HIV-1 Subtype Variability in Vif Derived from Molecular Clones Affects APOBEC3G-Mediated Host Restriction

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Key Words

HIV-1 subtype diversity · Vif · APOBEC3G · Viral infectivity

Abstract

Background: The host protein APOBEC3G (A3G) can limit HIV-1 replication. Its protective effect is overcome by the HIV-1 ‘viral infectivity factor’ (Vif), which targets A3G for proteasomal degradation. Although Vif is considered to be essential for HIV-1 replication, the effect of Vif variability among commonly used HIV-1 molecular clones of different genetic backgrounds on viral infectiousness and pathogenesis has not been fully determined. **Methods:** We cloned the intact Vif coding regions of available molecular clones of different subtypes into expression vectors. Δ vif full-length HIV-1 clonal variants were generated from corresponding subtype-specific full-length molecular clones. Replication-competent viruses were produced in 293T cells in the presence or absence of A3G, with Vif being supplied by the full-length HIV-1 clone or *in trans*. The extent of A3G-mediated restriction was then determined in a viral replication assay using a reporter cell line. **Results and Conclusions:** In the

absence of A3G, Vif subtype origin did not impact viral replication. In the presence of A3G the subtype origin of Vif had a differential effect on viral replication. Vif derived from a subtype C molecular clone was less effective at overcoming A3G-mediated inhibition than Vif derived from either subtype B or CRF02_AG molecular clones.

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Human APOBEC3G (A3G) is a member of the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like family of enzymes. A3G can restrict human immunodeficiency virus type-1 (HIV-1) infection by interfering with the synthesis of minus-strand viral cDNA via cytidine deamination that results in guanine (G) to adenine (A) mutations [1, 2]. The process of G-to-A mutations can lead to viral genomic instability through introduction of amino acid substitutions and premature stop codons. HIV-1 has evolved to overcome this restriction by expressing and packaging a virus-encoded protein termed viral infectivity factor (Vif), which induces the polyubiquitylation and subsequent proteasomal degradation of A3G [3, 4].

Given the rapid HIV-1 replication rate and high virus titers, genetically distinct allelic variants of *vif* can often be detected within infected individuals [5, 6]. Proviral DNA from some viruses that failed to neutralize A3G was shown to contain an abundance of G-to-A hypermutations [7], suggesting that sporadic inactivation of Vif can occur in vivo and that natural Vif variability might affect the course of HIV disease. HIV-1 subtype variability is extensive and ranges between 15–20 and 25–35% at the amino acid and nucleotide levels, respectively [8, 9]. The contribution of Vif amino acid variability among HIV-1 subtypes to viral pathogenesis is not fully understood. Indeed, others have shown that the subtype origin of Vif may play a role in determining the extent to which A3G-mediated restriction of HIV replication will occur [10, 11]. However, the impact of Vif variability among a number of commonly used full-length subtype-specific HIV-1 infectious molecular clones has not been investigated in the context of A3G-mediated restriction.

We performed a phylogenetic analysis to determine the extent of inter-subtype genetic differences present in Vif protein and to ensure that each molecular clone used in this study was representative of other Vif sequences of the same subtype. HIV-1 *vif* nucleotide sequences ($n = 1,004$), obtained through the Los Alamos HIV Database, were aligned and translated using VectorNTI software (Version 10.3.1; Invitrogen). Subtype-specific protein reference sequences were derived using Contig maker VectorNTI for subtypes A, B, C, G and A/G. Translated subtype-specific Vif sequences were realigned on VectorNTI and loaded into MEGA 4.0 software for phylogenetic analysis. The analysis revealed 80–85% similarity between subtypes B, C and CRF02_AG Vifs at the amino acid level (fig. 1). The Vif from molecular clone pAG_97 showed greater similarity to subtype A than G, which is in agreement with a recent report [12]. The Vif sequences investigated in this study are shown in figure 2; each was confirmed to be representative of its subtype of origin (fig. 1).

To assess the impact of A3G restriction on the replication of viruses derived from subtype-specific molecular clones, the TZM-bl luciferase reporter assay system was used as previously described [13]. Recombinant viruses were produced by transfecting 293T cells with 9 μ g pAG_97 or pINDIE_C Δ vif, 3 μ g Vif and $\pm$ 3 μ g pcDNA3.1-APOBEC3G using Lipofectamine 2000 (Invitrogen). Empty pcDNA3.1/V5-HisA was used as a filler control. A total of 16 μ g of DNA was transfected, except in the Vif titration experiment where 5 μ g of Vif

was transfected. Supernatants were harvested 48 h post-transfection, clarified by centrifugation, passed through a 0.45- μ m filter and tested for reverse transcriptase (RT) activity. A subtype C viral backbone was employed to establish the assay based on current understanding of subtype B viruses and A3G restriction. To obtain % HIV-1 replication, we normalized the measured luciferase activity for each virus tested to the assay maximum output point set by the virus derived from pNL4-3 produced in the presence of A3G. The results of figure 3a clearly distinguish between replication of full-length and Δ vif subtype C viruses produced in the presence or absence of A3G. While in the absence of A3G both the full-length subtype C and Δ vif subtype C viruses supported HIV replication, a marked reduction in HIV-1 replication was observed for Δ vif subtype C virus produced in the presence of A3G (fig. 3a). This suggests that Vif encoded by the full-length subtype C clone was able to overcome A3G restriction which restored viral replication to levels significantly higher than seen with Δ vif subtype C (fig. 3a, dashed lines, $p \leq 0.0001$).

We next assessed the replication of Δ vif subtype C viruses complemented with Vif *in trans* from different viral subtypes. In the absence of A3G, the subtype source of Vif did not impact levels of viral replication (fig. 3b). Next we titrated the amount of Vif supplied *in trans* for virus production. With a subtype C virus, increasing the amount of Vif derived from either subtype B or C from 1 to 5 μ g resulted in an increase in levels of viral replication (fig. 3c and d, respectively). Supplementation of Δ vif subtype C with 5 μ g of Vif *in trans* from either subtype B or C resulted in comparable replication of the Vif-deficient viruses (fig. 3c, d). Accordingly, an optimal concentration of Vif was set at 5 μ g and maintained in subsequent analyses.

Vif from 3 distinct subtypes complemented Δ vif subtype C clonal virus replication in the absence of A3G at levels between 70 and 90% of maximum values (fig. 4a, solid lines). In the presence of A3G, a notable reduction in HIV-1 replication was observed at levels ranging from 20 to 30% of maximum (fig. 4a, dashed lines). The presence of Vif from the subtype B and CRF02_AG clones afforded a greater advantage to virus replication in the presence of A3G compared to Vif from subtype C clone (fig. 4a). A similar trend was observed using recombinant viruses derived from the CRF02_AG backbone (fig. 4b). In the absence of A3G, Vif from subtype C and CRF02_AG complemented Δ vif AG virus replication to 60 and 40% of maximum, respectively (fig. 4b, solid lines). However, the Vif-complemented Δ vif AG viruses,

Fig. 1. Phylogenetic analysis of Vif amino acid sequences. Phylogenetic analysis of available HIV-1 *vif* nucleotide sequences ($n = 1,004$) and subtype-specific molecular clones used in this study. The *vif* nucleotide sequences were aligned and translated using VectorNTI software. The resultant amino acid sequences were realigned in VectorNTI and the phylogenetic analysis was performed using the

MEGA 4 program. Subtype Contig (consensus) sequences were derived from all available *vif* nucleotide sequences belonging to their respective subtype or circulating recombinant forms (CRF). Homology among subtypes B, C and CRF02_AG ranged between 80 and 85% at the amino acid level. Vif amino acid sequences from HIV-1 groups N and O were also included in this phylogenetic analysis.

produced in the presence of A3G, showed reduced levels of replication, ranging from 5 to 20% of maximum (fig. 4b, dashed lines). Furthermore, Vif from the subtype B and CRF02_AG clones overcame A3G-mediated restriction more effectively than Vif from subtype C as shown by a greater replicative advantage.

In summary, we have shown that recombinant viruses generated from different genetic backbones, in the presence of A3G, exhibited different abilities to replicate

in TZM-bl cells. We have also shown that Vif-containing viruses derived from pNL/ADA or pAG_97 were able to overcome A3G restriction more efficiently than Vif derived from pINDIE_C (fig. 4a, b). Our study supports and extends earlier works that used chimeric proteins, that reported that Vif proteins derived from HIV-1 clinical and viral isolates of different subtypes varied in their activities against A3G [10, 11]. In contrast to our results, others noted that Vif derived from subtype C had higher

	10	20	30	40	50	60	70	80	90	100
n14-3	MENRWQVMIV	WQVDRMRINT	WKRLVKHHMY	ISRKAKDWFY	RHHVESTNPK	ISSEVHIPLG	DAKLVTITYW	GLHTGERDWH	LGQGVSIIEWR	KKRYSTQVDP
B										
C	L.....	K.R.....	NS.....	V.R.G.....	D.RH.....	V.....	E.R.....	K.....	Q.....	H.....
CRF_AG	I.....	K.....	NS.....	K.....	G.....	RH.....	V.....	R.....	VR.....	QR.....

	110	120	130	140	150	160	170	180	190	
n14-3	DLADQLIHLH	YFDCFSESAL	RNTILGRIVS	PRCEYQAGHN	KVGSLLQYLAL	AALIKPKQIK	PPLPSVRKLT	EDRWNKPKQT	KGHRGSHTMN	GH*
B										*
C	G.....M.....	AD.....	KA.....	H.....	I.....	D.....	T.....	K.....	IK.....	V.....
CRF_AG	H.....	D.....	KA.....	EV.....	Q.....	PE.....	K.....	VT.....	TKT.....	K.....

Fig. 2. HIV-1 Vif amino acid sequence alignment of the Vif clones used in this study. pNL4-3 (n14-3) was used as a reference sequence for alignment. The Vif amino acid sequences of these molecular clones pNL/ADA (B), pINDIE_C (C) and pAG_97

(CRF_AG) were derived by translating in house sequencing data of vifs that were cloned into the mammalian expression vectors. Amino acid sequences were aligned using ClustalW alignment in Bioedit.

Fig. 3. Replication of HIV-1 in the presence and absence of A3G. **a** A3G-TZM-bl HIV-1 replication assay. Vif-deficient (Δ vif) clones were generated by introducing nonsense mutations using a site-directed mutagenesis kit (Stratagene) at codons 26 and 27 of vif. The resultant clones were sequenced to confirm the presence of the stop codons. Replication of subtype C viruses derived from the pINDIE_C full-length (C) or vif-deleted (Δ C) molecular clones were evaluated in the absence and presence of A3G. Luciferase readout was used to determine the extent of HIV-1 replication. **b** Replication of vif-deleted recombinant HIV-1 in the TZM-bl assay. Vif coding sequences from subtype-specific molecular clones were cloned into BamHI and EcoRI (NEB) digested pcDNA3.1/V5-HisA (Invitrogen). A 5' His-tag was introduced into the clones

using SDM. Clones were confirmed by digestion, sequencing and Western blotting. Vif derived from each of subtype B (B_{vif}), C (C_{vif}), or CRF02_AG (AG_{vif}) molecular clones was supplied *in trans* to complement the Δ vif (Δ C) molecular clone during virus production. All viruses produced were in A3G-free conditions. **c, d** Dose response of vif-deleted pINDIE_C (Δ C) to A3G. Recombinant virus was generated using Δ C backbone with Vif supplied *in trans* on an expression plasmid from subtype C (C_{vif}), shown in **c**, or B (B_{vif}), shown in **d**. To obtain % HIV-1 replication we normalized the measured luciferase activity for each virus tested to the assay maximum output point set by virus, pNL4-3 (produced in the presence of A3G). All data depict the results of 3 independent experiments performed in duplicate $\pm$ SD.

Fig. 4. Impact of Vif variability on A3G-mediated antiviral activity and HIV-1 replication. Effect of Vif subtype variability on A3G-mediated antiviral activity using the ΔC (a), or the ΔAG viral backbone (b) where Vif was supplemented *in trans* from subtype B (B_{vif}), C (C_{vif}) or CRF02_AG (AG_{vif}). The solid lines

represent replication of viruses produced in the absence of A3G. The dashed lines represent replication of viruses produced in the presence of A3G. Data depict the results of 3 independent experiments performed in duplicate $\pm$ SD.

anti-A3G activity than those from subtypes A, B, D and CRF01_AE and CRF02_AG viruses that were tested [11]. In our study, Vif derived from the molecular subtype C clone was the least efficient among the Vifs tested in overcoming A3G restriction (fig. 4a, b). Previous work showed that Vif proteins derived from three distinct subtype C viral isolates varied in anti-A3G activity. Variations in the anti-A3G activity of Vif may be due to amino acid sequence differences between the subtype C Vif molecules used in these studies. In fact, Vif derived from the pINDIE_C clone, used here, shares similarity to Vif derived from the C1 isolate that had the lowest infectivity among viruses belonging to subtypes A, B, D, F and G that were studied [10]. The impact of Vif sequence variations on the Vif-A3G axis remains to be determined. Another possible reason for differences in Vif anti-A3G activity reported by us and others may be the experimental system used to evaluate this activity. Here, we employed a physiological system in which the viral backbone is full-length and subtype-specific, whereas others employed a read-out system that may be less rigorous than the assay described here or have produced the recombinant virions on a subtype B backbone [10, 11]. Additionally, all our recombinant clones retained their subtype-specific envelopes and displayed R5 tropism, in contrast to the pseudotyped virus approach used by others [11]. Furthermore, the target cells used in this study (TZM-bl) express both co-receptors required

for HIV-1 entry. This experimental design permits us to study how Vif can overcome A3G-mediated restriction in the course of HIV-1 replication.

Analysis of Vif structural and functional domains revealed that the greatest degree of variation was present in the Vif nuclear localization inhibitory sequence and phosphorylation sites (table 1) [14, 15]. Phosphorylation is known to play an important role in Vif function and regulation of HIV-1 replication [16]. Phosphorylation of Vif serine-144, part of the SLQYLA domain, inhibits elongin C binding and this interferes with A3G ubiquitination [4]. However, the impact of Vif phosphorylation on HIV-1 replication or infectivity at other phosphorylation sites has yet to be determined. We also observed additional variability at specific locations outside the previously defined conserved domains (table 1). A number of these variations were present in Vif sequences derived from the molecular clones that we employed (fig. 2). Further evaluation of such sequence variations will be necessary to mechanistically understand the basis for Vif subtype variability in the context of A3G restriction.

HIV-1 heterogeneity is due in part to lack of RT proof-reading ability and the high rate of HIV-1 replication and recombination. The ability of A3G to introduce mutations into viral DNA may also contribute to viral diversity and impact viral pathogenesis. High-level inhibition of A3G may reduce the HIV mutation rate and generate a more genetically stable virus population, which may

Table 1. Analysis of Vif functional and structural domains among different HIV-1 subtypes and CRFs

Subtype	5,11,21 38W ^a	HH 42–43 ^b	90–93 ^c	S95, T96, S165, T170 and T188 ^d	H108, C114, C133 and H139 ^e	144–149 ^f	161–164 ^g
A _(n = 80) ¹	– ²	–	R90K, K91LQ, K92Rge, R93kgs ³	S95NR, T170Av, T188s	–	–	–
B _(n = 410)	W21mt W38r	H42pq	R90Kwe, K91ERt, K92REG, R93KGst	S95NRT, S165sn, T170a, T188Sip	C114wr	S144Tf	L163Fv
C _(n = 364)	W11r	H42n	R90ksi, K91LWm, K92RG, R93KGSe	S95NR, T170VA, T188I	–	–	–
D _(n = 49)	–	–	K91t, K92Rg, R93k	S95n	–	–	–
F _(n = 16)	–	–	K91Q, K92G, R93kn	S95R, T170V	–	–	–
G _(n = 7)	–	H42p	R90k, K91Q, K92R, R93ke	T170a	–	–	–
H J K _(n = 7)	–	–	K91lq, K92er, R93k	S95Rjk, T170V	–	S144Th	L163Vk
AE _(n = 53)	–	–	R90kg, K91Qr, K92RG, R93kent	S95NGT, T188p	H108r, C133w	–	P161l
AG _(n = 18)	–	–	R90K, K91Q, K92R, R93kgs	S95wi, T170A, T188Sp	–	–	P164a

^a Tryptophan-rich stretch – involved in A3G and A3F binding. ^b Hydrophobic motif involved in A3G interaction (also requires amino acids 14–17 for interacting with A3G). ^c NLIS: nuclear localization inhibitory signal, RKKR. ^d Phosphorylation sites. ^e Zinc-binding motif HCCH. ^f Viral BC-Box, SLQYLA motif, elonginC binding. ^g Vif dimerization domain, PPLP.

¹ n: number of sequences analyzed. ² (–): no change in sequence was observed. ³ Lightface type indicates original amino acid at the designated location; underlined type indicates sequence variation from lightface type; Variations found in ≥5 sequences are in capital, and variations found in ≤5 are in lower case.

render it more susceptible to elimination by the immune system [17, 18]. On the other hand, A3G may increase the mutation rate in HIV, such that error catastrophe occurs from which the virus cannot recover. Subtype variations within Vif may play a role in this dynamic.

In recent years, several small molecules that can help A3G to evade Vif have been described [19, 20]. These molecules antagonize Vif by targeting it for proteasomal degradation and increase A3G incorporation into the virion, thus increasing the HIV mutation rate. Our findings suggest that Vif variability may have an impact on the Vif-A3G axis. The development of Vif antagonists should be approached with caution in order to maintain the HIV mutation rate in a range that will not provide a replicative benefit to the virus, which may in turn lead to increased HIV-1 pathogenesis.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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