The requirement for Vif of SIVmac is cell-type dependent

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The vif gene (viral infectivity factor) of the human and simian immunodeficiency viruses (HIV and SIV) is present in almost all members of the lentivirus group of retroviruses. This gene is highly conserved among different HIV and SIV isolates and is therefore presumed to play an important role in pathogenesis. To analyse the role of Vif in SIV, three SIVmac mutants have been constructed by introducing site-specific mutations or deletions into vif of the pathogenic molecular clone SIVmac239. The effect of Vif on viral replication in T cells was examined by transfecting equal amounts of either vif-positive or vif-negative viral DNA into SupT1, CEM-SS and H9 cells. Reverse transcriptase assay of supernatants from transfected cultures revealed that both SupT1 and CEM-SS cell lines supported replication of all three vif mutants to a level comparable to the parental vif-positive virus, whereas vif mutants did not replicate in H9 cells. Our results demonstrate that the requirement for Vif in SIVmac replication is cell-type dependent and that sequences near both the N and C termini are required for its function. Vif-defective SIVmac239, produced in transfected SupT1 and CEM-SS cells, failed to infect primary T lymphocytes, whereas both vif-positive and vif-defective viruses established productive infection in CEMx174 cells. These findings in primary cells suggest that Vif plays an important role in viral replication in vivo.

Introduction

Human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) as well as the related animal virus, simian immunodeficiency virus (SIV) encode not only the retroviral gag, pol and env genes but also additional genes which include the regulatory genes (tat and rev) and accessory genes (vif, vpu, vpr, and nef for HIV-1 and vif, vpx, vpr and nef for HIV-2 and SIV). These accessory genes are designated ‘non-essential’ because they are dispensable for viral replication in some in vitro tissue culture systems (Subbramanian & Cohen, 1994). However, the conservation of these genes in different viral isolates suggests a fundamental role in vivo (i.e., infection of the susceptible host).

The HIV-1 vif (virion infectivity factor) gene encodes a 23 kDa protein (Kan et al., 1986; Lee et al., 1986) which is a late gene product whose expression is Rev dependent (Garrett et al., 1991; Schwartz et al., 1991). The vif gene is highly conserved among different HIV isolates and is present in all members of the lentivirus group with the exception of equine infectious anemia virus (Oberste & Gonda, 1992). Initial studies have shown that vif-defective HIV-1 genomes introduced into permissive cells by transfection are transcribed and translated, and virions are released to a level comparable to cells transfected with the wild-type viral genome (Strebel et al., 1987; Sakai et al., 1993). However, vif-defective cell-free virions are 100–1000-fold less infectious (Fisher et al., 1987; Strebel et al., 1987). The decrease in infectivity of vif-defective HIV-1 particles is cell-type specific (Gabuzda et al., 1992). Studies on HIV-1 have indicated that vif mutant viruses derived from the T cell line CEM-SS are severely impaired in their ability to complete proviral DNA synthesis in non-permissive cells (Von Schwedler et al., 1993). The goal of this study was to analyse the role of Vif in SIV replication in T cell lines as well as primary lymphoid cells.

Methods

Construction of vif mutations in SIVmac239. All mutations were introduced into the proviral clone pMA239 (Fig. 1) provided by Dr A. Adachi (University of Tokyo). The SIVmac239Δvif mutant was constructed by deleting nucleotides between the BglII site at 5698 and the SacI site at 5845. A plasmid subclone containing a DNA fragment from the XbaI site at 4986 to the SacI at 6016 was first digested with BglII, filled in with the Klenow fragment of DNA polymerase I, then ligated to the blunt end of a SacI site. As a result, a Δvif construct containing a 147 bp deletion and a translational frame shift within the Vif coding region was generated. To determine the significance of Vif domains on function, two additional vif mutants were produced. The SIVmac239-2S vif mutant was constructed by introducing two consecutive stop codons to the N terminus at nucleotide (nt) 5670 by polymerase chain reaction (PCR). The SIVmac239-2C vif mutant was

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Fig. 1. Construction of three SIVmac239 vif mutants. The top of this figure is a diagrammatic representation of the SIVmac239 genome. Positions of restriction enzyme sites in the vif gene are shown. Locations of deletions and mutations in the clones of SIVmac239Δvif, SIVmac239-vif2S and SIVmac239-vif2C are also shown. The GenBank accession number for SIVmac239 is M33262.

constructed by introducing two consecutive stop codons near the C terminus at nt 6010 using oligonucleotides. Briefly, a plasmid subclone, containing a DNA fragment from the XbaI site at nt 4986 to the SacI site at nt 6016 was digested with Avai at nt 6007 and SacI at nt 6016 and replaced with oligonucleotides containing two consecutive stop codons; this mutant is truncated by 228 bp in the vif gene. All mutations were confirmed by DNA sequencing.

Because wild-type SIVmac239 does not replicate efficiently in H9 cells due to the full-length transmembrane domain of env (Kodama et al., 1989; Johnston et al., 1993), chimeras of SIVmac239 wt and SIVmac239Avif with the HIV-1s33 env gene were constructed. Briefly, the 5’ portion of pMA239 from the EcoRI site in the cellular flanking sequence to the SpeI site at nt 6707 was ligated to a DNA fragment of pSHIV-33/S (from SpeI site to EcoRI site; Luciw et al., 1995) containing the env gene of HIV-1s33 (York-Higgins et al., 1990).

Cells. The CEMx174 cell line is a human hybrid T/B cell line provided by J. Hoxie (University of Pennsylvania). CEM-SS, SupT1 and H9 T lymphoid cell lines were obtained from the AIDS Research and Reference Program, National Institutes of Health (Bethesda, Maryland). All T cell lines were maintained in RPMI medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum (FCS) and antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin). Because SIVmac239 replicates well in human peripheral blood mononuclear cells (huPBMC), these cells were used as the source of primary T lymphocytes. Importantly, huPBMC show prolonged growth in complete RPMI medium supplemented with human interleukin-2 (IL-2) compared to rhesus monkey PBMC. Human PBMCs were obtained from healthy seronegative blood donors (provided by Irwin Memorial Blood Bank, San Francisco, California), stimulated with phytohaemagglutinin (3 µg/ml), and maintained in RPMI1640 medium supplemented with 10% FCS and 10% interleukin-2 (Collaborative Research) and antibiotics. Rhesus PBMCs obtained from two healthy, seronegative macaques (#23803 and #25874) were stimulated and maintained in the same way as for the human PBMCs.

Transfections. Cell-free wild-type and vif-defective viruses were prepared by transfecting SupT1, CEM-SS and H9 cells with 5 µg of wild-type and vif-defective proviral plasmid by the electroporation method (200 V and 960 µF). Transfected cultures were monitored at 3-4 day intervals for virus production by mini-reverse transcriptase
Fig. 2. Replication of vif-positive and vif-negative SIVmac239 in CD4+ human T cell lines. (a) SupT1 and (b) CEM-SS (shown in this order) were transfected with 5 μg of wild-type SIVmac239 plasmid DNA (□) or with 5 μg of each of the vif mutants SIVmac239Δ (●), SIVmac239-2S (○) and SIVmac239-2C (▲) plasmid DNA using a Gene Pulser (Bio-Rad) at 960 μF, 200 V. Viral replication was monitored by measuring RT activity in supernatants of transfected cell cultures. Solid squares (■) represent RT activity of culture supernatants of mock-transfected cells. The results in this figure are representative of at least three independent experiments.

Fig. 3. Replication of vif-positive and vif-negative SIVmac239 viral DNA or chimeric wild-type SHIV Spru and SHIV Spru Δvif viral DNA in H9 cells. Replication of viruses was monitored by measuring RT activity in supernatants of transfected cell cultures. The results shown are representative of at least three independent experiments.

(RT) assay (Wiley et al., 1988). Supernatants harvested at the peak of RT activity were used for infections of CEMx174 cells, huPBMC and rhesus macaque PBMC.

Preparation of virus stocks. The viral supernatants harvested at the peak of RT activity were filtered through 0.45 μm filter and frozen in aliquots. The TCID₅₀ of these virus stocks were determined in CEMx174 cells in microtitre plates. Titres were calculated by the method of Reed & Muench (Burleson et al., 1992).

Infection of cells. For the analysis of viral growth kinetics, CEMx174 cells, huPBMC and rhesus macaque PBMC were infected with cell-free vif-defective and wild-type viruses derived from transfected SupT1 or CEM-SS cells at an m.o.i. of 0.01. Infections were accomplished by incubating 2 x 10⁴ cells with each virus at room temperature on a platform shaker for 2 h. Cells were then washed three times with medium to remove residual free virus. Replication of viruses on CEMx174 cells was monitored by measuring the level of RT activity in supernatants.
Fig. 4. For legend see facing page.
the supernatants of infected cultures. Replication of the viruses on huPBMC and rhesus macaque PBMC was monitored by measuring the level of p27<sup>env</sup> by ELISA of samples of infected culture supernatants.

For the analysis of viral DNA synthesis in infected cells, 2 x 10<sup>5</sup> huPBMC were infected with viruses at an m.o.i. of 0.05. Equal amounts of RT of cell-free wild-type and mutant viruses were treated with 500 U/ml of RNase-free DNase (Boehringer Mannheim Biochemical) in the presence of 10 mM-Mg<sub>Cl</sub>₂ for 1 h at 37 °C. Heat-inactivated virus controls were prepared by incubating wild-type virus at 56 °C for 1 h before treatment with DNase. Infected cells were removed at different time points, washed three times with PBS, pelleted and resuspended with 50 µl of lysis buffer (10 mM-Tris-HCl pH 8.3, 0.45% NP40, 0.45% Tween 20 and 50 µg/ml proteinase K). The cell suspensions were incubated at 56 °C for 3 h. Proteinase K was inactivated by boiling these lysates for 10 min.

**Polymerase chain reaction (PCR) analysis.** To compare the amounts of viral DNA synthesized by vif mutants and wild-type virus in huPBMC for a single round of viral replication, lysates of infected huPBMC taken within 48 h post-infection (p.i.) were subjected to PCR amplification using primers that flank the primer binding site (PBS) of SIVmac239. Primer SIV265 5' AGTCGCTCTGCGGAGAGGCTGG 3' specific to the leader sequence (nt 1228-1249) employed for PCR reactions detect only full-length or nearly complete proviral DNA (Zack et al., 1990). Primer SIV265 was 39P-end-labelled and 20 pmol of each primer was used for the PCR reaction. An oligonucleotide pair complementary to the first exon of the human β-globin gene (Law et al., 1980) was included in each reaction as an internal standard for the amount of DNA present in the PCR amplifications. PCR was carried out in 50 µl reaction mixture containing 0.125 mM final concentration of each of the four deoxynucleoside triphosphates, 50 mM-KCl, 10 mM-Tris-HCl pH 8.3, 1.5 mM-Mg<sub>Cl</sub>₂, 2 U Taq DNA polymerase (Perkin Elmer) and 20 µl of cell lysate. The reaction mixtures were overlaid with 50 gl of mineral oil and subjected to denaturation at 94 °C for 2 min in a Perkin Elmer Cetus thermocycler, followed by 30 cycles of amplification with denaturation at 94 °C for 1.5 min, annealing and polymerization at 72 °C for 3 min and a final 7 min extension at 72 °C. Amplified products were analysed by electrophoresis on 5% non-denaturing polyacrylamide gels and visualized by autoradiography of the dried gels.

**Results**

**Vif is dispensable for replication of SIVmac239 in SupT1 and CEM-SS cells**

The effect of vif on SIVmac239 replication in T cells was examined by transfecting equal amounts of either vif-positive or vif-negative viral DNA (Fig. 1) into SupT1 and CEM-SS human T lymphocyte cell lines. Virus replication was monitored by measuring the level of RT activity in the culture supernatants. Both SupT1 and CEM-SS cell lines supported replication of all three vif-defective mutants of SIVmac239 to a level comparable to the parental vif-positive virus (Fig. 2a, b). Thus, these cell lines may contain a factor(s) that complements for the Vif defect in SIVmac or lack an inhibitor of virus growth, normally counterbalanced by Vif.

**Vif is required for replication of SIVmac239 in H9 cells**

SIVmac239 does not replicate efficiently in some human T cell lines including H9 due to the full-length transmembrane domain of the envelope glycoprotein (Kodama et al., 1989; Johnston et al., 1993). To assess the ability of vif-negative SIVmac239 viral DNA to replicate in H9 cells, SIV/HIV-1 (SHIV) chimeric viruses were generated. SHIVs<sub>SF2</sub> has tat, rev and env gp160 of HIV-1<sub>SF2</sub> and all other genes of SIVmac239 (Luciw et al., 1995). The deletion form of SIVmac239 vif (Fig. 1) was used to substitute for the full-length SIVmac239 vif gene in SHIV<sub>SF2</sub>; this vif deletion viral DNA is designated SHIV<sub>SF2</sub>Δvif. No RT activity was detected in H9 cells transfected with SIVmac239Δvif viral DNA plasmid (Fig. 3a) or chimeric SHIV<sub>SF2</sub>Δvif viral DNA plasmid (Fig. 3b), although the parental virus SIVmac239 (Fig. 3a) or SHIV<sub>SF2</sub>Δvif (Fig. 3b) replicated well. Thus, Vif is required for SIVmac239 replication in H9 cells. A delay in SIVmac239 replication was observed in H9 cells during the early period (Fig. 3a). Presumably, a variant virus with premature stop codon in the gp41 gene is produced during this period of infection.

**Vif is required for infection of SIVmac239 in primary T lymphocytes as cell-free virus**

The requirement for Vif for the establishment of infection in primary lymphocytes with cell-free virus was examined by infecting primary cultures of huPBMC and rhesus macaque PBMC with equivalent amounts of vif-positive and vif-negative virus stocks prepared from transfected SupT1 or CEM-SS cell lines. For this experiment, the permissive CEMx174 cell line served as a positive control for replication of mutant and wild-type viruses. Both vif-

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**Fig. 4. Infection of PBMC with wild-type SIVmac239 virus or vif mutant viruses from transfected SupT1 (a, c and d) and CEM-SS cells (b). Human PBMC (a, b) and rhesus macaque PBMC (c, d) were infected with cell-free wild-type SIVmac239 (□) virus or vif mutant virus SIVmac239Δvif (●) at an m.o.i. of 0.01. Viral replication was monitored by measuring the amount of p27 in the supernatants of infected cultures. Solid squares (■) represent the results of mock infection. The permissive CEMx174 cell line served as a positive control for replication of mutant and wild-type viruses. CEMx174 cells were infected with cell-free wild-type SIVmac239 (□) virus or vif mutants SIVmac239Δvif (●), SIVmac239-2S (○) and SIVmac239-2C (▲) at a m.o.i. of 001. Viral replication was monitored by measuring the RT activity in the supernatants of infected cultures. The results in this figure are representative of at least nine independent experiments.**
positive and vif-negative viruses established productive infection in CEMx174 cells (Fig. 4). However, vif-defective viruses failed to replicate in primary huPBMC and rhesus PBMC cultures; the wild-type SIVmac239 control replicated in these primary T lymphocytes (Fig. 4). These findings, demonstrating the requirement for Vif in primary cells, suggest that Vif plays an important role in viral replication in vivo.

**Proviral DNA synthesis by vif mutants after entry into target cells**

Because vif-defective viruses from transfected SupT1 and CEM-SS cells failed to establish infection in primary T lymphocyte cultures (PBMC), the block to viral replication was examined. To focus on early stages of infection, PCR amplification was used to measure the ability of vif-defective virus to complete the first round of proviral DNA synthesis. Virus stocks were prepared from supernatants of SupT1 and CEM-SS cultures transfected with vif-positive and vif-negative cloned viral DNA; these virus stocks were used to infect cultures of huPBMC. Whole cell lysates were analysed by PCR amplification with primers specific for nearly complete or complete proviral DNA.

Analysis of DNA from cells exposed to heat-inactivated virus demonstrates very little, if any, detectable proviral DNA (Fig. 5a, b). Similar amounts of proviral DNA were observed from 6–27 h p.i. in huPBMC infected with either vif-positive or vif-negative viruses (Fig. 5). This finding shows that vif-defective viruses produced from SupT1 (Fig. 5a) and CEM-SS (Fig. 5b) cells are able to complete the first round of proviral DNA synthesis in non-permissive primary T lymphocyte cultures.

**Discussion**

The aim of this study was to analyse the role of vif in SIVmac replication. Our results demonstrate that the requirement for vif is cell-type specific, as has been observed for HIV-1 (Gabuzda et al., 1992) and SIV (Gibbs et al., 1994; Park et al., 1994). H9 cells do not support replication of vif-defective SIVmac239. How-
ever, both SupT1 and CEM-SS cell lines are permissive for vif-defective SIVmac239. This could be due to the presence of compensatory cellular components in SupT1 and CEM-SS cells that complement for the function of Vif protein or the absence of an inhibitor of virus growth, which is normally counterbalanced by Vif. It has been suggested that Vif may play a role in viral protein processing, assembly or virus maturation. The study by Guy et al. (1991) suggests that HIV-1 Vif possesses a cysteine protease activity and is involved in the processing of the subunits of Env glycoprotein. Another report on HIV-1 demonstrated that the incorporation of envelope glycoprotein into viral particles is greatly impaired in the absence of Vif (Sakai et al., 1993). Recent studies by Hoglund et al. (1994) showed that virions produced by vif-defective HIV-1 genomes had an aberrant core; thus, Vif may play a role in the final stages of viral assembly.

Although SIVmac vif-defective viruses did not produce infectious progeny viruses in primary T lymphocytes, these vif mutants derived from SupT1 and CEM-SS were able to complete the first round of proviral DNA synthesis in a fashion similar to vif-positive viruses. The CEM-SS cell line is considered to be semi-permissive for HIV-1 Vif mutant and vif-defective HIV-1 derived from CEM-SS does not complete proviral DNA synthesis in non-permissive cell lines (Von Schwedler et al., 1993).

Our study also demonstrates that vif is required for the establishment of SIVmac infection in peripheral blood T lymphocytes; vif-defective viruses from transfected SupT1 and CEM-SS cells failed to establish infection in primary cultures of huPBMC and rhesus macaque PBMC which supported replication of wild-type SIVmac239 virus. Other investigators have also observed that deletion mutants of SIVmac239 were restricted for replication in primary lymphocyte cultures (Gibbs et al., 1994; Park et al., 1994). HIV-1 genomes with vif mutations also are restricted for replication in primary T lymphocytes (Gabuzda et al., 1994). This finding indicates that Vif is likely to be essential for infection of lymphocytes and perhaps other cell types in the susceptible host.

Both vif mutants SIVmac239-2S, containing stop codons at the N terminus (nt 5670), and SIVmac239-2C, containing stop codons at the C terminus (nt 6010), were not able to replicate in the H9 cell line (Fig. 3a); this finding indicates that sequences near both the N terminus and the C terminus of Vif are essential for its function. Further studies are required to determine the precise step(s) in viral replication governed by Vif and to define a functional domain(s) in this viral protein.

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References


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