Expression of feline immunodeficiency virus Vif is associated with reduced viral mutation rates without restoration of replication of vif mutant viruses

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Abstract

The vif gene of lentiviruses has been demonstrated to be essential for efficient viral replication in many cell types. Although the Vif protein of feline immunodeficiency virus (FIV) displays limited homology to HIV-1 Vif, the role of vif in FIV replication is not known. We have examined the requirements of vif for replication of a FIV strain isolated from a non-domestic felid, Otocolobus manul (FIV-Oma). In agreement with others, we find that replication of FIV vif mutant molecular clones in CrFK cells is highly attenuated. Initial attempts to rescue vif mutant viruses in trans were limited by lack of detectable wild-type Vif expression from DNA constructs. We demonstrate that FIV-Oma Vif expression can be increased by re-synthesis of the gene to remove splice donor and acceptor sites as well as improving codon usage to a mammalian codon optimized model. Cellular localization of resynthesized Vif (Vif-RS) is cytoplasmic. Clonal stable transfectants expressing HA-tagged Vif-RS do not restore replication levels of vif mutant virus. However, in such cell lines, G-to-A mutation rates in replicating wild-type viruses are reduced.

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Keywords: FIV; Vif; Lentivirus; APOBEC3G; FIV-Oma; Virus mutation; Retrovirus

Introduction

The viral infectivity factor (vif) is an accessory gene identified in all lentiviruses with the exception of equine infectious anemia virus (EIAV) (Kawakami et al., 1987; Oberste and Gonda, 1992). Although sequence similarities among Vif proteins are limited between lentiviruses (Oberste and Gonda, 1992), deletion or mutation of vif in human immunodeficiency virus type 1 (HIV-1) (Fisher et al., 1987; Gabuzda et al., 1992, 1994; Gibbs et al., 1994a; Strebel et al., 1987) and type-2 (HIV-2) (Michaels et al., 1993; Shibata et al., 1990), simian immunodeficiency virus (SIV) (Gibbs et al., 1994b; Shibata et al., 1990), caprine arthritis encephalitis virus (CAEV) (Har- mache et al., 1996), maedi-visna virus (MVV) (Kristbjornsdottir et al., 2004) and feline immunodeficiency virus (FIV) (Lockridge et al., 1999; Tomonaga et al., 1992) cause significant defects in viral replication in many primary lymphoid and myeloid cell lines termed “non-permissive” cells. However, in a subset of cell lines termed “permissive,” HIV-1 vif appears dispensable for viral replication (Courcoul et al., 1995; Fan and Peden, 1992; Fisher et al., 1987; Maki et al., 1992). The conflicting requirement of vif in viral replication has been resolved from studies differentiating cellular factors involved in HIV-1 viral replication in permissive and non-permissive cell types (Madani and Kabat, 1998; Simon et al., 1998; Sheey et al., 2002). A cellular protein, APOBEC3G, expressed exclusively in non-permissive cells, has been identified as the negative regulator of HIV-1 and SIV-1 vif-defective viral replication (Madani and Kabat, 1998; Sheey et al., 2002; Simon et al., 1998; Stopak et al., 2003; Yu et al., 2004a, 2004b). APOBEC3G, a member of the apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like (APOBEC) family of cytidine deaminases, is specifically packaged into HIV-1 virions and then blocks productive infection by catalyzing the deamination of deoxycytidine to deoxyuridine on the DNA minus strand during reverse transcription (Harris et al., 2003; Jarmuz et al., 2002; Lecossier et al., 2003; Mangeat et

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HIV-1 Vif abrogates the attenuation of HIV-1 vif mutant replication in non-permissive cells by recruiting an E3-ubiquitin ligase to APOBEC3G, thereby leading to its polyubiquitination, proteasomal degradation, and exclusion from assembling virus particles (Conticello et al., 2003; Marin et al., 2003; Sheehy et al., 2003; Yu et al., 2003). In addition, HIV-1 Vif inhibits the translation of APOBEC3G mRNA, further reducing levels of the enzyme within the cell (Stopak et al., 2003). Other members of the human APOBEC family, APOBEC3B and APOBEC3F, have also been associated with the inhibition of HIV-1 and HIV-2 replication (Bishop et al., 2004; Liddament et al., 2004; Wiegand et al., 2004; Zheng et al., 2004).

The vif gene of feline immunodeficiency virus (FIV) is similar in size and genome location to the vif gene of primate lentiviruses (Tomonaga et al., 1992). It encodes a protein that is highly hydrophilic and basic from spliced viral transcripts containing rev-responsive elements. Similar to primate lentiviruses, the C-terminus of FIV Vif contains a consensus (LQY/L/R/A) BC box motif. In HIV-1 Vif, this motif and an additional upstream zinc-finger motif has been demonstrated to complex with Cullin5, Elongin B, Elongin C, and Rbx1 to form a ubiquitin ligase (E3) complex which targets APOBEC3G and APOBEC3F for degradation (Mehele et al., 2006; Shirakawa et al., 2006; Yu et al., 2004a, 2004b). The conservation of the BC box motif in FIV Vif suggests that APOBEC3G inhibition may be a conserved function of lentivirus Vif proteins. In support of this, mutation of FIV Vif, including point mutations in the BC box, results in impaired cell-free infection of feline peripheral blood mononuclear cells (PBMCs) and established feline cell lines such as G355-5, CrFK, and MYA-1 (Lockridge et al., 1999; Shacklett and Luciw, 1994; Tomonaga et al., 1992). Additionally, FIV vif has been shown to be necessary for efficient viral replication during in vivo infections of cats (Inoshima et al., 1996). Although a cell line permissive for replication of vif-defective FIV strains has yet to be identified, Tomonaga et al. (1992) have shown that virus produced from CrFK cells transfected with a vif mutant molecular clone of FIV could infect a CD4+ T-cell line (Mya 1) by co-cultivation of T-cells with virus producing CrFK cells. Thus, in this system, Vif expression appears necessary to achieve cell-free infectivity, but dispensable for virus spread via cell-to-cell contact.

To further elucidate the role of vif in the FIV lifecycle, we have attempted to rescue infection of FIV vif mutant virus by expression of Vif in trans. A molecular clone of FIV-Oma, derived from a highly cytopathic FIV strain from a Pallas cat (Barr et al., 1995, 1997) was utilized in this study due to this isolate's replication efficiency and syncytium formation in CrFK cells compared to domestic FIV isolates (Barr et al., 1995). In this study, we have found that mutant FIV-Oma infectious clones with frameshift and deletion mutations in vif, replicate at lower levels compared to wild-type virus in CrFK cells. Replication of vif mutant Vif could not be restored by expression of wild-type Vif in trans. However, levels of G-to-A mutations in proviral DNA are reduced by infection of wild-type virus in a Vif-expressing cell line. Thus, FIV Vif may function similarly to HIV-1 Vif in preventing APOBEC-induced mutagenesis of proviral DNA during reverse transcription.

Results

vif mutant virus replication is attenuated

Using the infectious clone of the FIV Oma strain, pOma3, as starting material, we generated two different vif mutant viruses (Fig. 1A). The first, termed FIV VifΔ, was constructed by deleting the sequence between two unique Stul sites within the vif open reading frame. This deletion mutant removes 302 bp within the vif open reading frame (positions 325–626), however maintains predicted splice donor and acceptor sites within the resultant vif transcript. Additionally, a frameshift as a result of the deletion of the wild-type Vif protein adds eight missense codons after amino acid 109 and prematurely terminates translation. A second vif mutant, FIV Vif-fs, was created by insertion of a TA dinucleotide between base pairs 66 and 67 that forms a translation stop codon after amino acid 22. Since this mutant retains the complete vif sequence, it is unlikely that processing of the downstream subgenomic transcripts is altered in this mutant.

To assess the replication efficiency of vif mutant viruses, we transfected CrFK cells with molecular clones for wild-type virus (pOma3) and the two vif mutant viruses (FIV VifΔ and FIV Vif-fs). Monitoring of cell culture supernatant for reverse transcriptase (RT) activity, an indication of viral particle release, shows a peak in RT activity at day 11 for cells transfected with the wild-type virus that subsided at day 14, at which point cells displayed cytopathic effects (CPE) associated with viral replication (Fig. 1B). Supernatant from cells transfected with FIV Vif-fs displayed detectable RT activity at levels approximately eightfold lower than the wild-type virus at day 11. These data suggest that low levels of viral particles are released from cells transfected with the FIV Vif-fs mutant clone; however, formation of viral particles or their release is impaired due to the vif mutation. RT activity above background could not be detected in supernatant of cells transfected with the FIV VifΔ virus (Fig. 1B), indicating that viral replication is completely restricted in this clone. CPE were not observed in CrFK cells transfected with either vif-mutant molecular clone (data not shown).

Expression of tagged Oma3 Vif is not detectable in mammalian cells

To evaluate the role of vif in the FIV viral life cycle, we sought to generate lines of CrFK cells that express Vif. During the course of these experiments, achieving detectable levels of Vif protein expression proved problematic. In addition, FIV-Oma Vif was poorly antigenic in rabbits (data not shown) and, therefore, had to be fused to an epitope tag in order to visualize cellular localization and expression. In a first set of experiments, we cloned vif as a C-terminal fusion to enhanced green fluorescent protein (EGFP), referred to as EGFP-Vif, to monitor in vivo expression levels. In these experiments, transfection of
control EGFP and EGFP-Vif expression vectors into CrFK cells produced detectable levels of fluorescence at 24 h post-transfection. However, when the expressed protein product was evaluated by western blot using an antibody against EGFP, the EGFP-Vif fusion protein exhibited a similar molecular weight as control EGFP (Fig. 2A; compare lanes 2 and 3). To test the ability of the EGFP-Vif construct to be properly translated, we expressed the EGFP-Vif fusion in bacteria. After a 2-h induction, western blots showed the EGFP-Vif fusion protein at approximately 70 kDa (Fig. 2A, lane 4). Thus, although the EGFP-Vif fusion protein was properly cloned and capable of being translated in bacteria, it appears to be poorly expressed in eukaryotic cells.

One possible explanation for the poor expression and truncation of Vif fusion constructs is the improper splicing of transcripts containing the vif open reading frame. The vif transcript sequence contains a predicted splice donor and acceptor sites after positions 24 and 689 bp within the vif open reading frame with the potential to produce a truncated 11 a.a. protein product. The VifΔ virus is deleted between two Stul sites at 325 and 626 bp within vif open reading frame. The resultant transcript maintains sequences at the 3’ of the vif transcript (denoted by line) including the predicted splice acceptor site. The mutated open reading frame is predicted to express a 117 a.a. protein containing 109 a.a. from the N-terminus of wild-type Vif and 8 a.a. from an alternative reading frame. The Vif-fs virus incorporates an extra base pair at position 67 of the vif open reading frame causing a frame shift (fs) that terminates translation before amino acid 22. (B) Replication kinetics of FIV-Oma wild-type and mutant strains FIV VifΔ and FIV Vif-fs in CrFK cells. Replication of virus was measured by reverse transcriptase activity in aliquots of 10 μL of media up to 14 days post-transfection. Data presented represent the average RT activity for three independent transfected wells at each time-point.

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**Resynthesis of codon-optimized vif**

Examination of the vif sequence from positions 5429 to 6187 bp of pOma3 shows that codon usage is most similar to
that of prokaryotes, containing a high percentage of third codon position adenines (As) as opposed to guanines (Gs). Synonymous codon changes that better reflect mammalian codon usage and at the same time eliminated the splice donor and acceptor consensus sequences were made to improve Vif expression. The 252 codon vif gene was resynthesized with 42 synonymous codon changes using 21 overlapping oligonucleotides (Table 1). The reassembled vif gene was named vif-RS, and the sequence of this synthetic gene is shown in Fig. 3.

**Cellular localization of Vif**

vif-RS was cloned as a C-terminal fusion to EGFP and the resultant expression construct was transfected into CrFK cells. A protein consistent with the predicted molecular weight of the EGFP-Vif-RS fusion protein was detected by western blot (Fig. 2B, lane 2). To control for possible artifacts in Vif localization due to fusion to EGFP, a second Vif-RS fusion was made that replaces the EGFP reporter with an N-terminal 3X-HA epitope. Western blots of both stably and transiently transfected cells showed a reactive species near the predicted 35 kDa molecular weight of the HA-Vif-RS fusion (data not shown). The cellular localization of the HA-Vif-RS fusion was tested in CrFK cells stably transfected with the HA-Vif-RS expression vector. Immunofluorescence using an antibody for the HA epitope tag detected expression of HA-Vif-RS dispersed evenly throughout the cytoplasm; however, expression was excluded from the nucleus (Figs. 4C, D). CrFK cells stably transfected with HA-Vif-RS and infected with Vif-Oma virus demonstrate cytoplasmic localization of both HA-Vif-RS (right panel) and FIV p24 Gag (left panel) (Figs. 4E, F). Similar cytoplasmic staining for HA-Vif-RS has been observed in transiently transfected MDCK cells as well as in CrFK cells using fixation with either paraformaldehyde or methanol (data not shown).

**HA-Vif-RS expression in trans does not rescue vif mutant FIV replication**

The ability of Vif expression in trans to rescue attenuation of vif mutant FIV strains was tested in CrFK cells stably transfected with HA-Vif-RS. Two separate HA-Vif-RS-expressing clones were transfected with molecular clones for wild-type FIV-Oma, or vif mutant FIV VifΔ or FIV Vif-fs to

**Table 1**

<table>
<thead>
<tr>
<th>Primer positions/ vif gene resynthesis primer sequences</th>
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<tr>
<td>VIF 1F (55nt)</td>
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<td>GGAATCCATGAGGGTGAGAAGGATTGGAAGAGTCCCGCTCTCTTATCAAGTGC</td>
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<tr>
<td>VIF 2 (55nt)</td>
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<tr>
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<tr>
<td>VIF5 (55nt)</td>
</tr>
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<tr>
<td>VIF6 (55nt)</td>
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initiate infection. Cell supernatant was monitored for RT activity for the ability of the Vif-expressing cells to rescue \textit{vif} mutant viruses. During a 14 day period, neither HA-Vif-RS-expressing cell lines was capable of restoring infectivity of either \textit{vif} mutant virus (data not shown). Wild-type FIV-Oma virus replicated with slower kinetics in the two HA-Vif-RS-expressing cell lines compared to control CrFK cells; RT activity was first detected at day 13 post-infection (p.i.) in infected control CrFK cells versus day 18 p.i. and day 22 p.i. in the two HA-Vif-RS-expressing clonal cell lines (Fig. 5). Additionally, virus replicating in HA-Vif-RS-expressing cell lines displayed higher RT activity and delayed CPE compared to replication in control cells (Fig. 5). At the termination of the experiments due to virus-induced cell death at day 18 p.i. in control CrFK cells and at day 25 in HA-Vif-RS CrFK clones, HA-Vif-RS-expressing cells displayed 2.3–3 times the RT activity of control cells.

\textbf{Vif expression in trans reduces mutation of pOma3 provirus in infection}

If the FIV Vif protein acts in a similar manner to HIV Vif in blocking the packaging of cellular APOBEC3G, we hypothesized that Vif expression in \textit{trans} might reduce deamination derived mutations in proviral DNA. We initiated infection of control CrFK cells and the two HA-Vif-RS-expressing clonal cell lines by infection with cell-free supernatant from pOma3 transfected cells. Plus-strand virion DNA was isolated from pelleted viral particles and the frequency of viral mutations was detected by PCR amplification of a 592 bp region in \textit{pol} containing 118 Gs from each culture at 7 and 10 days p.i. PCR-amplified fragments were cloned and multiple clones were DNA sequenced. The resultant sequences were analyzed for G-to-A base changes indicative of cytidine deamination of the DNA minus strand. In control FIV infected CrFK cells, G-to-A conversions were detected, averaging 2.18 and 2.61 sites per 118 Gs in the amplicon at day 7 and day 10, respectively (Table 2). This compares to an average mutation rate of 0.27 and 0.16 changes per site at day 7 and day 10 for all other positions in the 592 bp \textit{pol} region. The frequency of G-to-A exchanges in individual clones ranged from 0 to 7 at day 7 and 0 to 6 at day 10. In contrast, G-to-A exchanges were reduced in viral DNA to an average of 0.13 and 0 sites per 118 guanine positions in two separate HA-Vif-RS-expressing cell lines at day 7 (Table 2). At day 10, a slight increase in G-to-A conversions was detected, averaging 0.5 to 0.38 sites per 118 Gs in FIV-Oma infected Vif-expressing cell lines. Individual clones contained between 0 and 2 G-to-A exchanges per sequence at day 10. Mutation rates of non-guanine positions in FIV \textit{pol} ranged from 0.28 to 0.38 changes at day 7 and 0.21 to 0.23 changes at day 10 per 474 positions analyzed.

\textbf{Discussion}

In this study, we sought to better define the role of \textit{vif} in the FIV viral life-cycle in light of recent studies in primate lentiviruses demonstrating a role for Vif in preventing APOBEC3G-induced cytosine deamination during minus strand synthesis. Similar to previous reports with different FIV isolates (Lockridge et al., 1999; Shacklett and Luciw, 1994; Tomonaga et al., 1992), replication of several \textit{vif} mutant viruses of a non-domestic cat isolate of FIV (FIV-Oma) displayed highly attenuated replication in CrFK cells compared to wild-type virus. As opposed to the primate lentiviruses (Blanc et al., 1993; Simon et al., 1995), replication of \textit{vif} mutant forms of FIV-Oma could not be restored by infection of cell lines stably expressing Vif in \textit{trans}. However, wild-type viruses replicating in Vif-expressing cell lines displayed fewer G-to-A mutations, suggesting that FIV Vif may act in a manner similar to primate
lentiviruses in preventing APOBEC3G-induced mutation of nascent viral DNAs.

We have observed differences in the replication kinetics of two separate FIV-Oma \textit{vif} mutant viruses. The first mutant, containing a premature stop codon after amino acid 21 (FIV Vif-fs) maintained low levels of detectable RT activity in the supernatant of transfected cells that slightly increased over the course of 2 weeks. It is possible that the FIV Vif-fs virus is capable of maintaining low levels of virus production by direct cell-to-cell transmission previously reported for other FIV \textit{vif} mutant viruses (Shacklett and Luciw, 1994; Tomonaga et al., 1992). However, FIV Vif-fs does not appear capable of initiating cell-free infection as filtered supernatant from transfected CrFK cells was unable to initiate \textit{de novo} infection in CrFK cells (data not shown). A second \textit{vif} mutant, FIV VifΔ, a 117 amino acid truncated form of FIV Vif, had undetectable RT activity suggesting that both cell-to-cell and cell-free transmission are completely restricted. As the FIV VifΔ mutant possesses a deletion from positions 325 to 626 bp of \textit{vif}, we cannot rule out that the phenotype of the FIV VifΔ mutant is attributed to the disruption of viral regulatory sequences within the mutation site affecting the

Fig. 4. Immunofluorescence of CrFK cells stably transfected with HA-Vif-RS using an anti-HA antibody indicates HA-Vif-RS expression in cytoplasmic (C) and excluded from the DAPI stained nucleus (D). Control CrFK similarly stained with anti-HA antibody indicates that expression is specific for the stably transfected cell line (A, B). CrFK cells infected with FIV-Oma and stably transfected with the HA-Vif-RS expression construct (E, F). Antibody to FIV p24 detects FIV infection of a syncytium (E) that also expresses HA-Vif-RS detected with an anti-HA antibody (F). Expression of both proteins appears confined to the cytoplasm.
expression of downstream genes *orf2*, *env*, and *rev*. However, *vif* transcripts in both mutant viruses retain predicted splice donor and acceptor sites (Fig. 1A). Thus, additional regulatory sites within the deleted region might be disrupted as a result of the 302 bp deletion in the FIV VifΔ mutant.

Our attempts to rescue the phenotype of the FIV-Oma vif mutants were hindered by poor expression of Vif in cell culture. High levels of HIV-1 Vif expression have also been difficult to achieve due to inefficient nuclear export of *vif* transcripts, poor mammalian codon usage, and high turnover rate of Vif protein (Fujita et al., 2004; Nguyen et al., 2004; Wang et al., 2005). Restricted *vif*-expression levels may represent a selective advantage for retroviral replication. Akari et al. (2004) suggested that high protein turnover rates of HIV-1 Vif prevent the improper processing of Gag polyproteins during virus maturation. Cells over-expressing HIV-1 Vif accumulate Gag processing intermediates and exhibit impaired replication rates compared to cells expressing Vif at physiological levels (Akari et al., 2004). Interestingly, we found that wild-type FIV-Oma replication in FIV Vif-expressing cells displays delayed replication kinetics and attains higher supernatant RT activity before the onset of virus-induced cytopathic effects. This suggests that high levels of FIV Vif expression in our system may have negative consequences on the replication of wild-type FIV.

In the course of our studies, we found that aberrant RNA splicing of *vif* transcripts contributed to the truncation of Vif fusion protein products. Aberrant *vif* splicing is conceivably a consequence of removing *vif* gene expression from the context of the viral genome and *rev* dependency. This became apparent when comparing the splicing pattern of *vif* transcripts during wild-type FIV infection and in cells where Vif was expressed independently of the FIV genome (Supplementary Fig. 1). When expressed alone, about one-half of the detected *vif* transcripts utilized splice donor and acceptor sites contained within the *vif* open reading frame. This splicing event is predicted to truncate the *vif* open reading frame and create a premature stop codon leading to the expression of an 11 amino acid peptide. During FIV infection of CrFK cells, the majority of the subgenomic *vif* transcripts comprise the full-length open reading frame. Although internally spliced *vif* transcripts were detected during FIV infection, the low intensity of these bands suggests that these represent a minor fraction of the *vif*-containing RNAs.

Our data that FIV-Oma Vif expression is confined to the cytoplasm correlate with reports in HIV-1 (Goncalves et al., 1994, 1995; Simon et al., 1997) and MVV (Audoly et al., 1992) infected cells. Expression in the cytoplasm would be consistent with a role of the Vif protein in regulating FIV virus maturation or modifying the infectivity of the virus after entry into a host cell by preventing APOBEC3G incorporation into virions. The cytoplasmic localization of HA-tagged Vif in cells infected with FIV-Oma contradicts a previous report showing FIV Vif expression in the nucleus. In this report, Chatterji et al. (2000) demonstrated that the subcellular localization of Vif in a feline astrocyte cell line, G355, infected with the FIV 34TF10 strain, differed depending upon the cellular fixation method prior to immunostaining; Vif localization appeared cytoplasmic in cells fixed with methanol and nuclear in cells fixed with 2% formaldehyde. Our results indicate that FIV-Oma Vif expression is invariably cytoplasmic in CrFK cells using either methanol or paraformaldehyde fixation. Thus, FIV Vif subcellular localization may vary between domestic and non-domestic cat strains of FIV or may differ depending upon the cell type in which it is expressed.

A surprising finding in our study is that Vif expression in trans was unable to rescue the replication of *vif* mutant viruses. This result differs from published studies demonstrating that HIV-1 Vif expression can abrogate the replication block for *vif*-

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**Table 2**

Frequency of G-to-A base changes detected in FIV-Oma DNA genomes during infection of control CrFK cells and two clones of CrFK cells expressing Vif-RS

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Day 7</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean G-to-A changesa (range)</td>
<td>Mean other base changesb</td>
</tr>
<tr>
<td>CrFK control</td>
<td>2.18 (0-7)</td>
<td>0.27</td>
</tr>
<tr>
<td>CrFK Vif-RS Clone 1</td>
<td>0.13 (0-2)</td>
<td>0.28</td>
</tr>
<tr>
<td>CrFK Vif-RS Clone 2</td>
<td>0 (0)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

*a* Mean G-to-A exchanges were detected in the indicated number of sequenced clones from a possible 118 Gs within the analyzed region. The range of values from sequenced amplicons is noted in parenthesis.

*b* Mean base changes detected in 474 non-guanine positions.
mutated viruses in non-permissive cells by preventing APOBEC3G incorporation into virions (Harris and Liddament, 2004). As members of the APOBEC3 family appear conserved throughout vertebrate evolution, Vif proteins of lentiviruses from primates (Harris and Liddament, 2004) and sheep (Kristbjörnsdóttir et al., 2004) have co-evolved to counteract the host anti-viral mechanism. Recent studies by Foss et al. (2006) and Lochelt et al. (2005) indicate that the APOBEC3-mediated mechanism of retroviral inhibition is conserved in felines. In support of this, the feline foamy virus Bet protein has been demonstrated to bind a feline APOBEC3 orthologue (fe3) and inhibit the cytidine deamination of the spumavirus genome (Lochelt et al., 2005). Our finding that fewer G-to-A mutations in wild-type FIV-Oma replicating in CrFK cells stably overexpressing FIV-Oma Vif suggests that FIV Vif is capable of preventing APOBEC-induced mutations in cells from domestic cats. However, the inability of Vif expression in trans to restore infectivity of vif mutant viruses suggests that additional functions of FIV Vif are not complimented in our system. It is possible that the spatial or temporal expression of Vif in our studies may not adequately reflect the native behavior of a multifunctional protein. Analysis of the interaction of FIV Vif and the feline APOBEC3 orthologue (fe3) will facilitate a further understanding of the function of FIV Vif in viral replication.

Materials and methods

Cell lines and virus

The FIV-Oma strain isolated from Pallas’ cat used in this study has been previously described (Barr et al., 1995, 1997), pOma3 is an infectious molecular clone derived from the FIV-Oma isolate. The sequence of the pOma3 proviral clone has been deposited in GenBank, accession number AY713445.

CrFK cells (ATCC CCL-94) were grown in 70% MEM-H, 30% L-15 and 10% FBS supplemented with 25 mM HEPES and 50 μg/mL gentamicin. MDCK cells (ATCC CCL-34) were grown in MEM medium containing 10% FBS and 50 μg/mL gentamicin. Both cell lines were grown in 37 °C incubators with 5% CO2. All transfections were done using Lipofectamine and the feline APOBEC3 orthologue (fe3) will facilitate a further understanding of the function of FIV Vif in viral replication.

Generation of vif mutant FIV strains

FIV pOma3 VifΔ molecular clone was constructed by digesting pOma3 with SstI and self-ligating the two unique enzyme sites within the vif open reading frame. FIV Vif-fs was made by site-directed mutagenesis of pOma3 using primers forward 5′-GGGGACCTAGAAGGCTATAGCATTAGCAT-TATGCTCTATAGG-3′ and reverse 5′-CCCTATAGGACATAGCATGCTCTTATAGG-3′ with the QuikChange kit from Stratagene. The primers insert a TA dinucleotide (sequence underlined) at 67 bp within the vif open reading frame causing the mutation of a SapI enzyme site and forming premature termination codons. Following the construction of both mutant molecular clones, DNA sequencing verified that no additional sequence changes were introduced during the cloning process. Infectivity of the molecular clones was measured by a reverse transcriptase assay, using procedures previously described (Zou et al., 1997). Aliquots from three independent transfections were measured for RT activity at each time point.

Vif resynthesis

FIV-Oma vif gene was resynthesized using the primers shown in Table 1. Amplification in 100 μL was as follows: primer vif 1 and vif 2 at 1 nM, Taq polymerase ten cycles of 94 °C for 30 s, 40 °C for 30 s and 72 °C for 30 s then five cycles of 94 °C for 30 s and 72 °C for 30 s. PCR products were electrophoresed in 2% Nusieve gels, and bands were gel-purified and added to the second amplification using adjacent primer sets (e.g. vif 1 and vif 3). After synthesis with primers vif 1–6, vif 5F-12 and vif 11F-19, products were cloned in to pCR2.1Topo TA vector and multiple clones were sequenced. Reassembly of resynthesized vif gene was accomplished by
cloning vif 1–6 cut with BamHI and EcoRI into pBluescript SK (−), followed by digestion of the resultant clone and vif 5F-12 with SfiI and EcoRI and ligation. The vif 11F-19 clone and the Bluescript clone of vif 1–12 were then digested with EcoNI and XhoI and fragments were ligated. The resultant vif clone was named vif-RS. All DNA sequencing was performed at the core facility at Cornell University Bioresource Center (Ithaca, NY).

For mammalian expression, the vif-RS digested with BamHI and XhoI was subcloned into pEGFP-C1 cut with BgIII and SalI, to create an N-terminal EGFP fusion, EGFP-Vif-RS. The same fragment was cloned into pCDNA4hismaxC cut with BamHI and XhoI. This plasmid was then digested with SnaBI and BamHI and ligated with a fragment containing a 3XHA tag derived from the plasmid (a generous gift from Dr. Jun-Lin Guan, Cornell University) digested with similar enzymes. This process exchanges the CMV promoter and Xpress epitope of pCDNA4hismaxC with the CMV promoter and 3X HA tag of pKH3-FAK to create the HA-Vif-RS expression construct.

Western blots and immunofluorescence

Cell lysates used for western blots were dissolved in Nupage (Invitrogen) sample buffer, boiled and run on Nupage 4–12% Bis–Tris gels in MOPS buffer and electroblotted to nitrocellulose as described in the manufacturer’s protocol. Membranes were probed with antibodies to anti-HA (Upstate), anti-GFP (Invitrogen) and anti-FIV p24 (Biodesign International) followed by incubation with species-specific secondary antibodies conjugated to alkaline phosphatase. Immunoreactive bands were detected with BCIP/NBT staining (KPL).

For indirect immunofluorescence staining, CrFK or MDCK cells expressing HA-Vif-RS were grown to approximately 50% confluency on 12-mm-diameter coverslips pretreated with 10 mg of poly-L-lysine per milliliter to aid cell attachment and spreading. For FIV Gag localization, CrFK cells were transfected with the FIV-Oma infectious clone pOMa3 using Lipofectamine reagent (Invitrogen). Five days post-transfection, equal volumes of supernatant were used to infect 1×10⁶ control CrFK cells as well as two clonal CrFK cell lines expressing HA-Vif-RS. Infection of each line was verified using indirect immunofluorescence staining procedures described above. At 7 and 10 days post-infection, viral pellets were prepared as previously described (Kristbjornsdottir et al., 2004). Virion incorporated DNA was isolated with a DNA Blood Mini Kit (Qiagen). Aliquots of 100 ng of DNA were utilized in PCR reactions with a high fidelity Taq polymerase (Invitrogen) using forward primers 5'-TAAGCAATGGGCCTCTTATCAAA-3' and reverse primers 5'-TAAAGCAATGGCCTCTATCAA-3' and conditions as follows: 94 °C for 30 s, 50 °C for 1 min and a 68 °C extension for 1 min, for 35 cycles. The resultant PCR products encompassing positions 2585–3177 within the pol region of pOMa3 were cloned into the pCR2.1 TA cloning vector. Fifteen independent cloned products were DNA sequenced using M13F and M13R primers and analyzed for DNA mutations.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2006.11.005.

References


RNase protection

pvif/TA9 (opposite orientation of the PCR-cloned vif insert to pvif/TA1) was linearized with DraI in order to make an antisense transcript containing 138 nt from the 3' end of vif and 78 nt of vector sequence (216 nt total). In vitro transcription was performed with T7 RNA polymerase, using Ambion’s RPA III kit. Total RNA from transfected and infected CrFK cells was prepared with the Rneasy kit (Qiagen). 8×10⁴ cpm of in vitro transcripts was annealed overnight to 10 μg of total RNA. A 10-bp ladder (Invitrogen) was labeled by a kinase-exchange reaction with γ-ATP. Samples were electrophoresed on Novex 7% acrylamide TBE/urea gels and exposed to X-ray film.

Analysis of mutations in replicating FIV

CrFK cells were transfected with the FIV-Oma infectious clone pOMa3 using Lipofectamine reagent (Invitrogen). Five days post-transfection, equal volumes of supernatant were used to infect 1×10⁶ control CrFK cells as well as two clonal CrFK cell lines expressing HA-Vif-RS. Infection of each line was verified using indirect immunofluorescence staining procedures described above. At 7 and 10 days post-infection, viral pellets were prepared as previously described (Kristbjornsdottir et al., 2004). Virion incorporated DNA was isolated with a DNA Blood Mini Kit (Qiagen). Aliquots of 100 ng of DNA were utilized in PCR reactions with a high fidelity Taq polymerase (Invitrogen) using forward primers 5'-TAAGCAATGGGCCTCTTATCAAA-3' and reverse primers 5'-TAAAGCAATGGCCTCTATCAA-3' and conditions as follows: 94 °C for 30 s, 50 °C for 1 min and a 68 °C extension for 1 min, for 35 cycles. The resultant PCR products encompassing positions 2585–3177 within the pol region of pOMa3 were cloned into the pCR2.1 TA cloning vector. Fifteen independent cloned products were DNA sequenced using M13F and M13R primers and analyzed for DNA mutations.

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