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Human APOBEC3B is a potent inhibitor of HIV-1 infectivity and is resistant to HIV-1 Vif

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Abstract

While the human antiretroviral defense factors APOBEC3F and APOBEC3G are potent inhibitors of the replication of HIV-1 mutants lacking a functional *vif* gene, the Vif protein expressed by wild-type HIV-1 blocks the function of both host cell proteins. Here, we report that a third human protein, APOBEC3B, is able to suppress the infectivity of both Vif-deficient and wild-type HIV-1 with equal efficiency. APOBEC3B, which shows ~58% sequence identity to both APOBEC3F and APOBEC3G, shares the ability of these other human proteins to bind the nucleocapsid domain of HIV-1 Gag specifically and to thereby package into progeny virion particles. However, APOBEC3B differs from APOBEC3F and APOBEC3G in that it is unable to bind to HIV-1 Vif in co-expressing cells and is therefore efficiently packaged into HIV-1 virions regardless of Vif expression. Unfortunately, APOBEC3B also differs from APOBEC3F and APOBEC3G in that it is not normally expressed in the lymphoid cells that serve as targets for HIV-1 infection. These studies therefore raise the possibility that activation of the endogenous *APOBEC3B* gene in primary human lymphoid cells could form a novel and effective strategy for inhibition of HIV-1 replication in vivo.

Keywords: APOBEC3B; Intrinsic immunity; HIV; Retrovirus

Introduction

Intrinsic immunity has recently been identified as a significant factor in preventing retroviral infection, particularly across species barriers (Bieniasz, 2004). One important form of intrinsic immunity is provided by the APOBEC3 family of proteins, and particularly by human APOBEC3G (hA3G) and APOBEC3F (hA3F), which are able to block infection of human cells by not only Vif-deficient mutants of human immunodeficiency virus type 1 (HIV-1), but also by wild-type forms of African green monkey simian immunodeficiency virus (SIVagm) and murine leukemia virus (MLV) (Bishop et al., 2004; Bogerd et al., 2004; Kobayashi et al., 2004; Liddament et al., 2004; Mangeat et al., 2003; Mariani et al., 2003; Schröfelbauer et al., 2004; Sheehy et al., 2002; Wiegand et al., 2004; Zheng et al., 2004). Moreover, hA3G and hA3F have also recently been shown to block the retrotransposition of several endogenous retrotransposons (Dutko et al., 2005; Esnault et al., 2005).

In addition to hA3G and hA3F, the human genome also contains genes encoding five other potential members of the APOBEC3 protein family, all of which are found at a single locus on chromosome 22 (Jarmuz et al., 2002). However, of these five genes, only three, i.e. APOBEC3A (hA3A), APOBEC3B (hA3B) and APOBEC3C (hA3C) have so far been shown to be expressed in human cells and/or tissues (Bishop et al., 2004; Jarmuz et al., 2002; Sheehy et al.,

Abbreviations: APOBEC, apolipoprotein B mRNA editing enzyme catalytic polypeptide; AGM, African green monkey; HA, influenza virus hemagglutinin; HIV, human immunodeficiency virus; MLV, murine leukemia virus; NC, nucleocapsid; PBMC, peripheral blood mononuclear cells; SIV, simian immunodeficiency virus.

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2002; Wiegand et al., 2004; Yu et al., 2004). Of these, hA3A has as yet not been reported to be able to inhibit the infectivity of any retrovirus tested (Bishop et al., 2004; Wiegand et al., 2004). In contrast, hA3C has been reported to be a potent inhibitor of Vif-deficient forms of both SIVagm and SIV from rhesus macaque (SIVmac), although wild-type SIVagm and SIVmac were not affected (Yu et al., 2004). However, hA3C has proven to be at best a weak inhibitor of either wild-type or Vif-deficient HIV-1 (Bishop et al., 2004; Doehle et al., 2005; Langlois et al., 2005; Wiegand et al., 2004; Yu et al., 2004). Finally, hA3B was reported by Bishop et al. (2004) to be able to inhibit both wild-type and Vif-deficient HIV-1 infectivity while Yu et al. (2004) reported that hA3B had at most a modest effect on HIV-1 infectivity, although hA3B did potently inhibit the infectivity of wild-type forms of both SIVagm and SIVmac.

Here, we report that hA3B is, in fact, a quite potent inhibitor of the infectivity of both wild-type and Vif-deficient HIV-1. Like hA3G, hA3B is specifically incorporated into virions due to an interaction with the HIV-1 nucleocapsid protein but, unlike hA3G, this incorporation is not reduced by HIV-1 Vif expression and hA3B does not bind to Vif in vivo. The inability of HIV-1 Vif to attenuate the inhibition of viral infectivity induced by hA3B, while inhibition by either hA3G or hA3F is effectively controlled, appears to reflect the fact that hA3B, unlike hA3G or hA3F, is not expressed at detectable levels in human lymphoid tissues.

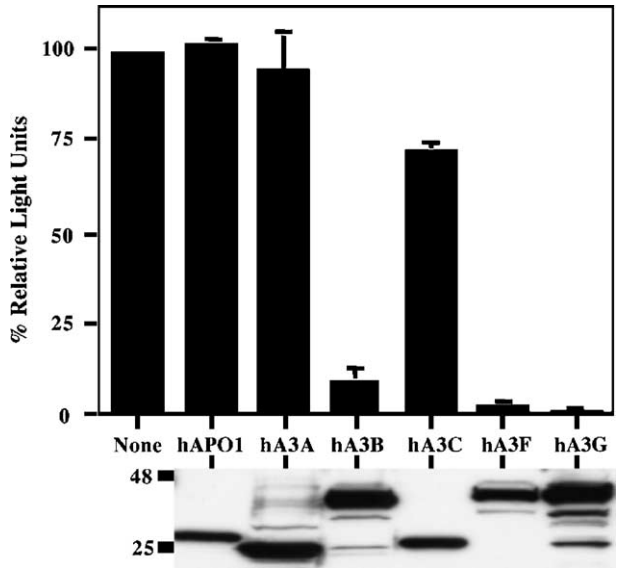

Fig. 1. Human APOBEC3B inhibits Vif-deficient HIV-1. The HIV-1 proviral expression plasmid pNL-HXB-LUC-ΔVif (1.5 μg) was co-transfected into 293T cells along with 125 ng of one of the human APOBEC3 expression plasmids indicated, or the parental pcDNA3 plasmid (none) as a control. Supernatant media were collected 44 h later and filtered. An aliquot was then analyzed by ELISA for p24 Gag levels while the remainder was used to infect naive 293T cells expressing viral receptors. No significant differences in supernatant p24 Gag levels were observed (data not shown). Induced luciferase levels were determined 24 h after infection. Data are presented as a percentage of the luciferase activity observed in cells infected with HIV-1ΔVif virus produced in APOBEC3-negative cells (none) (average of 3350 light units per control sample). Error bars represent the standard deviation observed in 3 independent experiments. The lower panel shows a Western blot analyzing the expression of the various APOBEC proteins in transfected cells, using an antibody specific for the carboxy-terminal HA tag. This tag, which is present in three copies, affects the migration of these proteins, which is slower than predicted by their untagged size.

Results

Human APOBEC3B inhibits HIV-1 infectivity and is resistant to HIV-1 and HIV-2 Vif

Human APOBEC3 proteins can be divided into two classes (Jarmuz et al., 2002) based on whether they are ~42 kDa in size and contain two copies of the cytidine deaminase active site first defined in human APOBEC1 (hAPO1), e.g.: hA3G, hA3F and hA3B, or are ~21 kDa in size with one copy of the active site, e.g.: hA3A and hA3C. Interestingly, for hA3G, hA3F, hA3A and hA3C, this subdivision also correlates with their ability to inhibit the infectivity of Vif-deficient HIV-1. Specifically, as previously reported and reproduced in Fig. 1, hA3G and hA3F are potent inhibitors of Vif-deficient HIV-1 infectivity while hA3A and hA3C, as well as hAPO1, have little or no effect (Bishop et al., 2004; Liddament et al., 2004; Sheehy et al., 2002; Wiegand et al., 2004; Yu et al., 2004). This difference is not due to differences in the level of expression of these proteins in transfected cells (Fig. 1).

Human APOBEC3B also contains two copies of the cytidine deaminase active site and shows a high degree of sequence homology to both hA3G (57% identity) and hA3F (59% identity). We were therefore interested in testing whether hA3B would also inhibit Vif-deficient HIV-1. As shown in Fig. 1, hA3B expression indeed strongly inhibited the infectivity of HIV-1 virions produced in its presence, although this inhibition was perhaps somewhat less severe than noted with hA3G or hA3F. Sequence analysis of HIV-1 reverse transcripts from cells infected with HIV-1ΔVif virus produced in the presence of hA3B demonstrated extensive G to A hypermutation of the viral sense strand. Specifically, sequencing 12,563 bp of DNA, obtained by PCR of total DNA from infected cells using primers specific for part of the viral *env* gene, revealed 272 G to A changes out of 3502 predicted G residues, i.e., an editing rate of 7.8%. In contrast, sequencing 4795 bp of HIV-1 *env* gene sequence obtained using DNA from cells infected by HIV-1ΔVif produced in the absence of any exogenous APOBEC protein revealed only 1 G to A change, a mutation rate of <0.1%. Analysis of the sequence context of the hA3B-mediated editing events (Table 1) showed that the edited deoxycytidine residues located on the antisense strand were flanked 5′ by either a T or, less frequently, a C residue, with purines strongly disfavored. No strong consensus sequence for hA3B editing was noted at the −2 or +1 position, although

Table 1
Consensus editing site for the hA3B protein

hA3B	−3	−2	−1	0	+1
A	17	33	1	0	33
C	28	23	23	100	26
G	28	13	< 1	0	2
T	27	31	76	0	39
Consensus	?	?	T	C	?

This table presents the observed sequence context of deoxycytidine residues edited by hA3B when hA3B was packaged into HIV-1ΔVif virions produced in its presence, based on a compilation of G to A transitions observed in the viral coding strand. A consensus sequence is presented at the bottom, with lack of consensus indicated by a question mark.

G residues were clearly underrepresented at both locations. These data are largely consistent with data previously reported by Bishop et al. (2004) documenting a strong preference for a pyrimidine residue at the −1 position relative to the edited deoxycytidine residue.

The HIV-1 and HIV-2 Vif proteins specifically rescue the inhibition of HIV-1 infectivity induced by hA3G or hA3F (Conticello et al., 2003; Kao et al., 2003; Liddament et al., 2004; Mariani et al., 2003; Mehle et al., 2004; Sheehy et al., 2002, 2003; Stopak et al., 2003; Wiegand et al., 2004; Yu et al., 2003). To test whether either of these Vif proteins would rescue the infectivity of HIV-1 virions produced in the presence of hA3B, we co-transfected 293T cells with the proviral indicator construct pNL-HXB-LUC-ΔVif and either phA3B-HA or phA3G-HA, as a positive control, in the presence or absence of plasmids expressing HIV-1 or HIV-2 Vif (Fig. 2A). While both Vif proteins effectively rescued the infectivity of HIV-1 virions produced in the presence of hA3G, as expected, neither protein was able to enhance the infectivity of HIV-1 virions produced in the presence of hA3B (Fig. 2A). This phenotype is reminiscent of African green monkey APOBEC3G (agmA3G) or murine APOBEC3 (mA3), both of which inhibit the infectivity of Vif-deficient or wild-type HIV-1 with equal efficiency (Bogerd et al., 2004; Kobayashi et al., 2004; Mangeat et al., 2004; Mariani et al., 2003; Schröfelbauer et al., 2004; Simon et al., 1998). For both of these heterologous APOBEC3 proteins, this resistance to inactivation by HIV-1 Vif has been shown to correlate with the inability of HIV-1 Vif to bind these proteins in vivo (Bogerd et al., 2004; Mariani et al., 2003; Schröfelbauer et al., 2004).

To test whether this is also true of hA3B, we co-expressed HIV-1 Vif with HA-tagged hA3B, hA3G (as a positive control) or mA3 (as a negative control) in transfected 293T cells. 48 h after transfection, the cells were lysed and immunoprecipitated using an HA-epitope tag specific monoclonal antibody. The resultant precipitate was then analyzed by Western blot using a Vif-specific rabbit antiserum. As shown in Fig. 2B, Vif efficiently co-immunoprecipitated with hA3G but not with mA3, as expected. hA3B also failed to detectably interact with HIV-1 Vif, thus explaining the inability of Vif to reverse the inhibition of HIV-1 infectivity induced by this human protein (Fig. 2A).

hA3B is packaged into HIV-1 virions due to a specific interaction with HIV-1 nucleocapsid

Analysis of hA3G function has shown that hA3G specifically interacts with the NC domain of the HIV-1 Gag polyprotein (Alce and Popik, 2004; Cen et al., 2004; Schäfer et al., 2004; Zennou et al., 2004). Moreover, this interaction is essential for the specific incorporation of hA3G into HIV-1 virion particles. We therefore next asked if hA3B would also be specifically packaged into HIV-1 virions and whether this packaging, if observed, would be affected by HIV-1 or HIV-2 Vif. As shown in Fig. 3, hA3A, which does not inhibit HIV-1 infectivity (Fig. 1) is not detectably incorporated into HIV-1 virions. In contrast, hA3G is efficiently incorporated into HIV-1 virions and this incorporation is, as predicted, prevented by HIV-1 or HIV-2 Vif (Fig. 3). This inhibition correlates with a reduction in the level of expression of hA3G in the virus producer cells

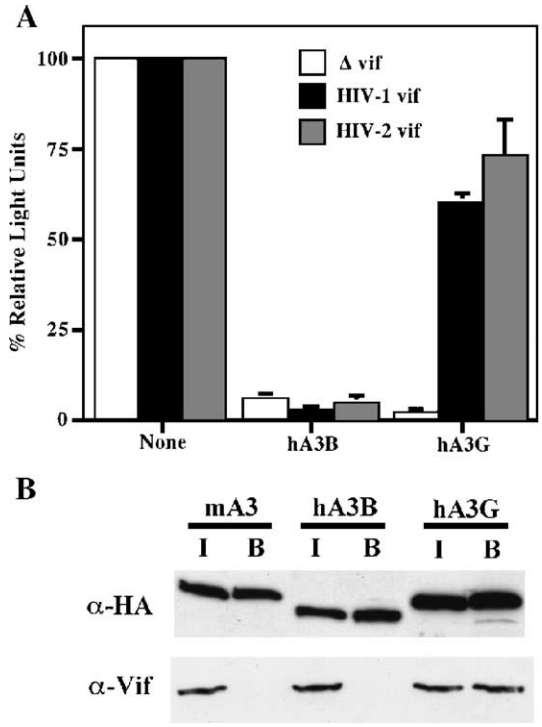

Fig. 2. hAPOBEC3B inhibition of HIV-1 infectivity is not relieved by HIV-1 or HIV-2 Vif. (A) pNL-HXB-LUC-ΔVif (1.5 μg) was co-transfected into 293T cells along with plasmids expressing phA3B-HA, phA3G-HA or the parental plasmid pcDNA3 (125 ng). Additionally, 250 ng of a plasmid encoding HIV-1 Vif, HIV-2 Vif, or a negative control plasmid (pgΔVif) was added to the transfection. Data are presented as in Fig. 1, with an average of 4240 light units observed in the controls (none). (B) 293T cells were co-transfected with plasmids expressing hA3B-HA, hA3G-HA, or mA3-HA and an HIV-1 Vif expression plasmid. 48 h post-transfection, cells were lysed and an aliquot subjected to immunoprecipitation using a monoclonal antibody specific for the HA epitope. 10% of the input lysate and 25% of the bound fraction were then subjected to Western analysis detecting the HA epitope (top panel) or the HIV-1 Vif protein (bottom panel).

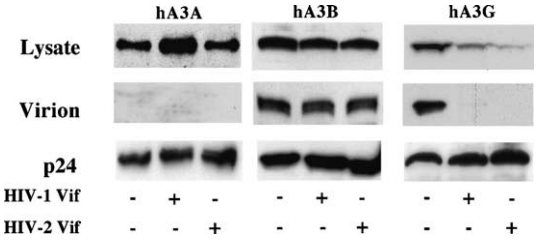

Fig. 3. hA3B packaging into HIV-1 virions is not inhibited by HIV-1 or HIV-2 Vif. 293T cells were co-transfected as in Fig. 2A, with the exception that the Env-deficient HIV-1 proviral expression plasmid pNL4-3ΔVifΔEnv was substituted for pNL-HXB-Luc-ΔVif. 48 h later, virions were collected by centrifugation of the supernatant media, and subjected to Western analysis along with a lysate of the producer cells. HA epitope tagged APOBEC3 proteins present in either the producer cell lysate or the disrupted virions were detected using a monoclonal antibody specific for the HA tag. A monoclonal antibody specific for p24 Gag was used to detect HIV-1 p24 capsid protein present in the released virions and to ensure consistent loading of samples.

due to the Vif-induced targeting of hA3G for proteolytic degradation (Conticello et al., 2003; Kao et al., 2003; Marin et al., 2003; Mehle et al., 2004; Sheehy et al., 2002, 2003; Stopak et al., 2003; Yu et al., 2003). In the case of hA3B, readily detectable levels were again detected in progeny virion particles. However, unlike the case with hA3G, neither HIV-1 nor HIV-2 Vif had any effect on the level of hA3B virion incorporation.

As noted above, hA3G incorporation into HIV-virion particles is dependent on the NC domain of Gag. Previously, a mutant form of HIV-1 Gag has been reported, called Gag Z_{WT}, in which the NC domain has been deleted and replaced with a leucine zipper domain from the yeast GCN4 protein (Accola et al., 2000). This substitution rescues HIV-1 virion production although these virions are, of course, defective. Previously, we and others have shown that the Z_{WT} form of HIV-1 Gag no longer supports the incorporation of the hA3G protein into virion particles, as confirmed in Fig. 4A (Schäfer et al., 2004; Zennou et al., 2004). Moreover, Gag Z_{WT} was also unable to incorporate the hA3B protein, thus implying that hA3B incorporation was likely also due to a specific interaction with NC.

To confirm that this was indeed the case, we prepared a recombinant, ^{35}S-methionine labeled form of the carboxy terminal domain of HIV-1 Gag, consisting of NC linked to the adjacent p6 protein, by in vitro translation (Schäfer et al., 2004). In parallel, we expressed HA-tagged forms of hA3A, hA3B, hA3F and hA3G at high levels in transfected 293T cells and then collected these proteins using an HA affinity matrix, as previously described (Schäfer et al., 2004). Each loaded HA affinity matrix was then incubated with the ^{35}S-labeled NC/p6 protein, extensively washed and bound proteins detected by gel electrophoresis and fluorography. As shown in Fig. 4B, we observed readily detectable levels of the NC/p6 protein bound to the HA affinity matrices pre-loaded with hA3G or hA3F. In contrast, the HA affinity matrix pre-loaded with hA3A failed to interact with NC/p6. Finally, the matrix pre-loaded with hA3B also was able to bind the NC/p6 protein (Fig. 4B). Together, these data argue that hA3B, like hA3G and hA3F, is able to specifically interact with the HIV-1 NC protein and that this interaction is critical for the specific packaging of hA3B into HIV-1 virion particles.

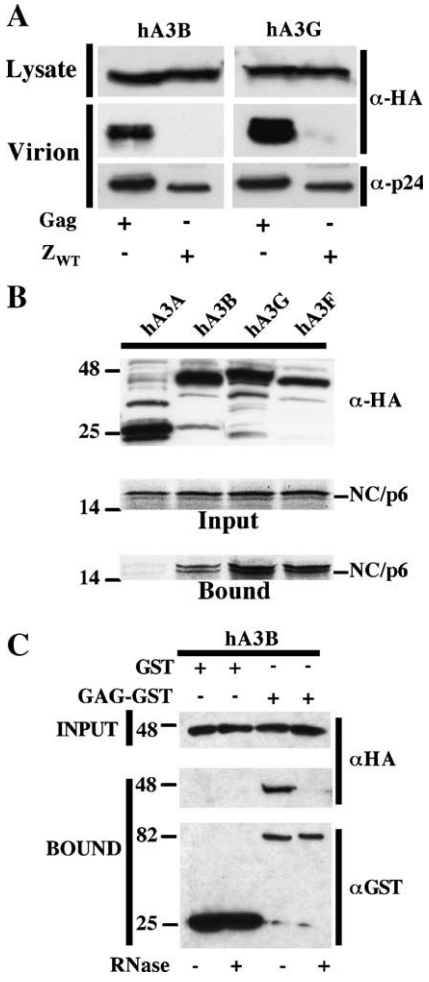

Fig. 4. hA3B packaging into HIV-1 virions requires RNA and the nucleocapsid region of HIV-1 Gag. (A) 293T cells were co-transfected with HA-tagged hAPOBEC3 expression plasmids as well as with the codon optimized HIV-1 Gag construct psynGag or a mutant version (Z_{WT}) of this plasmid in which the NC region has been replaced with a leucine zipper. Producer cell lysates and virions were collected and analyzed as in Fig. 3. (B) Analysis of in vitro binding of hA3A-HA, hA3B-HA, hA3F-HA or hA3G-HA to the NC/p6 region of the HIV-1 Gag protein. ^{35}S-methionine-labeled NC/p6 was incubated with an HA affinity matrix pre-loaded with HA-tagged APOBEC3 proteins obtained from transfected 293T cells. The top panel shows a Western analysis of an aliquot of the 293T cell lysates, collected using the HA-matrix, using a monoclonal antibody specific for the HA epitope tag. The middle panel shows an aliquot of the input ^{35}S-labeled, in vitro translated NC/p6 prior to incubation with the HA-matrix. The bottom panel shows the level of NC/p6 bound by each APOBEC3-HA protein after extensive washing and elution from the HA affinity matrix. (C) Co-immunoprecipitation of HIV-1 Gag with hA3B is dependent on RNA. 293T cells were transfected with plasmids expressing hA3B-HA, GST and/or HIV-1 Gag-GST. The effect of RNAse A treatment on the ability of hA3B to bind the GST or HIV-1 Gag-GST proteins present in cell lysates was determined as previously described (Dutko et al., 2005).

An interesting aspect of hA3G binding to the HIV-1 Gag protein is that it requires the presence of non-specific, i.e., cellular RNA (Schäfer et al., 2004; Zennou et al., 2004). To test whether this is also true of the HIV-1 Gag:hA3B interaction, we co-transfected cells with a plasmid expressing HA-tagged hA3B and a plasmid expressing either GST or a full-length HIV-1 Gag-GST fusion protein. After 48 h, the transfected cells were lysed and the clarified lysate incubated in the presence or absence of RNAse A, as previously described (Dutko et al., 2005). After incubation, GST, and the HIV-1 Gag-GST fusion protein, were recovered using glutathione-sepharose beads and bound proteins resolved by gel electrophoresis (Dutko et al., 2005). As shown in Fig. 4C, the HIV-1 Gag-GST fusion protein bound hA3B specifically in vivo but this interaction was disrupted by RNAse A treatment. Therefore, specific binding of hA3B by HIV-1 Gag is similar to hA3G binding in that both are RNA-dependent.

hA3B is expressed at very low levels in human tissues

T cells and, indeed all human cells, can be divided into two categories based on whether they are able to support the replication of Vif-deficient HIV-1 (permissive cells) or unable to do so (non-permissive cells) (Bieniasz, 2004). Previous analysis has shown that non-permissive T cells, such as the cell lines HUT78, H9 and CEM, as well as primary peripheral blood mononuclear cells (PBMCs), co-express both hA3G and hA3F (Bishop et al., 2004; Sheehy et al., 2002; Wiegand et al., 2004). In contrast, permissive cells, such as SupT1 and CEM-SS cells, express neither hA3G nor hA3F. We therefore asked if the non-permissive cell lines H9 and CEM, or the permissive cell line CEM-SS, expressed hA3B mRNA using a previously described semi-quantitative RT-PCR assay (Wiegand et al., 2004). As positive controls, we included RNA derived from two human cell lines, the chronic myelogenous leukemia cell line K562 and the colorectal adenocarcinoma cell line SW480, that had been previously reported to express hA3B mRNA (Jarmuz et al., 2002). For this RT-PCR analysis, we used previously characterized primers specific for hA3G, hA3F or hA3B (Bishop et al., 2004; Wiegand et al., 2004).

As shown in Fig. 5A, hA3G and hA3F mRNA was observed at readily detectable levels in the non-permissive T cell lines H9 and CEM, as previously reported (Bishop et al., 2004; Wiegand et al., 2004; Sheehy et al., 2002), as well as in the K562 and SW480 cell lines. No hA3G or hA3F mRNA was detected in the permissive cell line CEM-SS, as expected. In contrast, we detected only a low level of hA3B mRNA in SW480 cells and even less in the cell lines K562 or CEM. No hA3B mRNA was detected in H9 or CEM-SS cells. This difference was not due to the inefficiency of the hA3B primers used, as shown by PCR using cDNA expression plasmids for hA3B, hA3F and hA3G (Fig. 5A, right lane).

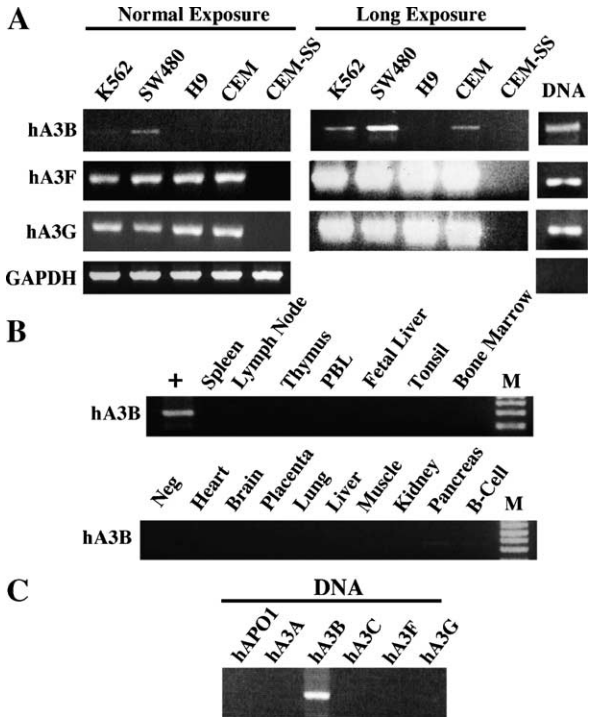

Fig. 5. hA3B mRNA is not detectable in primary tissues that co-express hA3G and hA3F. (A) Weak expression of hA3B mRNA in a number of transformed human cell culture lines. Total RNA extracted from the cell lines indicated was subjected to RT followed by 35 cycles of PCR using gene specific primers for either hA3B, hA3F or hA3G. Primers specific for GADPH were added to samples for 30 rounds of PCR and were used as a control. The right lane used an equimolar mixture of the expression plasmids encoding hA3B, hA3F or hA3G and serves as a positive control for PCR amplification by each APOBEC primer set, and as a negative control for the GAPDH primers. (B) hA3B mRNA was not detected in a range of primary human tissue samples. Top panel represents a pre-normalized cDNA tissue panel of human lymphoid tissues. Bottom panel samples are from a commercial human cDNA panel. Both hA3G and hA3F mRNA were readily detected in most of these tissue-specific mRNA samples, as previously described (Wiegand et al., 2004). +, hA3B plasmid DNA; Neg, no cDNA added; M, marker; PBL, peripheral blood lymphocytes. (C) Specificity control for the hA3B primers used. hA3B primers were tested using the hAPOBEC3 expression plasmids used in this study.

Given the low or undetectable level of expression of hA3B in the cell lines tested, we next asked if hA3B mRNA would be detectable in primary human tissues, including a range of lymphoid tissues. Importantly, while we have previously shown that almost all of these tissues express readily detectable levels of hA3G and hA3F mRNA (Wiegand et al., 2004), no hA3B mRNA was detected in this analysis. We therefore conclude that hA3B mRNA is either expressed in human tissues not analyzed in this panel or is induced in response to stimuli that have yet to be defined.

Discussion

In this report, we have analyzed the ability of hA3B to inhibit the infectivity of HIV-1 in culture. hA3B is closely

related to two other human proteins, hA3G and hA3F, that have been shown to potently inhibit the replication of Vif-deficient mutants of HIV-1 but that have little effect on the replication of wild-type HIV-1 (Bishop et al., 2004; Jarmuz et al., 2002; Sheehy et al., 2002; Wiegand et al., 2004; Zheng et al., 2004). In contrast, we here show that hA3B is equally effective at inhibiting both wild-type and Vif-deficient HIV-1 (Figs. 1 and 2). While perhaps slightly less effective than hA3G or hA3F at inhibiting Vif-deficient HIV-1, the level of inhibition by hA3B of both wild-type and Vif-deficient HIV-1 was still 10- to 20-fold in this analysis (Figs. 1 and 2) and involved hypermutation of the HIV-1 genome (Table 1). The inability of HIV-1 Vif to block the inhibition of HIV-1 infectivity induced by hA3B (Fig. 2A) correlated with the inability of HIV-1 Vif to bind to hA3B in vivo (Fig. 2B) and to reduce the incorporation of hA3B into HIV-1 virions (Fig. 3). However, hA3B was similar to hA3G in that incorporation into HIV-1 virions was mediated by a specific, RNA-dependent interaction of hA3B with the NC domain of the Gag polyprotein (Fig. 4).

The inability of HIV-1 Vif to counter the inhibition of HIV-1 infectivity induced by hA3B might appear difficult to understand, given that HIV-1 obviously replicates efficiently in many human cell types both in vivo and in culture. However, RT-PCR analysis demonstrated that hA3B, unlike hA3G and hA3F, is not expressed at detectable levels in any human tissue analyzed, including lymphoid tissues, although low levels were detected in a small number of cancer cell lines (Fig. 5). Therefore, HIV-1 may never encounter significant levels of hA3B in its normal target cell populations.

The phenotype of hA3B reported here is closely similar to the phenotypes previously reported for mA3 and agmA3G, both of which are effective inhibitors of wild-type HIV-1 replication and both of which also fail to interact with, or respond to, HIV-1 Vif (Bishop et al., 2004; Bogerd et al., 2004; Mangeat et al., 2004; Mariani et al., 2003; Schröfelbauer et al., 2004). In the case of mA3 and agmA3G, it seems reasonable to hypothesize that HIV-1 Vif has failed to evolve the ability to neutralize these antiretroviral defense factors because they are expressed in a heterologous, non-target species. Similarly, in the case of hA3B, HIV-1 Vif and HIV-2 Vif have presumably failed to evolve the ability to neutralize this human antiretroviral defense factor because it is not expressed in the human tissues targeted by these viruses.

In the case of agmA3G, we and others have previously reported that the inability of agmA3G to respond to HIV-1 Vif, or to bind HIV-1 Vif in vivo, is due to a single amino acid difference between hA3G and agmA3G at position 128, which is an aspartic acid in hA3G and a lysine in agmA3G (Bogerd et al., 2004; Mangeat et al., 2004; Schröfelbauer et al., 2004). Analysis of the hA3B sequence shows that this residue is a glutamic acid. In the case of hA3G, the conservative substitution of aspartic acid with glutamic acid at position 128 does not significantly compromise the ability of HIV-1 Vif to block hA3G function (Bogerd et al., 2004), so the residue(s) in hA3B that block HIV-1 Vif binding must presumably be located elsewhere. Given the close similarity of hA3G to hA3B in sequence (57% identity, 68% similarity), it may prove informative to map this residue(s) in detail.

Two groups have previously addressed the ability of hA3B to inhibit HIV-1 infectivity and reached apparently opposite conclusions. Specifically, Yu et al. (2004) reported that hA3B does not effectively inhibit HIV-1 infectivity while Bishop et al. (2004) reported that hA3B is able to inhibit HIV-1 infectivity in both the presence and absence of Vif. Close inspection of the data presented in these papers demonstrates, however, that both groups did detect 4- to 8-fold inhibition of wild-type HIV-1 infectivity by hA3B. Therefore, these different conclusions really represent a difference in emphasis and these earlier data actually agree quite closely with our results (Figs. 1 and 2) although we observed a somewhat more striking degree of inhibition by hA3B. The current report also extends these earlier data by showing that hA3B fails to interact with HIV-1 Vif (Fig. 2B) and is incorporated into HIV-1 virions due to an RNA-dependent interaction with the Gag NC domain (Figs. 3 and 4).

In this report, we present data arguing that hA3B mRNA is expressed at undetectable levels in all the primary human tissues examined and at low but detectable levels in the cell lines CEM, K562 and SW480. These data are closely comparable to data reported by Bishop et al. (2004), who failed to detect any hA3B mRNA expression in activated PBMCs or in the cell lines CEM-SS and H9 but did observe low level expression in CEM cells. In contrast, Yu et al. (2004) reported readily detectable levels of hA3B mRNA expression in activated $CD4^+$ T cells and small intestine and detectable expression in heart and macrophages. While the reason for the discrepancy between the data from Yu et al. (2004), and the data reported here and previously by Bishop et al. (2004) remains unknown, we note that we used the same hA3B-specific primers as Bishop et al. (2004) while Yu et al. (2004) used a distinct primer set.

The observation that wild-type HIV-1 is highly sensitive to inhibition by mA3 and agmA3G suggests that ectopic expression of these proteins could be used to block HIV-1 replication in vivo. Similarly, expression of hA3B in lymphoid cells susceptible to infection by HIV-1 should also effectively block HIV-1 replication, with the notable difference that *hA3B* is a normal human gene that happens not to be expressed in lymphoid cells. It therefore may be possible to activate the endogenous *hA3B* gene in lymphoid cells and therefore block HIV-1 replication. In the case of beta thalassemias, attempts to activate the expression of the otherwise silent fetal hemoglobin gene in adult cells using agents such as hydroxyurea, 5-azacytidine or butyric acid have been partially successful in activating fetal hemoglobin expression in culture (Schrier and Angelucci, 2005). It would therefore appear worthwhile to examine whether

analogous approaches might exert a similar effect on hA3B expression in primary $CD4^+$ T cells or macrophages in culture and, if so, whether this resulted in a significant inhibition in the level of HIV-1 replication.

Materials and methods

Construction of molecular clones

The HIV-1 proviral expression plasmids pNL-HXB-LUC-ΔVif and pNL4-3ΔVifΔEnv have been described (Bogerd et al., 2004; Wiegand et al., 2004), as have the expression plasmids phA3A-HA, phA3C-HA, phA3F-HA, phA3G-HA, phAPO1-HA, pmA3-HA, pgVif, pgVif2, pgΔVif and pK/HIVGAG-GST (Bogerd et al., 2004; Dutko et al., 2005; Simon et al., 1998). A plasmid expressing HA-tagged hA3B was constructed similarly by PCR amplification from a cDNA preparation of the human cell line K562 using the following primers:

5′ primer: 5′-GCGCGGTACCACCATGAATCCACA-GATCAGAAATCCG-3′;
3′ primer: 5′-GCGCCAATTGGTTTCCCTGATTCTG-GAGAATGG-3′.

Plasmid psynGag, encoding a codon optimized HIV-1 Gag protein, and psynGag-Z_{WT}, encoding a nucleocapsid (NC) deletion mutant of HIV-1 Gag, have been described elsewhere (Accola et al., 2000; Cowan et al., 2002; Schäfer et al., 2004).

Cell culture and analysis

K562, CEM, CEM-SS and H9 cells were cultured in RPMI media containing 10% fetal calf serum (FCS) while SW480 cells were cultured in Leibovitz L-15 media containing 10% FCS. 293T cells were cultured in DMEM containing 10% FCS and were transfected using the calcium phosphate method. HIV-1 infectivity assays were performed as described previously (Bogerd et al., 2004; Wiegand et al., 2004).

Virion packaging of APOBEC3 proteins was analyzed as previously described (Bogerd et al., 2004; Schäfer et al., 2004; Wiegand et al., 2004). Briefly, 293T cells were transfected with either pNL4-3ΔVifΔEnv, psynGag or psynGag-Z_{WT} and an APOBEC3 expression plasmid. 44 h later, the virus containing supernatant media were collected, filtered, and layered onto a 20% sucrose cushion. Virions were collected by centrifugation at 35,000 rpm for 1.5 h at 4 °C in a Beckman SW41 rotor. Pellets were lysed and analyzed by Western blot.

The level of editing of HIV-1 proviral DNA by hA3B was analyzed as previously described (Wiegand et al., 2004). Briefly, HIV-1ΔVif virus was produced in the presence or absence of hA3B as described for the HIV-1 infectivity assays (Fig. 1). Target cells were then infected with virus containing media and 16 h after infection total DNA was isolated using a DNeasy tissue kit (Qiagen). Isolated DNA was *Dpn*I treated and then used as the template to PCR amplify part of the HIV-1 *env* gene. Amplified fragments were cloned and sequenced.

Western blot analysis

Cell lysates, virion lysates and immunoprecipitates were subjected to gel electrophoresis and then transferred to nitrocellulose membrane. Membranes were probed with a mouse monoclonal antibody specific for the HIV-1 capsid protein (Chesebro et al., 1992), a mouse monoclonal antibody specific for the HA epitope tag (Covance), or a rabbit polyclonal antisera specific for the HIV-1 Vif protein (Goncalves et al., 1996). Reactive proteins were detected with Lumi-Light Western Blotting Substrate (Roche) as previously described (Bogerd et al., 2004; Wiegand et al., 2004).

In vitro binding assays

The ^{35}S-methionine-labeled NC/p6 domain of the HIV-1 Gag protein was synthesized by in vitro translation using a TNT coupled transcription/translation system, as described previously (Schäfer et al., 2004). In parallel, 293T cells were transfected with APOBEC3 protein expression plasmids and 48 h later, cultures were lysed and loaded onto an HA affinity matrix (Covance). After binding, samples were incubated with the in vitro translated, ^{35}S-labeled NC proteins, extensively washed and bound proteins detected by SDS-PAGE and fluorography.

mRNA expression analysis

Analysis of the tissue expression pattern of human mRNAs was performed using RT-PCR, as previously described (Wiegand et al., 2004), using either pre-normalized human multiple tissue cDNA panels (BD/Clontech) or isolated total RNA. The primers used for specific amplification of the hA3G, hA3F or hA3B cDNAs have been described (Bishop et al., 2004; Bogerd et al., 2004). Amplification reactions using the cDNA panels were performed according to the manufacturer's instructions. Total RNA was isolated from a variety of cell lines using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. All RNA preparations were treated with RQ1 RNase-free DNase (Promega) to remove any genomic DNA contamination. 10 μg of treated total RNA was subjected to reverse transcription using the StrataScript First Strand Synthesis System (Stratagene) using the provided oligo(dT) primers. PCR was subsequently performed using gene specific primers for 35 cycles of PCR under standard conditions, with the exception of GAPDH, which was only amplified for 30 cycles.

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