Lentivirus restriction by diverse primate APOBEC3A proteins

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ARTICLE INFO

Article history:
Received 7 February 2013
Returned to author for revisions 8 March 2013
Accepted 3 April 2013
Available online 4 May 2013

Abstract

Rhesus macaque APOBEC3A (rhA3A) is capable of restricting both simian–human immunodeficiency virus (SHIVΔvif) and human immunodeficiency virus (HIV-1Δvif) to a greater extent than hA3A. We constructed chimeric A3A proteins to define the domains required for differential lentivirus restriction. Substitution of amino acids 25–33 from rhA3A into hA3A was sufficient to restrict HIVΔvif to levels similar to rhA3A restriction of SHIVΔvif. We tested if differential lentivirus restriction is conserved between A3A from Old World monkey and hominid lineages. A3A from African green monkey restricted SHIVΔvif but not HIV-1Δvif and colobus monkey A3A restricted both wild type and SHIVΔvif and HIV-1Δvif. In contrast, the gibbon ape A3A restricted neither SHIVΔvif nor HIV-1Δvif. Restriction of SHIVΔvif and HIV-1Δvif by New World monkey A3A proteins was not conserved as the A3A from the squirrel monkey but not the northern owl monkey restricted SHIVΔvif. Finally, the colobus A3A protein appears to restrict by a novel post-entry mechanism.

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Introduction

The apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3 (APOBEC3; A3) family of proteins in humans have been shown to restrict the replication of many different retroviruses (Sheehy et al., 2002; Chiu and Greene, 2009). In humans and rhesus macaques, this family consists of seven members (A3A, A3B, A3C, A3D, A3F, A3G and A3H) (Dang et al., 2007; Hultquist et al., 2011; Jarmuz et al., 2002; Schmitt et al., 2011; Virgen et al., 2007). These proteins are cytidine deaminases that have canonical deaminase domains (H-x-E-x24-28-P-C-x-x-C) where the histidine and two cysteines coordinate a zinc cofactor, with the glutamate serving in proton transfer (MacGinnitie et al., 1995). The A3 proteins contain either a single cytidine deaminase domain (A3A, A3C, A3H) or duplicated domains (A3B, A3D, A3F, and A3G). Following entry of virus containing A3G, this enzyme causes deamination of cytidine to uridine during minus strand DNA synthesis, ultimately resulting in G-to-A mutations (Chelico et al., 2006; Yu et al., 2004).

HIV-1 has evolved a Vif protein to counter incorporation of select A3 proteins during virus maturation by interacting with and shunting these proteins to the proteasome via the Cul5/ElonginB/C/robX3 ubiquitin ligase (Liu et al., 2004; Marin et al., 2003; Mehel et al., 2004; Sheehy et al., 2003; Yu et al., 2003). Of the A3 proteins, the double deaminase domain proteins (A3B, A3D, A3F, and A3G) are most often associated with the restriction of HIVΔvif (Dang et al., 2007; Doehle et al., 2005; Sheehy et al., 2002; Wiegand et al., 2004). Human A3C, a single deaminase domain protein, is incorporated into both HIV-1 and HIV-1Δvif and is capable of inducing G-to-A mutations in the viral genome, although not to the same extent as A3G (Bourara et al., 2007; Kitamura et al., 2010; Langlois et al., 2005; Smith et al., 2010; Wang et al., 2008). Investigators have found that allelic differences of hA3H accounts for the ability of select hA3H proteins to potently inhibit HIV-1Δvif (Harari et al., 2009; Li et al., 2010; OhAinle et al., 2008; Ooms et al., 2010; Wang et al., 2011).

Virus restriction experiments on hA3A performed in epithelial cell lines such as HeLa or 293 cells indicate that hA3A does not inhibit HIV-1Δvif (Aguilar et al., 2008; Bishop et al., 2004; Golla-Gaur et al., 2007). However, hA3A can restrict parvoviruses and LINE-1 elements (Babushok et al., 2007; Bogerd et al., 2005a, 2006b; Brouha et al., 2003; Muckenfuss et al., 2006). Other studies showed that hA3A can inhibit the replication of adeno-associated virus type 2 (AAV-2) through a deaminase-independent mechanism, can activate the DNA damage response, cause preferential degradation of plasmid DNA, and inhibit human T-cell leukemia virus type 1 (HTLV-I) (Chen et al., 2006; Landry et al., 2011; Narvaiza et al., 2009; Ooms et al., 2012; Stenglein et al., 2010).
Recently, we showed that rhesus macaques also express an A3A protein (rhA3A) that restricted vif-deleted SHIV and to a lesser extent HIV-1Δvif (Schmitt et al., 2011). We showed that a three amino acid deletion in hA3A was partially responsible for the lack of restriction. Currently, we do not know whether the differential ability of rhA3A and hA3A to restrict lentiviruses is a conserved property of the corresponding Old World monkey (OWM) and hominid A3A lineages, respectively. Additionally, no information is available on whether A3A from New World monkeys (NWM), which are not known have endemic lentiviruses, are capable of restricting HIV-1 or SIV/SHIV. In this study, we further define the amino acid deletions/substitutions necessary to restore the virus restriction activity of hA3A. We show that the A3A from one NWM is capable of restricting SHIV. Finally, we present data that one OWM A3A protein from Colobus guereza can inhibit lentiviral replication by novel mechanism.

Results

The location of the HA-tag does not effect subcellular location

We determined if fusion of the HA-tag to the N-terminus or C-terminus of rhA3A affected the nucleocytoplasmic localization of the protein and restriction of SHIV. 293 cells were transfected with vectors expressing HA-rhA3G, HA-hA3A, hA3A-HA, HA-rhA3A, rhA3A-HA, and rhA3A-3XHA. At 48 h, cells were starved, radiolabeled for one hour and used in immunoprecipitation assays using an antibody against the HA-tag. As shown in Fig. 1, all A3 proteins were expressed as doublets, which was previously shown to be due the initiation of translation at an alternative methionine at position 12 (Theilen et al., 2010). Interestingly, placement of the HA-tag at the amino terminus of rhA3A (HA-rhA3A) resulted in a slight shift in the mobility compared to the rhA3A-HA. Previous studies have shown that hA3G is localized in the cytoplasm while rhA3G is found in both the cytoplasm and nucleus (Bogerd et al., 2006a, 2006b; Chen et al., 2006; Goila-Gaur et al., 2007; Muckenfuss et al., 2006; Schmitt et al., 2011). We examined the intracellular localization of the HA-tagged A3A proteins using immunofluorescence and confocal microscopy. We found that HA-rhA3G was observed exclusively in the cytoplasm as previously reported (Fig. 2) while HA-rhA3A, rhA3A-HA, and rhA3A-3XHA were detected in both the cytoplasm and nucleus as it co-localized with a eGFP-tagged nuclear marker (Fig. 2). Thus, any differences in the retroviral restriction properties of rhA3A proteins could not be explained by the changes in steady-state levels of the proteins or subcellular localization.

Virus restriction of the HA-tagged rhesus and human A3A proteins

We next determined if fusion of the HA-tag to the N- or C-terminus of rhA3A affected the restriction of SHIV. For virus restriction assays, 293 cells were transfected with vectors containing the SHIVΔvif or SHIVΔvifΔvpr genomes and vectors expressing either HA-hA3A, hA3A-HA, RH-hA3A, rhA3A-HA, rhA3A-3XHA, untagged rhA3A or vector alone. At 48 h the culture supernatants were collected and the infectious titers of the released viruses determined. As shown in Fig. 3, fusion of the HA-tag to the N-terminus of rhA3A resulted in approximately a 20-fold reduction in virus infectivity, which was similar to the results with the untagged rhA3A (Fig. 3). Fusion of a single HA-tag or a 3X HA-tag to the C-terminus of rhA3A resulted in a reduction of virus infectivity by 2–5-fold and 1–2-fold, respectively. All experiments were performed in triplicate and significance in the restriction of infectious SHIVΔvifΔvpr or SHIVΔvif was calculated with respect to the empty vector control using a Student’s two-tailed t-test (*). The reduction in infectivity was found to be significant for the HA-rhA3A and untagged rhA3A but not significant for the rhA3A-HA or rhA3A-3XHA. From these results, we conclude that HA-rhA3A more closely reflects the native rhA3A with respect to restriction of lentiviruses.

The N-terminal half of the rhA3A contains the necessary determinants for virus restriction

We previously showed that a 3 amino-acid indel (27SVN29 in rhA3A) is important for differential lentivirus restriction between rhA3A and hA3A (Schmitt et al., 2011). To determine the domains of rhA3A that could completely restore hA3A lentivirus restriction, we constructed a series of chimeric rhesus/human A3A proteins and evaluated their restriction properties against SHIVΔvif and HIV-1Δvif (Fig. 4). We analyzed the stability of these proteins by transfection of 293 cells with vectors expressing each chimeric protein or HA-rhA3A. At 48 h, transfected cells were radiolabeled for one hour and the HA-tagged A3 proteins immunoprecipitated with an anti-HA antibody as described in Fig. 1. Our results indicate that these chimeric proteins were readily expressed in 293 cells (Fig. 5). However, these chimeric proteins had divergent activities on SHIV infectivity with the HA-rh1−50hA3A efficiently restricting infectivity of SHIVΔvif and HIV-1Δvif while the HA-h1−50rhA3A did not restrict SHIVΔvif or HIVΔvif infectivity (Fig. 6A and B). From these results we conclude that N-terminal half of the rhA3A had the necessary amino acid substitutions for restriction of SHIVΔvif and HIV-1Δvif. We next analyzed chimeric proteins in which amino acids 1–33, 1–50 and 16–50 amino acids of hA3A were replaced with the analogous region from rhA3A (rh1−33hA3A, rh1−50hA3A, and rh16−50hA3A, respectively). When analyzed in restriction assays, rh1−33hA3A, rh1−50hA3A, and rh16−50hA3A all restricted the replication of SHIVΔvif and HIV-1Δvif (Fig. 6A and B). We further narrowed the amino acid changes required with another chimeric protein in which amino acids 25–33 of rhA3A were substituted into the same region of hA3A (rh25−33hA3A). Our analysis revealed that the rh25−33hA3A had a similar virus restriction profile to rhA3A (Fig. 6A and B). From these studies we conclude that the six amino acid substitutions in this region of rhA3A were sufficient to completely restore hA3A restriction activity against SHIVΔvif and HIV-1Δvif.

We also determined if rh25−33hA3A was incorporated in viral nucleocapsids. Previous studies have shown that hA3G but not hA3A was incorporated into viral nucleocapsids (Aguiar et al., 2008; Goila-Gaur et al., 2007; Schmitt et al., 2011). For these experiments, 293 cells were co-transfected with plasmids with either the HIV-1 or HIV-1Δvif genome and plasmids that expressed the Materials and methods section.
either HA-hA3G, HA-hA3A, or HA-rh25−33hA3A. At 48 h, virus-containing supernatants were collected and concentrated by pelleting through 20% sucrose. Concentrated viruses were resuspended in 1 ml of PBS and 50% of each were loaded onto a 20–60% sucrose step gradient and subjected to centrifugation in the presence of either 1% Triton-X-100 or PBS. Four fractions were collected with fraction S1 containing the soluble proteins at the top of the gradient, fraction S2 a buffer of 20% sucrose separating the soluble proteins from the viral particle and viral nucleocapsids, fraction S3 the interface of the 20%:60% fractions containing the viral particles and viral nucleocapsids, and fraction S4 at the bottom of the 60% fraction. The Env protein was predominantly in the S3 fraction in the absence of Triton-X-100 but was predominantly detected in S1 fraction in the presence of Triton-X-100 while the viral capsid protein was predominantly detected in S1 fraction in the presence of Triton-X-100 (Fig. 6C–E). Notably, as a structural model for hA3A is not yet available. We therefore constructed structural models of rhA3A and hA3A based on hA3G-CD2 (Holden et al., 2008) using the interactive SWISS-MODEL program (Fig. 7B and C). As shown in Fig. 7A and B, the 25DLSRGRHQ residues (Fig. 6D and E).

### Differential lentivirus restriction by rhA3A and hA3A maps exclusively to the AC-Loop1 domain

Molecular insights on the differential lentivirus restriction properties of rhA3A and hA3A may be gained through structural models. In a previous study, a structural model of hA3A based on the related C-terminal half of hA3G or hA3G-CD2 (Holden et al., 2008) was constructed (Bulliard et al., 2011). This study identified 11 residues critical for L1 retrotransposition, which were proposed to form the polynucleotide-accommodating groove of A3A (Bulliard et al., 2011). These 11 residues are highlighted in gray in the protein alignment between hA3A and rhA3A (Fig. 7A). Notably, a structural model of rhA3A is not yet available. We therefore constructed structural models of rhA3A and hA3A based on hA3G-CD2 (Holden et al., 2008) using the interactive SWISS-MODEL program (Fig. 7B and C). As shown in Fig. 7A and B, the 25DLSRGRHQ residues in rhA3A are predicted to completely reside within the Active Center-Loop1 (AC-Loop1) region of rhA3A. A space-filling model suggests that
the rhA3A amino acids 25–33 are located in a solvent-accessible area that is adjacent, but not within the proposed polynucleotide accommodating groove (Fig. 7C). Notably, the 27SVR29 insertion in rhA3A results in a substantial increase in

![Graph showing percentage virus infectivity compared to SHIV empty vector control.](image)

Fig. 3. The site of the HA-tag effects restriction of SHIVΔvif virion infectivity by rhA3A. 293 cells were transfected with 0.5 μg of vectors expressing HA-rhA3A, rhA3A-HA, rhA3A-3xHA, HA-rhA3G or empty vector and 1 μg of wild-type SHIV or SHIVΔvif molecular clones. At 48 h, the culture medium was harvested and virion infectivity measured by taking the ratio of infectious viral titers on TZM-bl cells. The experiments were performed at least four times and statistical differences with the untagged rhA3A control were evaluated using a two-tailed Student's t-test, with p < 0.05 (▲) considered significant. Shown is the mean percentage virion infectivity with the empty plasmid control normalized to 100%.

![Sequence of the chimeric rhA3A/hA3A proteins analyzed in this study. The sequences shown are based on the hA3A sequence. The differences in rhA3A are indicated below the hA3A sequence and the identity at positions designated with a dash.](image)

Fig. 4. Sequence of the chimeric rhA3A/hA3A proteins analyzed in this study. The sequences shown are based on the hA3A sequence. The differences in rhA3A are indicated below the hA3A sequence and the identity at positions designated with a dash.
the rhA3A AC-Loop1 molecular surface compared to hA3A (Fig. 7C). Thus, the AC-Loop1 region of A3A accounts for the differential lentivirus restriction properties of rhA3A and hA3A.

Comparison of lentivirus restriction by hominid, Old World monkey and New World monkey A3A proteins

It remains unclear whether the differential lentivirus restriction properties of rhA3A and hA3A are conserved in their respective OWM and hominid lineages, respectively. In addition, the lentivirus restriction properties of NWM A3As is unknown. We therefore evaluated the SHIV/SHIVΔvif and HIV-1/HIV-1Δvif restriction properties of A3As from additional OWM species, Chlorocebus aethiops (grivet monkey; agmA3A) and Colobus guereza (mantled guereza; colA3A), a lesser ape, Hylobates lar (common gibbon; gibA3A), and NWM species, Saimiri sciureus (squirrel monkey; sqmA3A) and Aotus trivirgatus (Northern owl monkey; nomA3A).

Alignment of the protein sequences from these different primate A3As revealed that the 3 amino acid AC-Loop1 indel in hA3A versus rhA3A was conserved in gibA3A and agmA3A/colA3A, respectively (Fig. 8A). Similar to the OWM A3As, the two NWM A3As have a 3 amino acid AC-Loop1 insertion, as well as a proline insertion at position 45 and a deletion of two amino acids at positions 108–109 within the deaminase domain motif (Fig. 8A).

As expected, phylogenetic analyses show that the A3A amino acid sequences clustered accordingly with their OWM, hominid and NWM lineages (Fig. 8B). Expression constructs were prepared and analyzed for expression in 293 cells to examine the stability of
these A3A proteins. Our results indicate that these proteins were expressed at comparable levels and had a similar Mr on SDS-PAGE to rhA3A (Fig. 9). We next analyzed the ability of these A3A proteins to restrict the replication of SHIVKUΔvif, SHIVΔvif, HIV-1, and HIV-1Δvif (Fig. 10A and B). For the NWM A3A proteins, we found that sqmA3A restricted the replication of SHIVΔvif but neither sqmA3A nor nomA3A restricted HIV-1 and HIV-1Δvif. Thus, the case for evolutionary conservation of lentivirus restriction for NWM A3As is inconclusive. Similar to hA3A, the gibbon A3A (gibA3A) protein did not restrict HIV-1Δvif but in contrast to hA3A, gibA3A had no significant activity SHIVΔvif (Fig. 10A). Within the OWM lineage, the agmA3A restricted SHIVΔvif and HIV-1Δvif but not parental SHIVKUΔvif or HIV-1. Interestingly, the colobus A3A protein (colA3A) potently restricted SHIVKUΔvif, HIV-1 and HIV-1Δvif (Fig. 10A and B). Thus, three OWM A3As exhibited greater lentivirus restriction of SHIVΔvif compared to the 2 hominin A3As, suggesting that the differential restriction of lentiviruses by A3A proteins are conserved within the OWM and hominid lineages.

A novel mechanism for lentivirus restriction by colA3A

The potent restriction of wild type and Δvif viruses may be the inability of SIV and HIV-1 Vif proteins to interact with the colA3A protein leading to its incorporation and its subsequent effect on infectivity. However, when we attempted to analyze virus for the presence of colA3A, we were unable to detect virus in our preparations. We also examined the intracellular localization of the colA3A. We found that similar to rhA3A and hA3A, colA3A was localized in both the cytoplasm and nucleus (data not shown). We next determined if there was a defect in virus release by transfection of 293 cells with vectors expressing hA3G, rhA3A, hA3A, agmA3A, gibA3A, nomA3A, sqmA3A, or colA3A and either the SHIVKUΔvif, HIV-1 or HIV-1Δvif genomes. At 48 h, the

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Fig. 7. Structural models of human versus rhesus macaque A3A. (A) Amino acid alignment of hA3A and rhA3A. Residues highlighted in red correspond to differences between hA3A and rhA3A. Note that there is a 3 amino-acid indel (dashes) in AC-Loop1. Catalytic site residues are highlighted in cyan, while residues in the polynucleotide groove (Bulliard et al., 2011) were highlighted in gray. Predicted α-helices (cylinders) and β-sheets (arrows) from the homology models are shown. (B) Homology models for hA3A and rhA3A based on the crystal structure of hA3G-CD2 (Holden et al., 2008). Magenta spheres indicate the position of the Zinc atom. AC-Loop1 is indicated in green. (C) Space-filling models. Residues shaded in gray correspond to 11 residues predicted to be in the A3A groove. Residues in green correspond to AC-Loop1, with amino acids that vary between rhA3A and hA3A in AC-Loop1 highlighted in red.
Fig. 8. The sequence and phylogenetic relationships of the seven A3A proteins analyzed in these studies. Panel A. The amino acid sequence of the human, lar gibbon, rhesus macaque, African green monkey, Colobus monkey, squirrel monkey, and northern owl monkey A3A. Panel B. Nearest neighbor joining tree of seven A3A proteins analyzed in this study. Bootstrap values were obtained from 1000 subreplicates. Species highlighted in black correspond to A3A proteins analyzed in the study.
culture supernatants were collected and cell lysates prepared to assess the levels of p27 or p24 expression. The results from five separate experiments indicate that the rhA3G, hA3G, rhA3A, hA3A, agmA3A, gibaA3A, sqmA3A, and nomA3A did not significantly restrict the release of p27 or p24 into the culture medium of cells transfected in the presence of either SHIVKU2–MC4 or HIV-1 (Fig. 11A and B, E and F). However, co-transfection of the vector expressing colA3A and SHIVKU2–MC4 or SHIVΔvif resulted in < 0.1% of the p27 protein (SHIVKU2–MC4: range of 0 to 1.2 ng/ml; SHIV-Δvif: mean of 0.18 ng/ml, range of 0.04–0.42 ng/ml) being released into the culture medium compared to the other 6 A3A proteins tested (SHIVKU2–MC4: mean of 158 ng/ml, range of 79–266 ng/ml; SHIVΔvif: mean of 123 ng/ml, range of 86–195 ng/ml) (Fig. 11A and B). Similar results were obtained when 293 cells were co-transfected with the vector expressing colA3A and either HIV-1 or HIVΔvif (Fig. 11E–H). In the presence of colA3A, HIV-1 and HIVΔvif released < 0.1% p24 (HIV-1: mean of 0.58 ng/ml, range of 0.21 to 0.91 ng/ml; HIV-1Δvif: mean of 0.48 ng/ml, range of 0.18–0.60 ng/ml) compared to the other A3A proteins (HIV-1: mean of 1110 ng/ml, range of 700–1272 ng/ml; HIV-1Δvif: mean of 2781 ng/ml; range of 1112–4985 ng/ml) (Fig. 11E and F). When the cell lysates from the same co-transfections were examined for p27 or p24 expression, very little capsid protein was detected compared to the other A3A proteins analyzed (Fig. 11C and D, G and H). Taken together, there was approximately a 1000-fold reduction in the amount of SIV p27 or HIV-1 p24 protein synthesized and released from cells co-transfected with colA3A.

We next determined if colA3A was inhibiting an early step of replication. 293 cells were first transfected with the viral genome followed by transfection of colA3A 24 h later. The culture medium and cell lysates were assessed for the presence of p24 antigen at 72 h following transfection of the viral genome. The results indicate that colA3A still restricted the replication of HIV-1 (Fig. 12). Similar findings were obtained for SHIVKU2–MC4 (data not shown). To rule out that these preparations might have a cryptic DNase activity, plasmids with the colA3A and each of the four viral genomes were incubated in the transfection solution for 4 h at 37 °C. The plasmids were then run on agarose gels to determine if there was any significant DNA degradation. We detected neither the degradation the vector expressing colA3A nor the viral genomes (data not shown). We also determined if somehow the viral genomes were not being transfected into cells. Cells were transfected with plasmids with the HIV-1 or SHIVKU2–MC4 genomes and colA3A. At 12 h post-transfection, DNA was isolated from cells and analyzed for presence of the viral genome. Our PCR results also indicated the presence of the two viral genomes (data not shown).

Colobus A3A does not restrict foreign plasmid DNA

A previous study provided data that hA3A was capable of restricting foreign plasmid DNA (Stenglein et al., 2010). We determined if colA3A was capable of restricting foreign DNA in cells. 293 cells were transfected with vectors expressing hA3A, rhA3A, or colA3A followed by transfection with the pcEGFP (expressing the enhanced green fluorescent protein, Clontech; expressed in pcDNA3.1(±)) or empty vector pcDNA3.1(±) 24 h later. At 24 h, cells were harvested and analyzed for the number of EGF positive cells by flow cytometry. Our results indicate that at 48 h hA3A caused a decrease in the number of EGF positive cells while rhA3A and colA3A did not cause a statistically significant reduction in the number of EGF positive cells compared to cells co-transfected with pcDNA3.1(±) and EGF or cells

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**Fig. 9.** The expression of the A3A proteins from the gibbon, Old World and New World monkeys. 293 cells were transfected with vectors expressing either HA-rhA3A, HA-hA3A, HA-gibaA3A, HA-agmA3A, HA-sqmA3A, or HA-nomA3A. At 48 h, cells were starved for methionine/cysteine and then labeled for 1 h with 35S-methionine/cysteine. The radiolabel was then removed washed, and incubated in cold excess methionine/cysteine for 6 h. The cells were lysed in 1X RIPA buffer, the lysates spun in a microfuge to remove the nuclei and the HA-tagged proteins immunoprecipitated overnight using an antibody against the HA-tag and Protein-A Sepharose beads. The beads were collected and washed three times with RIPA buffer and boiled in 2X sample reducing buffer. The proteins were separated using SDS-PAGE, and autoradiography used to visualize the proteins.

**Fig. 10.** The A3A proteins from other Old World and New World monkeys also restrict SHIVΔvif and HIV-1Δvif. 293 cells were transfected with vectors (0.5 μg) expressing either rhA3A, hA3A, rhA3G, gibaA3A, sqmA3A, omaA3A, agmA3A, or colA3A proteins and 1 μg wild-type SHIVKU2–MC4, SHIVΔvif, HIV-1 or HIV-1Δvif molecular clones. At 48 h, the culture medium was harvested and virion infectivity measured by taking the ratio of infectious viral titers on TZM-bl cells. The experiments were performed at least four times. Shown is the mean percentage virion infectivity with the empty plasmid control normalized to 100%. In all panels, error bars correspond to standard deviations from triplicate determinations and statistical differences with the wild-type control were evaluated using a two-tailed Student’s t-test, with p < 0.05 (∗) considered significant. Panel A. Infectivity of SHIVKU2–MC4 and SHIVΔvif in the presence of NWM, OWM, and hominid A3A proteins. Panel B. Infectivity of HIV-1 and HIV-1Δvif in the presence of NWM, OWM, and hominid A3A proteins.
Fig. 11. The colA3A inhibits SHIV and HIV-1 replication by a novel mechanism. Panels A–D. The colA3A restricts the synthesis and release of viral p27. 293 cells were transfected with vectors expressing either rhA3G, hA3A, rhA3A, gibA3A, agmA3A, colA3A, nomA3A, or sqmA3A and either SHIVKU−2MC4 and SHIVΔvif. At 48 h, the culture medium was collected and lysates prepared from cells. The levels of p27 in the medium (Panels A–B) and cell lysates (Panels C–D) were determined using commercial p27 kits. In all panels, error bars correspond to standard deviations from triplicate determinations and statistical differences with the wild-type control were evaluated using a two-tailed Student’s t-test, with p < 0.05 (▲) considered significant. Panels E–H. The colA3A restricts the synthesis and release of viral p24. 293 cells were transfected with vectors expressing either rhA3G, hA3A, rhA3A, gibA3A, agmA3A, colA3A, nomA3A, or sqmA3A and either HIV-1, or HIV-1Δvif genomes. At 48 h, the culture medium was collected and lysates prepared from cells. The levels of p27 in the medium (Panels E–F) and cell lysates (Panels G–H) were determined using commercial p24 kits. In all panels, error bars correspond to standard deviations from triplicate determinations and statistical differences with the wild-type control were evaluated using a two-tailed Student’s t-test, with p < 0.05 (▲) considered significant.
transfected with EGFP alone (Fig. 13). These results indicate that colA3A did not restrict foreign DNA (i.e., pcEGFP).

Discussion

The human APOBEC3 genes are widely accepted as potent antiretroviral genes, with the notable exception of human A3A (hA3A). Human A3A lacked activity against HIV-1Δvif but instead restricted LINE-1 retrotransposons and DNA viruses, suggesting that the hA3A may have diverged from its ancient antiretroviral function. To better understand this evolutionary process, we sought to document the lentivirus restriction properties of additional primate A3As.

In a previous report, we showed that the rhesus macaque A3A (rhA3A) potently restricted the replication of Δvif SHIV and HIV-1

Fig. 12. The colA3A restricts HIV-1 replication if transfected into cells 24 h after the viral genome. 293 cells were transfected with plasmids with either the HIV-1 or HIVΔvif genomes. At 24 h, cells were washed and transfected with vectors expressing either hA3G, rhA3A, colA3A or pcDNA3.1(+). At 72 h post-transfection, the culture medium was collected and the cell lysates prepared. The levels of p24 in the medium (Panel A) and cell lysates (Panel B) were assessed using commercially available p24 antigen capture kits. Infectious virus in the culture medium was determined using TZM-bl cell assays.
to a greater extent than hA3A (Schmitt et al., 2011). At first glance, our results appear to be discordant from other investigators who found no activity with rhA3A (Hultquist et al., 2011). However, there are important differences in the constructs used in the two studies. In our study, we fused the HA-tag to N-terminus of rhA3A while in the other study the investigators fused a 3X-HA tag to the C-terminus rhA3A. Based on our results presented here, it appears that the site of the HA-tag does influence the virus restriction properties of rhA3A, which may explain the discordant results from the two studies.

The differential restriction properties of hA3A and rhA3A against lentiviruses was striking, given that these proteins were 81% identical to each other. We therefore performed detailed chimeric studies and found that the addition of six amino acid substitutions (from 27SVR29 to 25DLSVR29 and K33Q) into hA3A increased the HIV-1Δvif restriction activity of hA3A to the same level as wild-type rhA3A restricted SHIVΔvif. Interestingly, structural models revealed that the rhA3A amino acids 25 to 33 map exclusively onto AC-Loop1, resulting in greater AC-Loop1 molecular surface in rhA3A compared to hA3A. Due to its predicted solvent-accessibility, this additional molecular surface may be an important region for protein–protein interactions. Thus, one possibility for the enhanced restriction activity of rhA3A may be due to the existence of additional protein–protein interactions that normally do not occur with hA3A. Recently, Vif was shown to recruit the transcription factor CBF-β to the ubiquitin ligase complex to enhance the degradation of hA3G but not hA3A (Jäger et al., 2011; Zhang et al., 2011). It will be of interest to determine if rh25–33hA3A is capable of interacting with CBF-β.

Since rhA3A can restrict SHIV/SIV to a greater extent than hA3A, it raised the question of whether A3A from other OWM and hominids behave similarly. We therefore characterized the antilentiviral properties of A3A from two additional OWM (agmA3A and colA3A) and one hominid (gibA3A). The results revealed that the OWM A3A proteins restricted SHIV Δvif and HIV-1 Δvif to a greater extent than hominid A3As. These data provide evidence for evolutionary conservation of hominid (lack) and OWM (potent) A3A lentivirus restriction. The reason for the loss of lentivirus restriction properties of the hominid A3A proteins remains open to speculation. One possibility is that the lack of lentivirus infection in hominids resulted in the neofunctionalization of A3A to restrict DNA viruses and/or L1 retrotransposons. While SIV is endemic in chimpanzees and gorillas in the wild, these SIV strains do not uniformly infect chimpanzees and gorillas across Africa (Santiago et al., 2002; van Heuverswyn et al., 2006). In fact, SIVcpz likely emerged more recently in these species following cross-species transmission and recombination of A strains from OWM (Bailes et al., 2003).

Another salient result was that the A3A protein from a colobus monkey restricted not only SHIVΔvif and HIVΔvif but also the parental viruses expressing intact Vif proteins. This result could be due to the inability of Vif proteins of SIV (more specifically, SIVmac239) and HIV-1 to counteract colA3A. While a sequence for SIVcol exists, neither an infectious virus nor an infectious molecular clone has been described. Furthermore, the SIVcol Vif protein sequence shares little identity to other lentiviruses (Courgnaud et al., 2001). It will be of interest to determine if the SIVcol Vif could antagonize colA3A although a recent study showed that SIVcol Vif was not expressed and hence is probably very unstable (Compton and Emerman, 2013). Surprisingly, analysis of cell lysates and culture supernatants from cells co-transfected with colA3A and SHIV/HIV revealed substantial inhibition of virion particle release. When we performed a temporal transfection in which the viral genome was transfected first into 293 cells and 24 h later transfected with the vector expressing colA3A, viral protein biosynthesis (p24) and release was severely reduced. These results suggest that colA3A may restrict by a unique post-entry mechanism in the producer cell. Based on the disruption of
p24 biosynthesis, we believe virus assembly is unlikely to be the target of the colA3A. Additionally, our preliminary studies suggest that restriction is likely through a deaminase-independent mechanism (data not shown). While beyond the scope of this study, colA3A may: (a) prevent integration of the viral genome; (b) interact with Tat or Rev which could decrease the synthesis of transcripts and/or export of unspliced and singly spliced to the cytoplasm; or (c) could interact with the TAR or RRE to decrease Tat and/or Rev functions.

The unique properties of colA3A contrasted from two other OWM A3As (agmA3A and rhA3A). Both agmA3A and rhA3A were incorporated into Vif-deficient lentivirus particles, and did not possess a post-entry restriction property. Interestingly, colA3A belongs to a subclade of OWM A3As that include mandrills and De Brazza monkeys. Further analysis of the A3A proteins from these monkey species, as well as other members of the Colobus or Pilocolobus genus should help determine if the post-entry restriction function of A3A is a unique property of this OWM subclade.

In this study, we also evaluated the lentivirus restriction properties of A3A from NW3, and found that 1 of 2 NW3 A3As (sqmA3A but not nomA3A) restricted SHIVΔvif. This suggested that the lentivirus restriction property of NW3 A3As may not be conserved. It is generally accepted that the NW3 (Intraorder Platyrhini) branched from the ancestors of the OWM and great apes (Intraorder Catarrhini) approximately 35 million years ago (Schrago and Russo, 2003; Schrago et al., 2007). To date, lentiviruses have not been isolated from the NW3, suggesting there was no selective pressure for the A3A to evolve restriction activity against lentiviruses. This suggests that the function of NW3 A3A may have been to restrict other retroviruses or other families of viruses. One potential candidate would be the human T-lymphotropic viruses, which are known to be sensitive to A3A restriction in the laboratory as previously described (Schmitt et al., 2010; 2011). A plasmid expressing HA-rhA3A and HA-hA3A was constructed in the laboratory as previously described (Schmitt et al., 2011; referred to in text as HIV-1) was used to construct a Δvif version (Schmitt et al., 2011; referred to in text as HIV-1Δvif) (pNL4-3; NIH AIDS Research and Reference Reagent Program). This plasmid was digested with PflM1, phenol: chloroform extracted, and ethanol precipitated. The 5′-protruding ends were filled using DNA Polymerase I Large Fragment (Klenow; Promega). The resulting Klenow fragment reaction produced blunt ended fragments that were purified and re-ligated. The resulting plasmid was sequenced and found to contain a deletion in pNL4-3 vif between base pairs 5301 to 5308. Therefore, only the first 79 amino acids of pNL4-3 Vif could be expressed. Plasmid pcDNA3.1(+)-HA-rh3A3G was kindly provided by Nathaniel Landau (New York University School of Medicine). Plasmids expressing HA-rhA3A and HA-hA3A were constructed in the laboratory as previously described (Schmitt et al., 2010; 2011). A plasmid expressing pcDNA3.1(+)-HA-hA3G used in these studies was provided by the NIH AIDS Reference and Reagent Program. The genes for rhA3A-HA, rhA3A-3XHA, and the A3A genes from the African green monkey (HA-aghA3A), C. guereza (HA-colA3A), Northern owl monkey (HA-nomA3A), squirrel monkey (HA-sqmA3A) and the Lars gibbon (gibA3A) were synthesized by Genscript, subcloned into pcDNA3.1(+) vector as previously done for rhA3A and haA3A and sequenced to ensure that the sequences were correct.

**Chimeric rh/hA3A proteins, Old and New World monkey A3A proteins and detection**

The chimeric rhesus/human A3A genes used in this study were synthesized by Genscript. Upon receipt, the sequence of the genes was verified followed by subcloning into pcDNA3.1(+) using standard cloning procedures. The chimeric proteins included (a) rh500tha3A, rh500ha3A, and rh500ha3A, in which the N-terminal 100, 50, 33 amino acids of ha3A were exchanged for the same amino acids from rhA3A; (b) rh150-ha3A and rh243-ha3A, in which amino acids 1–50 and 24–33 of ha3A were exchanged for the same amino acids from rhA3A; and (c) rh493-ha3A, in which the N-terminal 100 amino acids of rhA3A were exchanged for the same amino acids from ha3A. The additional homid, Old World monkey, and New World monkey A3A proteins were synthesized based on the sequences from Genbank and subcloned into the pcDNA3.1(+) vectors as we did for chimeric rh/hA3A proteins.

To assess the stability of these clones in the absence of virus, 293 cells were transfected using PEI transfection reagent (Ex-Gen 500; Fermentas) into a 6–well plate. At 48 h post-transfection, the cells were starved for methionine/cysteine for 2 h, and radiolabeled with 300 μCi/ml 35S-Translabel (methionine and cysteine, Perkin-Elmer, Waltham, MA). Cell lysates were prepared by lysing radiolabeled cells in radioimmunoprecipitation buffer (RIPA: 50 mM Tris-HCl, pH 7.5; 50 mM NaCl; 0.5% deoxycholate; 0.2% SDS; 10 mM EDTA) on ice, and the nuclei removed by centrifugation in a microcentrifuge. The proteins were immunoprecipitated using an anti-HA polyclonal antibody (sc-805; Santa Cruz) and collected on protein-A Sepharose. The samples resuspended in 2X sample reducing buffer, boiled, and the HA-containing A3 proteins analyzed by SDS-PAGE. Proteins were visualized by standard autoradiographic techniques.

**Materials and methods**

**Cells, viruses, and plasmids**

293 cells were used for transfections of vectors expressing various APOBEC3 proteins, full-length SHIV or HIV-1 (NL4-3). The TZM-bl cell line was used as an indicator cell line to measure the infectivity of viruses (Derdeyn et al., 2000; Wei et al., 2002). Both cell lines were maintained in Dulbecco’s minimal essential medium (DMEM) with 10% fetal bovine serum (R10FBS), 10 mM Hepes buffer, pH 7.3, and 100 U/ml penicillin and 100 μg/ml streptomycin and 5 μg gentamicin. The derivation of SHIVVsvΔvif (a pathogenic molecular clone; referred to in the text as SHIV) and SHIVΔvif have been previously described (Liu et al., 1999; Schmitt et al., 2010; 2011). A plasmid with the genome of HIV-1 strain NL4-3 (referred to in the text as HIV-1) was used to construct a Δvif version (Schmitt et al., 2011; referred to in text as HIV-1Δvif) (pNL4-3; NIH AIDS Research and Reference Reagent Program). This plasmid was digested with PflM1, phenol: chloroform extracted, and ethanol precipitated. The 5′-protruding ends were filled using DNA Polymerase I Large Fragment (Klenow; Promega). The resulting Klenow fragment reaction produced blunt ended fragments that were purified and re-ligated. The resulting plasmid was sequenced and found to contain a deletion in pNL4-3 vif between base pairs 5301 to 5308. Therefore, only the first 79 amino acids of pNL4-3 Vif could be expressed. Plasmid pcDNA3.1(+)-HA-rh3A3G was kindly provided by Nathaniel Landau (New York University School of Medicine). Plasmids expressing HA-rhA3A and HA-hA3A were constructed in the laboratory as previously described (Schmitt et al., 2010; 2011). A plasmid expressing pcDNA3.1(+)-HA-hA3G used in these studies was provided by the NIH AIDS Reference and Reagent Program. The genes for rhA3A-HA, rhA3A-3XHA, and the A3A genes from the African green monkey (HA-aghA3A), C. guereza (HA-colA3A), Northern owl monkey (HA-nomA3A), squirrel monkey (HA-sqmA3A) and the Lars gibbon (gibA3A) were synthesized by Genscript, subcloned into pcDNA3.1(+) vector as previously done for rhA3A and haA3A and sequenced to ensure that the sequences were correct.

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Subcellular localization of A3 proteins

293 cells were plated onto 13 mm cover slips in a 6 well plate and transiently co-transfected with vectors expressing HA-rhA3A, rhA3A-HA, rhA3A-3xHA, or HA-rhA3G, and a vector expressing a eGFP tagged nuclear localization marker using TurboFect (MBI Fermentas) according to the manufacturer's instructions. The cultures were maintained for 48 h and then fixed in 2% paraformaldehyde for 10 min, permeabilized in 0.1% Triton X-100, and washed in twice in 1X PBS (pH 7.4) for 5 min. Cover slips were then incubated with a rabbit polyclonal anti-HA antibody (HA-probe, Santa Cruz) for 1 h at ambient temperature in 1X PBS plus 1% BSA. Cover slips were washed twice with 1X PBS for 5 min and incubated with a Cy-5 conjugated secondary antibody (Abcam) in 1X PBS +1% BSA for 30 min. The cover slips were washed twice in 1X PBS for 5 min and mounted in a glycerol containing mounting media (Component A: antifade reagent in glycerol/PBS, Invitrogen). A Nikon A1 confocal microscope was used to collect 100 x images with a 2 x digital zoom, using EZ-C1 software. The pinhole was set to medium for all wavelengths used. Cy5 and eGFP were excited using 638 and 488 nm diode lasers, respectively, and the images were collected using a 670 nm filter for Cy5 and a 525/25 nm filter for eGFP.

Inhibition of SHIV and HIV-1 infectivity by A3A

SHIV, SHIVΔvif, HIV-1 or HIVΔvif infectious molecular clones (1 µg) were co-transfected (0.5 µg) with plasmids expressing rhA3A, hA3A, rhA3G, hA3G, the various rhesus/human chimeric protein or the various OWM, NWm and hominoid A3A proteins using Turbobect (Fermentas) in a 12-well plate. All plasmids (viral and A3) used in the assays are tested for the ability to transfect and express viral or A3 proteins before it is used in transfection studies. At 48 h post-transfection, the culture medium was harvested and clarified by low speed centrifugation. Equivalent amounts of p27 were serially diluted using 10-fold dilutions from 10^1 to 10^6 and used to inoculate TZM-bl cells. At 48 h post-inoculation, the media was removed, cells washed with PBS and the monolayer fixed using 1% formaldehyde-0.2% glutaraldehyde in PBS. The cells were washed and incubated in a solution for 2 h at 37 °C containing 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 4 mM magnesium chloride and 0.4 mg/ml of 1% formaldehyde for 10 min. The reaction was stopped and the infectious units (IU) per ml were calculated (Dereyns et al., 2000; Wei et al., 2002).

Production of HIV-1Δvif and SHIVΔvif virions in the presence of A3 proteins

293 cells were seeded onto a 12-well tissue culture plate and transfected with 1 µg of HIV-1, HIV-1Δvif, SHIV, or SHIVΔvif genomes and 0.5 µg of HA-tagged rhA3A, hA3A, or the other New World and Old World monkey A3A proteins. After 48 h, supernatants were collected and the cellular debris was removed by low speed centrifugation. The cells were lysed in 500 µl of 1X RIPA (50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.5% deoxycholate, 0.2% SDS, 10 mM EDTA) and the nuclei removed by high speed centrifugation. The amount of Gag p24 or p27 present in the supernatants and cell lysates was measured using a commercially available p24 and p27 ELISA kit (Zetaplex). The experiment was run in triplicate and the differences between the mean percentages were calculated using a two-tailed Student's t-test with p < 0.05 considered significant.

Analysis of A3 proteins in the nucleoprotein complex

In order to assess the incorporation of A3A proteins in the nucleoprotein complex (NPC), 293 cells seeded into a 6-well plate were co-transfected with HIV-1 or HIV-1Δvif (3 µg) proviral genomes and either HA-hA3G, HA-hA3A, or HA-rhA3A, or hA3GΔΔ3 or hA3AΔΔ3 (1.5 µg) using Turbofect (Fermentas). At 24 h post-transfection, cells were starved for methionine/cysteine for 2 h and radiolabeled with 300 µCi of [35S]-translabel (methionine and cysteine, Perkin-Elmer, Waltham, MA) for an additional 24 h. The virus-containing supernatants were harvested, cleared by low speed centrifugation, and concentrated by pelleting the virus through a 20% sucrose cushion using ultracentrifugation (SW41 rotor, 247,000 ×g, 1 h, 4 °C). The concentrated viral pellets were resuspended in 1X PBS (pH 7.4) and used on sucrose step-gradients with or without Triton-X-100. The sucrose step gradients were prepared as follows: 2.0 ml of a 60% sucrose solution (w/v, in 1X PBS) was placed into the bottom of a SW55Ti ultracentrifuge tube and overlaid with 2.0 ml of a 20% sucrose solution (w/v, in 1X PBS). Immediately prior to the addition of concentrated virus stocks, the step gradients were overlaid with 100 µl of either 1X PBS or 1% Triton-X-100 in 1X PBS. This procedure minimized the amount of time the detergent was exposed to the virions. Samples were then centrifuged in a SW55Ti rotor (Beckman) at 4 °C for 6 h at 247,000 ×g. Four fractions of 1.0 ml each were collected from the top of the gradient with fraction 1 containing soluble proteins, fraction 2 a buffer of 20% sucrose that separates soluble proteins from the viral particles or viral cores, fraction 3 that includes the interphase of 20%-60% sucrose where viral protein and viral cores accumulate, and fraction 4 containing the 60% sucrose fraction. Gradient fractions were divided in half, diluted to 1 ml with 1× RIPA buffer and subjected to immunoprecipitation assays using an HA-specific antibody (sc-805, Santa Cruz) for the A3 proteins or anti-HIV-1 patient serum for the capsid and matrix proteins.

Foreign DNA restriction by A3A

293 cells were seeded into 6-well tissue culture plates 24 h prior to transfection. Cells were first transfected with 3 µg of vectors expressing either HA-rhA3A, HA-hA3A, HA-hA3G, or pcDNA3.1(+)-vector using a polyethylenimine transfection reagent (ExGen™ 500, MBI Fermentas) according to the manufacturer's instructions. After 24 h, the cells were re-transfected with 3 µg of a vector expressing eGFP. Cells were incubated at 37 °C in 5% CO2 atmosphere for 24 h. The cells were removed from the plate using CaCl2/MgCl2-free PBS containing 10 mM EDTA. Cells were then fixed in 2% paraformaldehyde, for 5 min. The cells were washed twice in 1X PBS plus and analyzed using an LSRII flow cytometer. The mean fluorescence intensity (MFI) for eGFP positive cells was calculated. The MFI ratio and percentage of eGFP positive cells was calculated for each sample. Normalized ratios from six separate experiments were averaged and the standard error calculated. All groups were compared to pcDNA3.1(+)+ eGFP control using a Student's t-test with p < 0.05 considered significant.

References


