in the absence of Vif, and their deaminase target site specificity (23–33). A3F is regarded as a less potent anti-HIV factor than A3G. Vif engages A3G through residues located in the N-terminal region of A3G but binds to A3F via different sequences located in the C-terminal region of A3F. A3F appears less sensitive to Vif than A3G. The preferred target sequence for A3G-mediated deamination is 5'-CC, while A3F is 5'-TC (26, 30, 34, 35). The CD2 region mediates cytidine deamination in both A3G and A3F, whereas the CD1 domains may be involved in RNA binding (26, 28, 36). Alternative splicing of either A3G or A3F could result in the exclusion of one or more of these distinct domains, resulting in A3 proteins exhibiting distinct properties and functions.

To assess the role of alternative splicing in A3G/A3F biology and its potential effects on the antiviral activity of these proteins, we screened peripheral blood mononuclear cells...
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(PBMCs)6 from multiple uninfected donors for alternatively spliced forms of A3G and A3F and further analyzed their expression in physiologically relevant subsets of cells. Our findings suggest that alternative splicing leads to the production of variants of both A3G and A3F. Of note, while none of the A3G spliceforms exhibit antiviral activity, two alternatively spliced A3F gene products do display antiviral activity. These findings demonstrate that alternative splicing produces two A3F variants that Vif must attempt to counter.

EXPERIMENTAL PROCEDURES

Cells—293T and TZM-bl cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech) supplemented with 10% fetal bovine serum (FBS, Gembio) and penicillin/streptomycin. PBMCs were isolated by ficoll-hypaque density-gradient centrifugation of buffy coat preparations of heparinized venous blood from HIV seronegative donors (Stanford University Medical Center Blood Bank). PBMCs were maintained in RPMI 1640 medium supplemented with 10% FBS and penicillin/streptomycin. CD14 cells were isolated by positive selection using human CD14 microbeads (Miltenyi). CD14 monocytes were directly lysed for analysis or cultured in 6-well plates for 7 days in DMEM supplemented with 10% FBS and 10% human AB serum to promote differentiation of these cells into macrophages (Gembio). Differentiated macrophages were stimulated overnight with 1000 units/ml interferon-α. CD4 T cells were purified by positive selection from CD14: PBMCs with human CD4 microbeads (Miltenyi). CD4 T cells or total PBMCs were stimulated by culturing cells upright in T-75 flasks at a concentration of 2 × 106 or 10 × 106 cells/ml, respectively, in RPMI supplemented with 10% FBS, 10 μg/ml phytohemagglutinin, and 100 units/ml interleukin-2 (IL-2) for 60–72 h.

Antibodies—A3G polyclonal antibody and mouse monoclonal anti-p24 Gag have been previously described (20, 37). Vif anti-serum was a gift from Dr. Dana Gabuzda (NIH AIDS Research and Reference Reagent Program, HIV-1 HXB2). Anti-A3F antibody was a gift from Dr. Michael H. Malim (AIDS Research and Reference Reagent Program, human APOBEC3F (C-18), Catalog no. 11474) and used at a final dilution of 1:1000. Anti-FLAG mouse monoclonal M2 (Sigma) was used for immunoblotting at a final dilution of 1:1000.

Primers and Plasmids—A3G splice variants were detected using the following primers: A3G E1F, 5′-AGCGGCGCAAGGATGAAAGCCTC-3′; A3G E2R, 5′-CAGCCAGAGCCTTTCCGACGA-3′; A3G E3R, 5′-CGTTGGGCATATCCTCTGTTGACAC-3′; A3G E4R, 5′-TAAATATTAGGCAAGATTATCTCC-3′; A3G E5R, 5′-TCATGCGGTCCTCTGACCCAAGGG-3′; A3G E6R, 5′-TCCAGGAGGTAGAAGCAGTGTAAC-3′; A3G E7, 5′-CATCTATCCATCCGTTCGGTTC-3′; and A3G E8R, 5′-GAGACTGAGGCCATTCACTGAC-3′. A3F splice variants were detected with the A3G E1F primer because of the homology between A3F and A3G in this region and primer A3F E7R (5′-TCACTCGGAAATCTCTTGCA-3′). Splice variants were TOPO cloned (Invitrogen) and sequenced with universal primers. To generate A3G/A3F expression vectors, A3G/A3F splice variants were cloned into the pcMV3X FLAG vector (C-terminal FLAG tag, Sigma) or the pcI-neo expression vector (Promega, Madison, WI) to generate both tagged and untagged constructs. A3G and its spliceforms were cloned with A3GEE1F containing NotI or XbaI restriction sites (pcMV-FLAG3X) or A3GEE8R containing XbaI or NotI restriction sites (pcI-neo). A3F and its spliceforms were cloned with A3GE1F containing NotI or XbaI restriction sites (pcMV-FLAG3X) or A3FE7R containing XbaI or NotI restriction sites (pcI-neo).

Quantitative RT-PCR—Total RNA was isolated from cells with RNeasy Mini Columns (Qiagen). Total input RNA was normalized and reverse transcribed with random hexamers (Invitrogen) and Superscript II (Invitrogen). Real-time RT-PCR was performed with QuantiTect Probe PCR kits (Qiagen) on an ABI PRISM 7900HT sequence detection system (Applied Biosystems). Thermal cycling was carried out with a 15-min denaturation step at 94 °C, followed by 50 cycles of 15 s at 94 °C and 60 s at 60 °C. All real-time PCR assays were performed in triplicate. β-Actin was used as a reference control, and standard curves were made for each primer-probe set using A3G/A3F spliceform expression plasmids. Additionally, reactions were performed to monitor the specificity of splice variant detection. The real-time primer and probes are listed in supplemental Table S1.

Western Blot Analysis—Cells were lysed in radioimmune precipitation assay buffer containing 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 10 μl/ml protease inhibitor mixture (Sigma). Typically 5–10 μg of total protein lysate was loaded onto a 12.5% Criterion Tris-HCl polyacrylamide gel (Bio-Rad) for analysis of transfected 293T cells.

Vif-APOBEC3 Degradation Assays—293T cells were placed in a 24-well plate (60,000 cells/well) and allowed to adhere overnight. Cells were transfected with Effectene transfection reagent (Qiagen) according to the manufacturer’s protocol, using a total of 300 ng of DNA with indicated ratios of codon-optimized 3XFLAG-Vif or A3G/A3F splice variants in pcDNA or 3XFLAG vector. Cells were incubated for 48 h after transfection and lysed in 100 μl of radioimmune precipitation assay buffer. Total protein lysates were quantitated with a BCA assay (Pierce).

Virus Infectivity Assays—To generate viral stocks, pNL4–3 and pNL4–3 Δvif HIV-1 and indicated A3G/A3F expression plasmids (or empty pcDNA3.1 vector) were cotransfected into 293T cells at the indicated ratios with Effectene (Qiagen). Total DNA in the transfection mixtures was maintained at a constant level (600 ng/well for 6-well plates and 300 ng/g for 12-well plates). The medium was changed 6 h after transfection, and the cells were cultured for an additional 48–60 h prior to harvest. Viral supernatants were filtered, and p24-Gag content was quantitated by enzyme-linked immunosorbent assay (ELISA, PerkinElmer Life Sciences). TZM-bl cells were seeded in 96-well flat-bottomed plates (5,000–10,000 cells/well) 1 day before infection. For each virus tested, six independent wells of TZM-bl cells were infected for 36–48 h with viral supernatants containing 1 or 5 ng of p24-Gag. Cells were washed with PBS and lysed with 1× Glo lysis buffer (Promega, Madison, WI), and

6 The abbreviations used are: PBMC, peripheral blood mononuclear cells; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay.
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Luciferase units were quantitated with the Luciferase Assay System (Promega) and a Victor Luminescence Counter (PerkinElmer Life Sciences). Relative light units from six independent infections were averaged for each virus and normalized to infection levels with virus generated in the absence of the relevant APOBEC3 protein being tested.

In Vitro Deoxyctydine Deaminase Assay—Samples for analysis were generated from lysates of 293T cells transfected with empty vector, A3F-FLAG, or A3FΔ2–4-FLAG. Forty-eight hours after transfection, cell lysates were normalized based on total protein content and were immunoprecipitated with 50 μl of anti-FLAG beads (Sigma). The amount of A3F-FLAG in the input samples was confirmed by immunoblotting before analysis. DNA oligonucleotides (5′-[Cy5.5]GAAGGGAAGGAAGGAAGGAGTTAATTTGTGTAAATA-3′) containing target sites for A3F deamination (bold) and serially diluted input samples were incubated in 20 μl of 50 mM Tris buffer, pH 7.4, with RNase A (1 μg) at 37 °C for 4–5 h unless stated otherwise. Samples were then heat-denatured for 10 min at 90 °C. Any uracil bases generated by A3F were converted to abasic sites by treating the purified oligonucleotides with 1 unit of uracil DNA glycosylase (New England Biolabs) for 35 min at 37 °C. The reactions were subjected to alkaline hydrolysis by the addition of NaOH (final concentration, 0.2M) for 5–10 min at 90 °C. Cleavage products were resolved on 15% TBE-urea gels Criterion polyacrylamide gel (Bio-Rad) and visualized with a Personal FX Imager (Bio-Rad), for radiography or fluorescence. Probes and cleavage products were visualized with a LI-COR gel-imaging system.

Virion Incorporation Assays—293T cells were plated in 6-well plates (120,000 cells/well) and allowed to adhere overnight. Cells were transfected with 800 ng of total DNA per well with Effectene transfection reagent. Cells were transfected with pNL4–3 or pNL4–3ΔVif at indicated ratios with the A3G/A3F splicing variants. The medium was replaced 6 h after transfection, and the supernatant was recovered after an additional 48 h. Virions were purified by low-speed centrifugation (1,500 rpm) for 5 min and passed through a 0.22-μm filter, concentrated on Centriprep-20 by centrifugation at 3,000 rpm, underlayed with 8% iodixanol, and centrifuged at 20,000 rpm for 1 h at 4 °C. Supernatants were removed, and pellets were resuspended in 50 μl of radioimmune precipitation assay buffer. Typically 1 μl of lysate was serially diluted in PBS and used for p24-Gag ELISA analysis, performed according to the manufacturer’s protocol (PerkinElmer Life Sciences).

RESULTS

Identification of Alternatively Spliced A3G and A3F mRNAs—The human A3G gene contains eight exons, whereas human A3F contains seven exons. Alternative splicing of the pre-mRNAs from these genes could yield numerous permutations. To assess the appearance of A3G splice variants in an unbiased manner, we designed PCR primer pairs with the forward primer in the 5′-untranslated region (UTR) and reverse primers in each of the remaining exons (Fig. 1A). RT-PCR with these primers was performed using total RNA isolated from PBMCs of 10 uninfected, healthy anonymous donors. After separation on agarose gels, DNA products differing from the expected full-length size were excised, cloned, and sequenced (supplemental Fig. S1A). Multiple A3G splice variants were detected in all primary PBMCs. All contained both the first and last exons. Therefore, primers to detect A3F splice variants were designed with a forward primer in the first exon and a reverse primer in the last exon. Splice variants for A3F were also detected in PBMCs from all donors. Alternative splicing produced three splicing variants of A3G and two splicing variants of A3F (Fig. 1, A and B). New exon-exon junctions in the alternatively spliced mRNAs were found to be intact by sequence analysis. Messenger RNAs lacking exon 2 were detected for both A3G and A3F. This result is not surprising considering A3G and A3F are homologous in exons 1–2.

The predicted amino acid sequences of the splice variant A3G and A3F proteins were determined by cDNA sequencing. A3G and A3F proteins lacking nearly half of the normal number of amino acids found in their full-length counterparts yet retaining the N and C termini were detected (Fig. 1, C and D). These proteins were encoded by alternatively spliced A3G and A3F mRNAs lacking exons 3–5 and 2–4, respectively. The remaining splice variants were predicted to produce frame-shifted proteins where stop codons appeared near the translation initiation site, yielding only short peptide fragments. However, both A3G and A3F contain multiple in-frame downstream ATG sequences that could serve as alternate translation initiation sites. Use of these sites could result in the production of truncated forms of A3G and A3F (Fig. 1, C and D). Alternatively spliced A3G/A3F mRNAs were cloned into expression vectors either containing a C-terminal FLAG epitope tag or lacking any epitope tag. When A3GΔ2 or A3FΔ2 cDNAs were expressed in 293T cells, a series of A3G and A3F proteins was detected. The size of these protein products was consistent with the use of the alternative in-frame start codons located downstream (supplemental Fig. S1B). Detection of A3GΔ2–5 protein expression required transfection of 4-fold increased amounts of expression vector DNA, raising the possibility that this mRNA is poorly translated or that the resultant gene product is less stable (data not shown). Together, these findings highlight expression of a surprisingly diverse group of A3G and A3F splicing variants in vivo encoding smaller forms of the A3G and A3F antiviral proteins.

Alternatively Spliced A3G and A3F mRNAs Are Differentially Expressed with Little Inter-patient Variability—The experiments to identify splice variants in PBMCs yielded no information on their relative abundance, cell-type-specific expression, or variability in expression from patient-to-patient. Expression of full-length A3G and A3F mRNAs varies in different immune cell types and can be altered by exogenous stimuli (38, 39). To determine whether the alternative splicing of A3G/A3F mRNA is regulated in a similar manner, we designed quantitative real-time PCR (qRT-PCR) primers for each splice variant (except A3FΔ2) and for total A3G or A3F mRNA (Fig. 1, A and B). Four different primer/probe pairs for A3FΔ2 were tested for their ability to specifically detect the splice variant mRNA; however, due to their homology, each primer set also detected full-length A3F. Therefore all qRT-PCR data for A3FΔ2 were excluded. Levels of mRNA were measured in unstimulated and phytohe-
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bands were detected in HeLa cells, which lack expression of both A3G and A3F (data not shown). Consistent with the qRT-PCR data, spliceoform expression was typically much lower than the expression of the full-length isoforms with A3FΔ2 being the one exception. Importantly, A3FΔ2 was expressed at levels nearly comparable to full-length A3F in monocytes, macrophages, and IFN-α-treated macrophages. This high level of expression suggests that this 37-kDa variant may have an important biological role.

Of note, the A3G antibody cross-reacts with APOBEC3A. Because A3A expression is highly induced upon treatment of cells with interferon-α, it is likely that the high level of the small isoform in interferon-treated macrophages is actually A3A and not an A3G splice variant (38, 40). This result is consistent with our qRT-PCR analysis. Very little donor-to-donor variation was observed in the expression levels of alternatively spliced A3G and A3F mRNAs suggesting that splicing of these mRNAs is a ubiquitous, well conserved event.

A3G Splice Variants Do Not Interact with Vif and Lack Antiviral Activity—Although A3G spliceoforms were expressed at low levels in primary CD4 T cells, we evaluated their interaction with HIV Vif and tested their potential antiviral activity. To determine whether Vif can degrade the A3G splice variants, 293T cells were cotransfected with expression vector DNA encoding the appropriate A3G spliceoform and either wild-type Vif or Vif substituted in the SLQ domain (VifSLQ) (1:5 or 1:2 mass ratio); this latter Vif mutant fails to assemble normally with the Cul5-E3 ligase complex and thus does not promote proteasome-mediated degradation of either A3G or A3F (41). As expected, full-length A3G was degraded in the presence of wild-type Vif at both concentrations tested (Fig. 3A). A3GΔ2 was expressed poorly and generated multiple bands, consistent with initiation at multiple downstream ATG sites. However, neither the A3GΔ2 nor A3GΔ3–5 protein was sensitive to Vif-mediated degradation (Fig. 3A).

To determine whether A3G splice variants mediate antiviral activity independently of Vif, we performed single-round antiviral assays. To validate the system, virions were produced in the presence or absence of A3G. 293T cells were cotransfected with A3G and pNL4–3 or pNL4–3ΔVif at different ratios. Supernatants containing virions were purified and normalized for p24-Gag content using an ELISA, and comparable amounts of virus were used to infect TZM-bl reporter cells. Luciferase

magglutin/IL-2-stimulated PBMCs, CD4 T cells, monocytes, macrophages, and interferon-α (INF-α)-treated macrophages using cells isolated from four healthy donors (Fig. 2A). Consistent with previous reports, A3F was expressed at ∼10-fold lower levels than A3G in all cell types evaluated (38). All A3G/A3F splice variants were expressed at much lower levels than full-length A3G/A3F mRNA; A3GΔ2–5 was usually expressed at levels below the detection limit of the assay (data not shown).

Although mRNA spliceoforms could be detected in peripheral blood subsets it was still unclear whether these mRNAs were functional and resulted in the production of corresponding proteins. Because all of the splice variants retained the last exon encoding the C terminus of A3G or A3F, we used C-terminal antibodies specific for A3G and A3F in immunoblotting analyses to detect both the splice variant proteins and their wild-type counterparts. Total protein lysates from PBMCs, unstimulated and stimulated CD4 T cells, monocytes, macrophages, and IFN-α-treated macrophages were analyzed from at least four different donors. Representative blots from two donors are shown (Fig. 2B). Spliceoform bands from primary cells were compared with overexpressed proteins from 293T cell lysates. Bands corresponding to A3GΔ2, A3GΔ3–5, A3FΔ2, and A3FΔ2–4 were detected in multiple cell types. No
activity was measured ~36 h after infection. As anticipated, viral infectivity decreased as the amount of A3G increased relative to HIV-1 (Fig. 3B). The same assay was performed using the A3G splice variants. The A3G splice variants and viral plasmids were transfected at a 1:1 ratio to achieve maximal antiviral activity and to control for lower spliceoform protein expression. No antiviral activity was observed with any of the A3G spliceoforms in the absence or presence Vif (Fig. 3C). The low cellular expression and lack of antiviral activity suggest that A3G splice variants have little physiological relevance in the biology of HIV-1.

Spliceoform A3FΔ2–4 Is Highly Susceptible to Vif-mediated Degradation whereas A3FΔ2 Is Relatively Resistant—Unlike A3G, the Vif-interacting domain of A3F resides within the C-terminal portion of the protein (Fig. 1D) (30). Because both A3FΔ2 and A3FΔ2–4 proteins retain this domain, we evaluated...
their susceptibility to Vif-mediated degradation. As described above, 293T cells were cotransfected at a 1:5 or 1:2 ratio with expression vectors encoding the A3F spliceoforms and either wild-type Vif or the VifSLQ mutant. To control for protein expression differences, translation of A3F/H9004 was directed from the first downstream ATG codon producing a single variant of 37 kDa. Consistent with previous reports (30, 33), A3F was consistently less susceptible to Vif-mediated degradation than A3G. As expected, VifSLQ did not alter the levels of any of the A3F proteins (Fig. 4A). Of note, A3F/H9004 appeared less sensitive to Vif-mediated degradation than wild-type A3F, whereas the A3F/H9004–4 protein exhibited enhanced degradation in the presence of Vif (Fig. 4A). Thus, these splicing variants of A3F differ sharply in their sensitivity to Vif.

A3F/H9004 and A3F/H9004–4 Display Decreased Antiviral Activity Compared with Full-length A3F—Because the A3F splice variants differed in their susceptibility to Vif-mediated degradation, we evaluated their potential antiviral activities. TZM-bl indicator cells were again infected with virions produced in the presence of various ratios of wild-type A3F or the A3F/H9004 splice form. A3F/H9004 displayed enhanced degradation in the presence of Vif (Fig. 4A). Thus, these splicing variants of A3F differ sharply in their sensitivity to Vif.

A3FΔ2 and A3FΔ2–4 Display Decreased Antiviral Activity Compared with Full-length A3F—Because the A3F splice variants differed in their susceptibility to Vif-mediated degradation, we evaluated their potential antiviral activities. TZM-bl indicator cells were again infected with virions produced in the presence of various ratios of wild-type A3F or the splice variants. A dose-dependent increase in antiviral activity was observed with A3F, A3FΔ2, and A3FΔ2–4 in the absence of Vif; however, wild-type A3F was consistently more potent (Fig. 4B). The antiviral activity of the A3FΔ2 protein was only slightly decreased compared with full-length A3F. Consistent with this isofrom being less susceptible to Vif-mediated degradation there was little difference in its antiviral activity in the presence or absence of Vif. The A3FΔ2–4 protein produced modest antiviral activity in the absence of Vif, and as expected, this effect was abrogated in the presence of Vif (Fig. 4B). Thus, alternative splice removal of exon 2 of A3F produces a Vif-resistant protein that retains significant antiviral activity. Splice removal of exons 2–4 results in a Vif-sensitive protein that displays weaker antiviral activity.

Antiviral Activities of A3FΔ2 and A3FΔ2–4 Are Compromised by Low Enzymatic Activity and Decreased Incorporation into Virions—To determine whether the decrease in antiviral activity of A3FΔ2 and A3FΔ2–4 reflects changes in their intrinsic enzymatic activity, in vitro cytidine deaminase assays were performed. Immunoprecipitated A3F-FLAG, A3FΔ2-FLAG, and A3FΔ2–4-FLAG were serially diluted 5-fold and analyzed for deaminase activity. A3F exhibited deaminase activity at the highest dilution (1:1), and this activity remained detectable at dilutions greater than 1:25 (Fig. 5A). Western blot analysis of immunoprecipitated proteins confirmed that the poorer
activity was not due to differences in isoform expression. Thus, A3F\textsubscript{H9004} and A3F\textsubscript{H9004–4} exhibit reduced deoxycytidine deaminase activity compared with wild-type A3F. Although A3F\textsubscript{H9004} and A3F\textsubscript{H9004–4} each exhibited similar levels of deaminase activity, A3F\textsubscript{H9004} consistently displayed higher levels of antiviral activity. To further evaluate this difference, virions were produced in the presence of A3F\textsubscript{H9004} or A3F\textsubscript{H9004–4} and analyzed for the level of variant protein encapsidation. A3F\textsubscript{H9004} was incorporated into virions at slightly lower levels than full-length A3F (Fig. 5B, left panels). Interestingly, A3F\textsubscript{H9004–4} was incorporated into virions at markedly lower levels than full-length A3F or A3F\textsubscript{H9004} (Fig. 5B). A3F\textsubscript{H9004–4} incorporation was detected only after overexposure in a dose-dependent manner, consistent with its greatly reduced antiviral activity (Fig. 5B). Negative controls transfected with FLAG-tagged A3F or A3F\textsubscript{H9004–4} alone and lacking pNL4–3 yielded no signal with anti-FLAG in virion preparations, indicating the absence of microvesicle contamination in these studies (data not shown). Together, these findings suggest that alternative splicing compromises the deaminase activity of both A3F\textsubscript{H9004} and A3F\textsubscript{H9004–4} spliceforms and severely impairs the incorporation of A3F\textsubscript{H9004–4} into budding HIV-1 virions.

### DISCUSSION

Approximately 40–60% of human genes are alternatively spliced, adding great complexity to the genome (8–13). Alternative splicing can increase or decrease the antiviral activity of mouse, cat, and human APOBEC3 genes (4, 14–16). Although alternative splicing can play a significant role in APOBEC3 biology, its contribution to A3G and A3F function is unknown. Because therapeutic strategies aimed at inhibiting Vif’s attack on A3G and A3F are being tested, it is important to consider the biology of alternately spliced forms of A3G and A3F and their sensitivity to Vif. In this study, we identified novel splice variants of both A3G and A3F and determined their relative prevalence in primary cells that are natural targets of HIV infection in vivo. Analysis of these A3G/A3F spliceforms revealed distinct properties compared with their full-length counterparts. Three A3G spliceforms and two A3F spliceforms were detected in primary human PBMCs at the RNA level. Importantly, although there was very little donor-to-donor variability in expression of these isoforms, expression among various cell types varied considerably. Spliced forms of A3G or A3F were not specifically induced by the exogenous stimuli tested. How-
ever, T-cell activation consistently resulted in decreased expression of these splicing variants. A3G splice variant RNAs were expressed at much lower levels than full-length A3G mRNA. Expression of A3GΔ2 and A3GΔ3–5 was highest in unstimulated cell types (PBMCs, CD4 T cells, and monocytes). These spliceoforms did not interact with HIV-1 Vif and exhibited no antiviral activity. Thus, the function of alternatively spliced forms of A3G remains unclear. However, we conclude that these splice variants of A3G are unlikely to play a major role in HIV biology.

In contrast to A3G, A3FΔ2, and A3FΔ2–4 accounted for a significant proportion of total A3F protein. In cell types with lower full-length A3F expression, such as PBMCs and unstimulated CD4 T cells, expression of A3FΔ2–4 was notable. In CD14+/H11001-derived cell types (monocytes, macrophages, and IFN-treated macrophages), a significantly high level of A3FΔ2 expression was consistently detected, often at levels comparable to those of wild-type A3F. Importantly, deletion of exons 2–4 generated an A3F splice form that was more sensitive to Vif-mediated degradation than the full-length A3F protein. However, deletion of exon 2 alone in A3F resulted in increased resistance to Vif-induced proteolysis. These findings are consistent with studies indicating the Vif-interacting domain of A3F is located at the C terminus (30); however, they also suggest that portions of the N terminus of A3F may contain domains that negatively and positively regulate Vif-mediated degradation. Alternative splicing of A3F can thus produce splice variants displaying greater (A3FΔ2–4) or lesser (A3FΔ2) sensitivity to Vif.

Alternative splicing of A3F with the removal of exon 2 alone or exons 2–4 produces variant A3F proteins displaying reduced cytidine deaminase activity. Mutational analysis and domain swapping studies indicate that the CD2 of A3F encodes its deaminase activity and determines its nucleotide specificity (26, 28). A3FΔ2 and A3FΔ2–4 contain intact CD2 regions; however, their lower enzymatic activities likely reflect conformational changes in the proteins that compromise deoxycytidine deaminase activity. It has been hypothesized that cytidine deaminase-driven viral defense mechanisms may introduce inherent mutagenic risks to the host and could potentially generate mutations that are beneficial to the virus (42, 43). Expression of A3F isoforms with reduced enzymatic activity in multi-
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...ple cell types provides a potential mechanism for enhancing antiviral defense while minimizing deleterious host gene mutations. Additionally, both A3F and A3G spontaneously give rise to out-of-frame spliceforms lacking exon 2. This may be a conserved mechanism designed to limit the intracellular concentrations of A3G/A3F to optimal levels within certain cell types.

Both enzyme-dependent and enzyme-independent mechanisms have been proposed to play important roles in the antiviral activities of A3G/A3F (27–29, 33, 44–52). A3FΔ2 and A3FΔ2–4 exhibit reduced cytidine deaminase activity while maintaining modest antiviral activity, potentially consistent with reports that enzymatic activity is not fully required for antiviral activity (27–29, 50). An additional explanation for the attenuated antiviral activity of the A3F spliceforms relates to their reduced incorporation into virions. As shown by Western blot analysis of virions, encapsidation of A3FΔ2 was slightly reduced compared with full-length A3F while encapsidation of A3FΔ2–4 was decreased by more than 30-fold (1:1 ratio versus 1:30 ratio). Consistent with previous mutagenesis results, the low level of incorporation of A3FΔ2–4 into virions supports the notion that the N-terminal 184 amino acids play a distinct role in determining the overall level of virion incorporation but potentially contribute little to its antiviral activity (28). The identification of two naturally expressed A3F spliceforms that exhibit antiviral activity with variable susceptibility to Vif shows that the antiviral defense mounted by A3F is more complex than previously considered.

Alternative splicing of genes is one mechanism for broadening the functional repertoire of proteins emanating from a single gene. Recent host-pathogen conflict has resulted in expansion of the APOBEC3 gene locus from one member in mice to seven members in higher primates and humans (designated A3A–A3H). This study, plus those of others, suggests that the human A3 family encodes nearly double the number of proteins compared to out-of-frame spliceforms lacking exon 2. This may be a conserved mechanism designed to limit the intracellular concentrations of A3G/A3F to optimal levels within certain cell types.

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