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Report

APOBEC3A Is a Potent Inhibitor of Adeno-Associated Virus and Retrotransposons

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Summary

APOBEC3 proteins constitute a family of cytidine deaminases that provide intracellular resistance to retrovirus replication and transposition of endogenous retroelements [1]. One family member, APOBEC3A (hA3A), is an orphan, without any known antiviral activity. We show that hA3A is catalytically active and that it, but none of the other family members, potently inhibits replication of the parvovirus adeno-associated virus (AAV). hA3A was also a potent inhibitor of the endogenous LTR retroelements, MusD, IAP, and the non-LTR retroelement, LINE-1. Its function was dependent on the conserved amino acids of the hA3A active site, consistent with a role for cytidine deamination, although mutations in retroelement sequences were not found. These findings demonstrate the potent activity of hA3A, an APOBEC3 family member with no previously identified function. They also highlight the functional differences between APOBEC3 proteins. The APOBEC3 family members have distinct functions and may have evolved to resist various classes of genetic elements.

Results and Discussion

In the human, the APOBEC family consists of APOBEC1 (hA1), APOBEC2 (hA2), and APOBEC3A, B, C, D/E, F, G, and H (hA3A-H) [2, 3], while the mouse genome encodes APOBEC1 (mA1), APOBEC2 (mA2), and a single APOBEC3 (mA3). HIV-1 has evolved the accessory protein Vif to block the inhibitory effects of hA3F and hA3G [1, 4]. Virions produced by cells infected with *Δvif* HIV-1 package hA3G that catalyzes C→U deamination of the minus-strand reverse transcripts synthesized in the next round of infection [5–8]. Vif protects the virus by inducing ubiquitination and proteasomal degradation of hA3G [9–11]. Endogenous LTR retroelements such as the mouse intracisternal A particle (IAP) and MusD and human HERV and the non-LTR retroelements such as LINE-1 comprise a large portion of the mammalian genome (>42% in the human) and predate lentiviruses [12, 13]. The elements reverse transcribe their RNA genome and integrate through a process that is similar to retroviral replication although only a few are active and capable of retrotransposition [14, 15]. Retrotransposition of IAP and MusD (but not LINE-1) is blocked by hA3G [16, 17], and this is associated with G→A mutations.

Parvoviruses (family *Parvoviridae*) are small eukaryotic DNA viruses that infect humans and a variety of other animal species [18]. The Parvovirus genome consists of a linear single-stranded DNA (ssDNA) molecule containing ORFs encoding replication (Rep) and capsid (Cap) proteins, flanked by hairpins. Parvoviruses are prevalent in the human population, where they exist as an array of serotypes of high-sequence diversity [19, 20]. Adeno-associated virus (AAV) requires a helper virus and replicates in the nucleus through a single-stranded intermediate.

To test for antiretroviral activity of hA3A, wild-type and *Δvif* HIV-1 and SIV luciferase reporter viruses were prepared in transfected 293T cells in the presence or absence of APOBEC3 expression vectors, and their infectivity was determined on target cells by measurement of intracellular luciferase. Immunoblot analysis of the virions confirmed that hA3A was efficiently packaged into virions, yet in spite of its presence in the virion, hA3A had no effect on infectivity (Figures 1A and 1D). In contrast, hA3G potently inhibited *Δvif* HIV-1 and SIVagm (Figure 1A and [21]).

hA3A Is a Single-Strand-Specific DNA Cytidine Deaminase

hA3A contains a single copy of the cytidine deaminase active site sequence motif **$HXEX_{28}PCX_4C$** in which the histidine and two cysteines coordinate a Zn^{2+} and the glutamate serves as a proton shuttle in catalysis [2]. To test for hA3A cytidine deaminase activity, hA3A was packaged into HIV-1 virions and cytidine deaminase activity released from the virions upon detergent lysis was measured in vitro by incubation with a radiolabeled deoxyoligonucleotide that contained a single target cytosine [22]. The substrate preference of hA3A was determined on single-stranded, double-stranded DNA (DNA:DNA) or double-stranded hybrids (DNA:RNA) substrates. After incubation with virus lysate, the oligonucleotide was cleaved with uracil DNA glycosylase and treated at high pH, and the products were detected by autoradiography. hA3A was found to deaminate single-stranded DNA but not double-stranded DNA or DNA:RNA hybrids (Figure 1B). A small amount of the DNA:RNA hybrid appeared to be deaminated, probably as a result of a small amount of single-stranded probe in the preparation. Control virions prepared without APOBEC3 lacked deaminase activity.

To determine the role of the active site amino acids in catalysis, hA3A with a point mutation in the Zn^{2+} coordination residues H70R or C106S or the catalytic glutamic acid E72Q were tested for cytidine deaminase activity. The mutated hA3A molecules were packaged into

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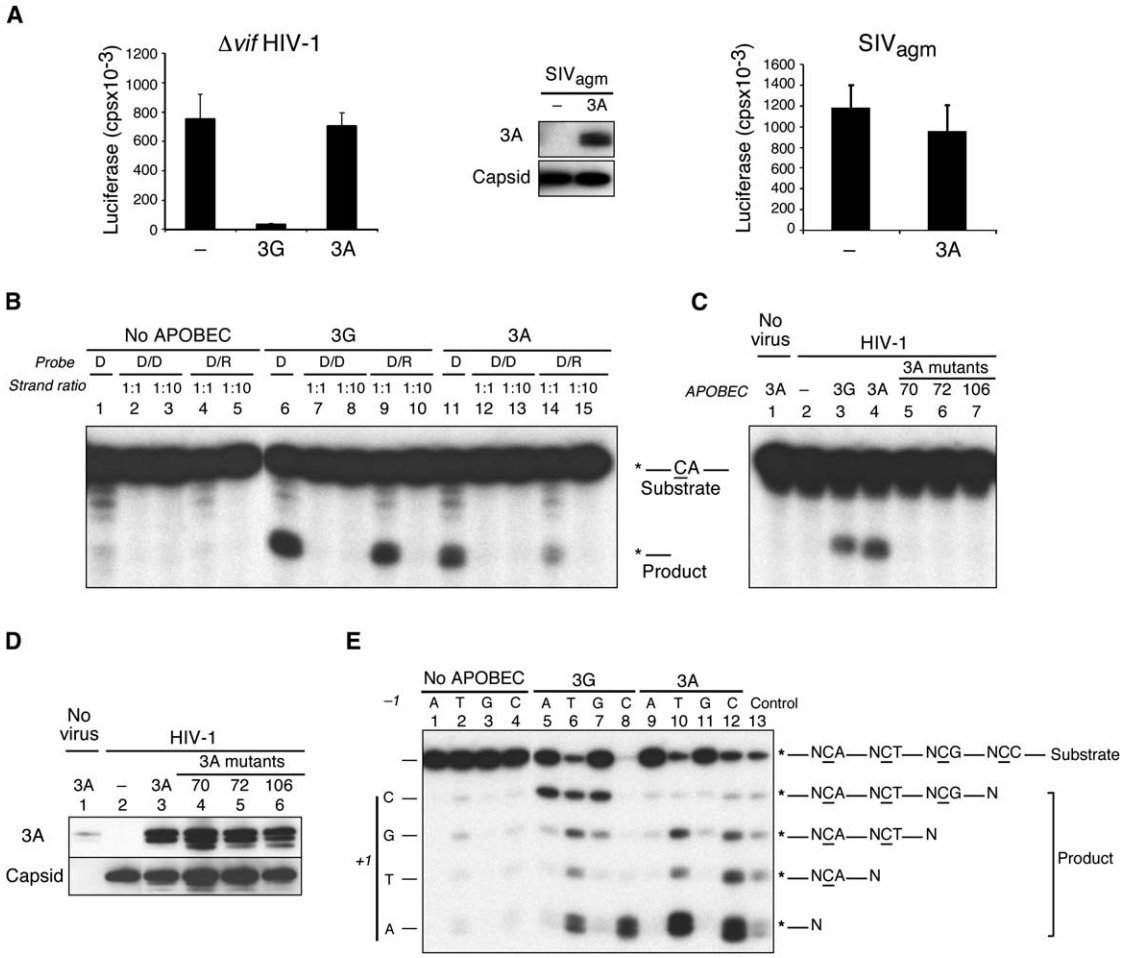

Figure 1. hA3A Is a Single Strand-Specific DNA Cytidine Deaminase

(A) hA3A does not inhibit HIV-1 or SIVagm. *Δvif* HIV-1 and SIV_{agm} luciferase reporter virus [5, 29] was produced in 293T cells cotransfected with or without hA3A or hA3G expression vector, and infectivity of the viruses (1 ng p24/27) was measured by luciferase activity in infected target cells (left and right). hA3A encapsidated in SIVagm virions and normalized for p27 was detected on an immunoblot probed with anti-HA and anti-CA mAb. Experiments were performed in triplicate and data are presented as the mean, with error bars reflecting the standard deviation.

(B) HIV-1 virions loaded with or without hA3A or hA3G were incubated in 0.1% Triton-X100 buffer. Cytidine deaminase activity released was measured by incubation with a 5′-^{32}P-labeled deoxyoligonucleotide substrate (CCT40; Figure S1) containing a target C. The deaminated molecules were cleaved by treatment with uracil DNA glycosylase followed by high pH, and the products were resolved by PAGE and visualized by autoradiography. The probe was single-stranded DNA (D), or hybridized to an unlabeled complementary DNA (D/D) or RNA (D/R) at the ratios indicated above. The substrate and deaminated product are indicated.

(C) Catalytic activity of hA3A active site mutants H70R, E72Q, and C106S was measured in the cytidine deaminase assay. Virions loaded with each mutant were prepared and cytidine deaminase activity was measured as in (B). Virions prepared without hA3A or without viral DNA were measured as controls.

(D) The HIV-1 virions contained similar amounts of hA3A active site mutant protein. Virion contents were measured on an immunoblot probed with anti-HA and anti-CA mAbs.

(E) The target sequence preference of the hA3A was determined on a panel of 5′-^{32}P-labeled deoxyoligonucleotide substrates, each containing four target sites. The –1 base is shown at the top of each lane and the +1 base is shown on the left. The structure of the cleaved products is shown on the right. The control (last lane) is an oligonucleotide with the same sequence at each target site to show the independence of target site position on cleavage frequency.

Δvif HIV-1 virions, and the cytidine deaminase activity released from the virions was measured. The results showed that the mutant hA3A proteins were inactive (Figure 1C). Immunoblot analysis confirmed that they were packaged at comparable levels (Figure 1D). To test the site sequence preference of hA3A, a panel of four oligonucleotides was designed that tests each of the 16 possible NCN trinucleotide sequence combinations (Figure 1E). The method was validated with hA3G, which was found to preferentially target the sequence CCA, consistent with its known specificity [7, 8, 22]. This method showed that hA3A prefers the sequence (T/C)CA.

hA3A Inhibits AAV

Since AAV replicates as single-stranded DNA in the nuclei of infected cells, we considered it as a potential APOBEC3 target. The effect of APOBEC3 proteins on parvovirus replication was determined by production of recombinant AAV (rAAV) in a transfection assay (Figure 2A). Plasmids required for rAAV replication and packaging were cotransfected into 293 cells together

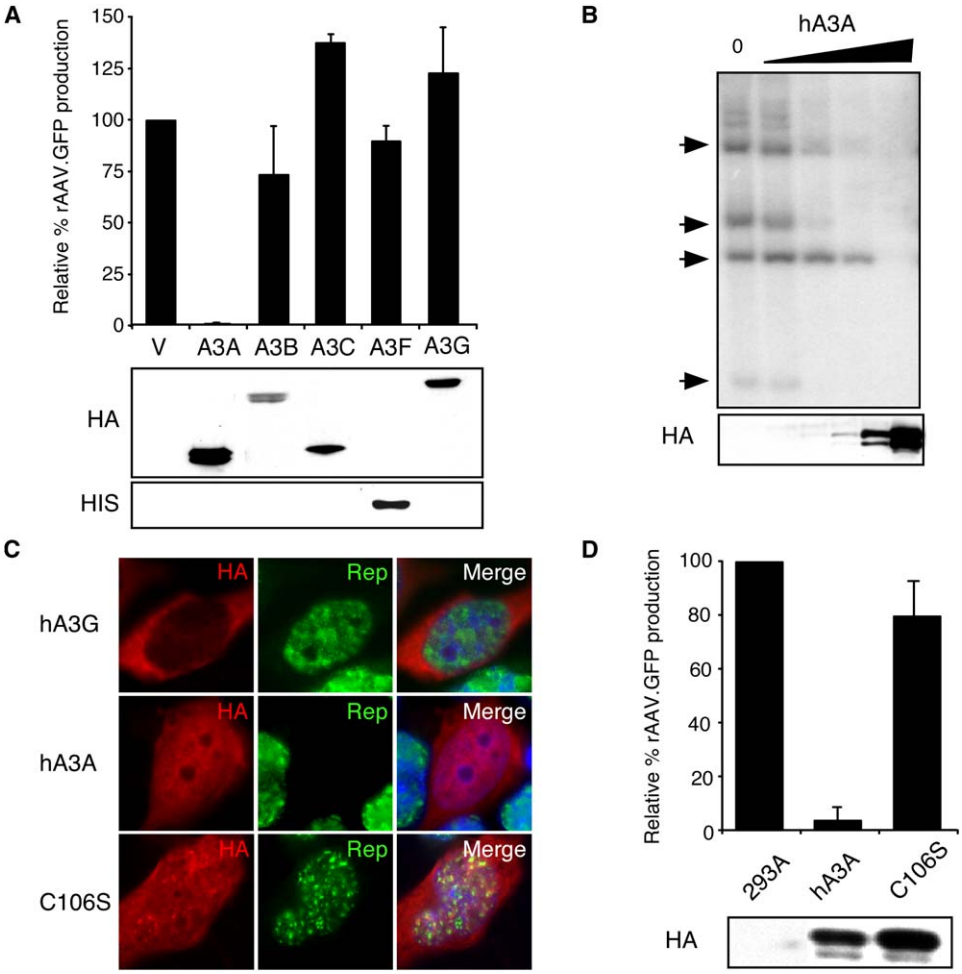

Figure 2. hA3A Inhibits AAV Replication

(A) Production of rAAV was inhibited by hA3A but was unaffected by other APOBEC proteins. 293 cells were transfected with plasmids required for rAAV.GFP production in the presence or absence of APOBEC3 expression vectors. Production of rAAV was assessed by transduction of target cells and quantitation by FACS. The data in (A) and (D) are the average of triplicate transfections normalized to vector only control. The panels below show immunoblots to detect HA-tagged hA3A, hA3B, hA3C, hA3G, and His-tagged hA3F. V, vector alone. Experiments were performed in triplicate and data are presented as the mean, with error bars reflecting the standard deviation.

(B) Southern blot detection of low molecular weight DNA extracted from cells transfected for rAAV production in the presence of increasing amounts (0, 0.001, 0.01, 0.1, 1.0 μg) of hA3A expression vector. The DNA was digested with Dpn I, separated by gel electrophoresis, and hybridized with a radiolabeled AAV probe. The arrows indicate AAV replication products. Shown below is an immunoblot to detect HA-tagged hA3A.

(C) Immunofluorescence to detect HA-tagged APOBEC and AAV (Rep) proteins in cells transfected with wild-type and mutant APOBEC and then infected with AAV and adenovirus. Rep from the input virus is undetectable in this assay, so positive Rep staining is indicative of AAV replication. Nuclei are stained with DAPI as indicated in the merged panels to the right.

(D) Production of rAAV in 293 cells is inhibited by wild-type but not mutant HA-tagged hA3A in stable cell lines. An immunoblot to detect HA-tagged hA3A is shown below. Experiments were performed in triplicate and data are presented as the mean, with error bars reflecting the standard deviation.

with APOBEC3 expression vectors. In this system, Rep and Cap are supplied in *trans* to enable replication of an AAV vector and packaging of the ssDNA genome. rAAV produced is quantitated by transduction of target cells with cell lysate. hA3A protein was found to completely block rAAV production. In contrast, hA3B, hA3C, hA3F, and hA3G had no effect. hA3A did not interfere with the production of helper proteins (see Figure S2A in the Supplemental Data available with this article online).

Analysis of the low molecular weight DNA extracted from the transfected cells revealed dramatic inhibition of rAAV replication by hA3A (Figure 2B). As little as 10 ng of plasmid hA3A inhibited replication and particle production (Figure S2B). None of the APOBEC3 proteins affected adenovirus or herpes simplex virus replication, suggesting a requirement for ssDNA (data not shown).

hA3A Is in the Nucleus and Blocks Formation of AAV Replication Centers

Replication of AAV by helper virus results in the formation of nuclear viral replication centers that can be detected with antibody to Rep [23, 24]. To visualize the effect of APOBEC3 proteins on AAV replication, cells were transfected with hA3G, hA3A, or hA3A C106S and then infected with wild-type AAV and adenovirus

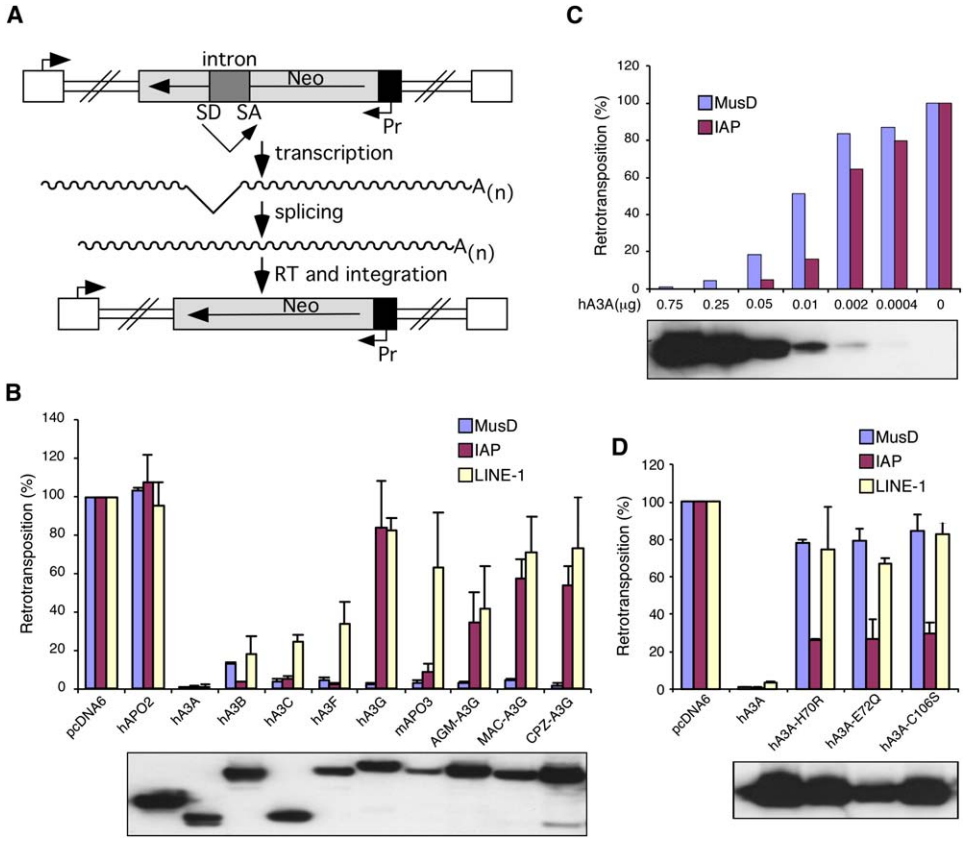

Figure 3. hA3A Is a Potent Inhibitor of MusD, IAP, and LINE-1 Retrotransposition

(A) The diagram shows the structure of the MusD and IAP *neo*^{r}-marked elements [25]. The reverse orientation *neo*^{r} gene is interrupted by an intron. Expression of functional *neo*^{r} requires splicing of the RNA transcript followed by reverse transcription. Retrotransposition events are scored as the number of G418-resistant colonies. Arrows indicate transcription initiation sites. SD, splice donor; SA, splice acceptor.

(B) Inhibition of MusD, IAP, and LINE-1 retrotransposition by APOBEC3 proteins. HeLa cells were transfected with MusD, IAP, or LINE-1 plasmids and the indicated APOBEC3 expression vector (1:1 mass ratio for MusD and IAP and 2:1 ratio for LINE-1) and selected in G418-containing medium. The data in (B)–(D) are normalized to the empty vector control (an average of 1002 for MusD, 268 for IAP, and 227 for LINE-1 per 10 cm dish), which was set to 100%. The data are averaged from at least three independent experiments. An immunoblot to detect expression of the HA-tagged APOBEC3 protein in the transfected cells is shown below each bar graph (B–D).

(C) Titration of hA3A against MusD and IAP. Cells were transfected with a fixed amount of MusD or IAP (0.75 μg) and the indicated mass of hA3A expression vector. The total mass of plasmid was held constant by addition of empty vector.

(D) Inhibition of retrotransposition by hA3A is dependent on the active site. H70R, E72Q, and C106S were cotransfected with MusD, IAP, or LINE-1. Experiments were performed in triplicate and data are presented as the mean, with error bars reflecting the standard deviation.

(Figure 2C). Numerous replication centers were seen in cells that expressed hA3G, but these were strikingly absent in cells that expressed hA3A. Analysis of APOBEC3 localization by immunofluorescence cells showed that hA3A was in the cytoplasm and nucleus, unlike hA3G, hA3F, and mA3, which were confined to the cytoplasm (Figure S3). hA3A C106S retained nuclear localization but had no effect on the number of viral replication centers (Figure 2C). Replication and production of rAAV in stable cell lines expressing hA3A was dramatically reduced, while the C106S mutant was similar to control (Figure 2D). These results suggested that the active site defect rather than an inability to localize to the nucleus accounted for the failure of the hA3A mutant to inhibit.

hA3A Inhibits LTR and Non-LTR Retroelements

APOBEC3 family members were tested for their ability to inhibit retrotransposition of IAP, MusD, and LINE-1 retroelements. Retrotransposition was measured by means of a culture system in which cells are transfected with a retroelement that contains a reverse orientation *neo*^{r} gene that is interrupted by an intron (Figure 3A) [21, 25]. Retrotransposition results in the integration of a spliced DNA copy of the element. Neo^r colonies are counted after selection in G418. Several APOBEC3 family members were found to inhibit retrotransposition, including hA3A, hA3B, hA3C, hA3F, agmA3G, macA3G, cpzA3G, and mA3 (Figure 3B). hA3A was the most active, inhibiting retrotransposition of the LTR (MusD and IAP) and non-LTR (LINE-1) elements. In contrast, hA3G, agmA3G, macA3G, and cpzA3G were nearly inactive against IAP. hA3G did not inhibit LINE-1, consistent with an earlier report [17], but hA3B, hA3C, and hA3F were partially effective. hAPO2, included as a control, had no effect. hA3A was expressed at a level comparable to the other APOBEC proteins (Figure 3B, below), and thus the results were not caused by overexpression. hA3A was potent, causing 50% inhibition of MusD at 0.01 μg and IAP at 0.002 μg (Figure 3C). The potency

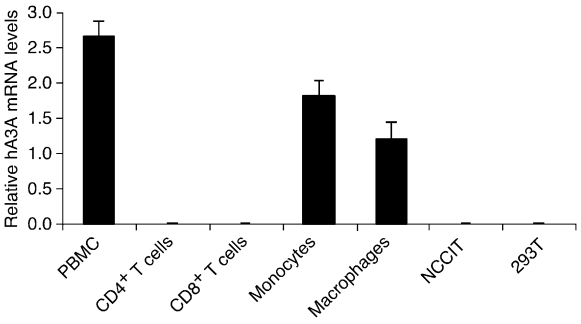

Figure 4. hA3A Is Expressed in Monocytes and Macrophages

Relative mRNA levels of hA3A in activated PBMCs, $CD4^+$, and $CD8^+$ T cells, monocytes, macrophages, NCCIT, and 293 cells were quantitated by real-time PCR and normalized according to 18S rRNA content. The data for primary cells are representative of separate experiments with cells obtained from three healthy donors. The data are presented as the average of triplicates ± the standard deviation.

of hA3A against AAV and retroelements was striking in contrast to its lack of activity against SIV, which is a particularly sensitive target of most APOBEC3 family members (Figure 1A and [21]).

The hA3A active site mutants were inactive against MusD and LINE-1 and had weak activity against IAP (Figure 3D). Whether the weak activity against IAP is caused by residual deaminase activity of the active site mutants or by weak deaminase-independent inhibition [26] cannot be determined from these results. Extensive sequence analysis of transposed IAP and MusD retroelements, surprisingly, failed to show hA3A induced G→A mutations (Figure S4).

Expression of hA3A mRNA in Human Cells

Real-time PCR analysis showed undetectable expression of hA3A in 293 cells and mitogen-activated primary CD4 and CD8 T cells, but significant quantities in monocytes and monocyte-derived macrophages (Figure 4). These results suggest that hA3A is expressed in cells in addition to keratinocytes [27] and that it could have wider expression in yet other cell types not included in this analysis.

Conclusions

The inhibition of AAV by hA3A is the first example of an APOBEC protein that targets a virus that replicates exclusively in the nucleus and without passing through an RNA intermediate. The ability of hA3A to enter the nucleus combined with its specificity for single-stranded DNA may contribute to its potency and to its broad range of targets. The activity of hA3A against LINE-1 is the first example of an APOBEC3 protein that acts on a non-LTR retroelement. Although hA3A is active against AAV and the retroelements, it is remarkable that it is inactive against lentiviruses.

The absence of hA3A-induced mutations is surprising and not yet understood. It may be that deamination causes rapid degradation of the target DNA, preventing PCR amplification of the mutated molecules. Alternatively, inhibition could be mediated by a deaminase-independent mechanism in which hA3A binds to single-stranded viral DNA, interfering with the completion of DNA synthesis. The requirement for a functional active site suggests a role for deamination; however, the single active site of hA3A could mediate both nucleic acid binding and catalysis [28].

It is unlikely that hA3A evolved to inhibit AAV, which is not known to be pathogenic in humans, but hA3A could serve to inhibit related pathogenic parvoviruses such as B19 [18]. Potential roles for hA3A in vivo will be dependent upon further analysis of its cell-type expression. It will be of interest to understand the features of the APOBEC3 family members that cause their different viral specificities, how the enzymes specifically target viral and retroviral-like sequences, and how the genome of the cell is protected from their activity.

Supplemental Data

Supplemental Data include four figures and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/5/480/DC1/.

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Note Added in Proof

Similar findings on the inhibition of IAP by hA3A were recently reported (Bogerd, H.P., Wiegand, H.L., Doehle, B.P., Lueders, K.K., and Cullen, B.R. (2006). APOBEC3A and APOBEC3B are potent inhibitors of LTR-retrotransposon function in human cells. Nucleic Acids Res. *34*, 89–95).