Human immunodeficiency virus type 1 Vif supports efficient primate lentivirus replication in rhesus monkey cells

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Human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) Vif share limited homology and display species-specific activity, leading to speculation that Vif sequences could determine the block in HIV-1 replication in rhesus monkeys. To address this issue, we engineered a novel SIV recombinant in which HIV-1 vif replaced SIV vif in a SIVmac239 background. Insertion of HIV-1 vif into the SIV vif locus did not produce a replication-competent virus. Therefore, we inserted HIV-1 vif sequences into the SIV nef locus, which produced a recombinant that, in the absence of SIV vif sequences, replicated similarly to wild-type SIVmac239 in rhesus monkey PBMC. From these studies we conclude that the HIV-1 replication block in rhesus monkeys is almost certainly not Vif determined. These studies also suggest that SHIV/Vif or derivative sequences could be utilized for structure/function studies of HIV-1 Vif in experimentally infected rhesus monkeys.

There is notable divergence between human immunodeficiency virus type 1 (HIV-1) and the simian immunodeficiency virus (SIV) SIVmac239 gene sequences, as well as differences in their gene complements, making the SIV/rhesus macaque model for human AIDS less than optimal (Desrosiers, 2001). The SIV model was made necessary by the fact that HIV-1 does not replicate to detectable levels in rhesus monkeys or cultured rhesus monkey PBMC (Desrosiers, 2001). Therefore, a great deal of effort has been invested in the development and characterization of recombinant SIVs that contain HIV-1 gene sequences, i.e. SHIVs, for direct investigation of the functions of HIV-1 sequences in experimentally infected rhesus monkeys. Thus far, three types of SHIVs, with different complements of HIV-1 sequences, have been documented that replicate efficiently in rhesus monkeys. One (RT/SHIV) replaced SIV reverse transcriptase (RT) (Soderberg et al., 2002; Uberla et al., 1995), a second (SHIVnef) replaced SIV nef/U3 (Alexander et al., 1999a; Kirchhoff et al., 1999) and a third (SHIVenv) replaced SIV vpr, tat, rev and env sequences with analogous HIV-1 sequences (Karlsson et al., 1997; Reimann et al., 1996a, b).

HIV-1 and SIV Vif have limited homology and demonstrate species-specific activity (Simon et al., 1998). It has been shown that in human cells, Vif from non-primate lentiviruses, i.e. visna virus, bovine immunodeficiency virus or feline immunodeficiency virus, did not restore efficient virus replication. Conversely, Vif from SIVmac239 did restore efficient replication of Vif-defective HIV-1 (Simon et al., 1995, 1998). However, the capacity of HIV-1 Vif to support Vif-defective SIV replication in rhesus monkey cells has not been tested. In this paper, we present data addressing this capacity.

In an attempt to determine whether HIV-1 Vif would function in an SIV background, we engineered a recombinant in which HIV-1 vif sequences replaced SIV vif sequences. Previously, in the construction of SHIV recombinants, HIV-1 sequences have been inserted into the SIV genome (containing a large deletion in SIV vif) genome (Gibbs et al., 1994) using the QuickChange site-directed mutagenesis kit (Stratagene). To facilitate insertion of the HIV-1 vif sequences, Nhel and SacII restriction sites were introduced at the 5’ and 3’ ends, respectively, of HIV-1 vif by PCR. The 5’ primer also introduced the consensus translation initiation sequences CCACC immediately 5’ of the initiation ATG to optimize Vif protein expression (Kozak, 1981, 1987). To ensure that the exact desired sequence was contained in these recombinants, the cloned DNA was subjected to fluorescent sequencing (ABI) using SIV- and HIV-1-specific primers. In order to test the viability of SHIV/Vif, these sequences were fused to 3’ SIV sequences and the DNA was introduced into CEM × 174Vif
cells (which express SIV Vif and can rescue replication of SIVmac239 lacking Vif; Gibbs et al., 1994) by DEAE-dextran-mediated transfection (Naidu et al., 1988). The cultures were maintained in RPMI 1640 (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL). The production of replication-competent virus was assessed by the appearance of cytopathic effects and the release of p27Gag into the culture supernatant, which was assayed by ELISA (Coulter). In these cultures, we did not observe the appearance of cytopathic effects (CPE) or the release of p27Gag into the culture supernatant for a period of 8 weeks post-transfection, whereas the parental SIVAVif replicated similarly to wild-type SIVmac239 in these cultures (data not shown). Furthermore, Western blot analysis using an SIV p17Gag monoclonal antibody (NIH AIDS Research and Reference Reagent Program) did not reveal the production of this structural protein in SHIV/Vif-transfected cells, although it was detected in SIVAVif-transfected cells (data not shown). These data suggest that the insertion of HIV-1 vif into the SIV vif locus disrupted an unknown element that was essential for SHIV/Vif replication.

We have previously shown that the nef locus of SIV is amenable to the insertion of foreign gene sequences without substantial inhibition of virus replication (Alexander et al., 1997, 1999a, b). We tested whether the insertion of HIV-1 vif into the nef locus would result in a replication competent SHIV (SHIV/NVif; Fig. 1). To facilitate insertion of the HIV-1 vif sequences into the previously described vector SIVΔNef XES (Alexander et al., 1997), XbaI and SacI restriction sites were introduced at the 5' and 3' ends, respectively, of HIV-1 vif by PCR. As with SHIV/Vif, the 5' primer also introduced the consensus translation initiation sequences to optimize Vif protein expression and the resultant cloned DNA was subjected to fluorescent sequencing. To test the viability of SHIV/NVif, 3' SHIV/NVif sequences were fused to 5' SIVΔVif sequences. The DNA was then transfected into CEM × 174 cells. 5' Wild-type SIVmac239 or 5' SIVΔVif were also fused to SIVΔNef sequences (Kestler et al., 1991) (to create SIVΔNef and SIVΔVifΔNef, respectively) prior to transfection and served as controls for these experiments. As an additional control, wild-type SIVmac239 5' and 3' sequences were fused and transfected into CEM × 174 cells. In these cultures, SIVΔNef-, SHIV/Nvif- and SIVmac239-transfected cells displayed CPE within the first week of transfection (data not shown) and p27Gag was detected in the culture supernatants soon after (data not shown). Neither sign of virus replication was detected in the SIVΔVifΔNef-transfected cells, although efficient virus production was detected in CEM × 174Vif cells transfected with this recombinant DNA (data not shown). These data indicated that, unlike the insertion of HIV-1 vif in the SIV vif locus, the insertion of HIV-1 vif into the SIV nef locus resulted in a viable SHIV/NVif recombinant.

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**Fig. 1.** Diagram of SIVmac239 and SHIV/NVif genomes. SIV sequences are in white and HIV-1 Vif sequences are in black.
We next determined the replicative capacity of SHIV/NVif in experimentally infected human cells. For these experiments, stocks of SIVmac239, SIVΔNef, SHIV/NVif or SIVΔVifΔNef (generated in CEM×174Vif cells) diluted to contain 1·0 ng p27Gag ml⁻¹ were used to infect 1·0×10⁶ CEM×174 cells. In these cultures, we observed that SIVmac239, SIVΔNef and SHIV/NVif replicated similarly, whereas SIVΔVifΔNef did not replicate to detectable levels (Fig. 2A). These data indicated that SHIV/NVif was not substantially inhibited in replication in comparison with wild-type SIV in a human cell line.

Having established that HIV-1 Vif supported efficient SHIV/NVif replication in human cells, we then tested whether HIV-1 Vif could interact with putative rhesus monkey factors to facilitate SHIV/NVif replication in rhesus monkey cells. For these experiments, PBMC were isolated from rhesus monkey blood obtained from the New England Regional Primate Research Center (NERPRC). Ten ml of heparinized blood was layered on to 5 ml of lymphocyte separation medium (ICN Biomedical), which was centrifuged for 30 min at 1500 r.p.m. The layer containing the PBMC was withdrawn, washed with RPMI 1640 and resuspended in RPMI 1640 supplemented with 10 % IL-2 (Advanced Biotechnologies), 20 % FBS and 5 μg phytohaemagglutinin (PHA) ml⁻¹ (Murex Biotech Limited). These cells were stimulated with PHA and IL-2 for 3 days prior to infection and were subsequently carried in RPMI 1640 supplemented with 10 % IL-2 and 20 % FBS. Stimulated cells (2·0×10⁶) were infected with stocks of SIVmac239, SIVΔNef, SHIV/NVif or SIVΔVifΔNef diluted to contain 10 ng p27Gag ml⁻¹. In these cultures we observed that SIVmac239, SIVΔNef and SHIV/NVif replicated similarly, whereas SIVΔVifΔNef did not replicate to detectable levels (Fig. 2B). These data indicated that SHIV/NVif was not substantially inhibited in replication in comparison with wild-type SIV in rhesus monkey cells.

Since HIV-1 Vif normally interacts with HIV-1 and human cellular partners, we investigated whether HIV-1 vif sequences were altered during the passage of SHIV/NVif in rhesus monkey PBMC to potentially improve interactions with putative SIV or rhesus monkey cellular partners. For these experiments, cellular DNA from the infected cells was isolated (on day 8 post-infection) using a previously described saturated NaCl technique (Alexander et al., 2000). A fragment of SHIV/NVif that contained the HIV-1 vif sequences was amplified using primers that annealed to SIV env and LTR sequences. The HIV-1 vif sequences were then determined by fluorescent sequencing using SIV- and HIV-1-specific primers, which revealed that they remained unchanged through short-term passage in rhesus monkey PBMC (data not shown). These data suggested that HIV-1 Vif interacted efficiently with putative viral and host cofactors.

The expression of HIV-1 Vif in a foreign locus could affect the efficiency of its expression and, hence, the efficiency of SHIV/NVif replication. Therefore, we examined the efficiency of HIV-1 Vif expression from the nef locus of SHIV/NVif to determine whether Vif expression was comparable in SHIV/NVif and wild-type HIV-1. For these experiments, 1×10⁶ CEM×174 cells were infected overnight with SHIV/NVif or HIV-1 diluted to contain 100 ng p27Gag or p24Gag, respectively. After infection, the cells were washed and resuspended in fresh media. On day 4 post-infection, culture supernatants were collected and the amount of p27Gag or p24Gag produced by the two cultures was quantified by ELISA (Coulter), which revealed that the SHIV/NVif-infected cells had produced 47 ng p27Gag and the HIV-1-infected cells had produced 55 ng p24Gag. The cell pellets from these infections were washed and resuspended in PBS (Gibco BRL). The cells were then lysed in SDS sample buffer and the protein concentration from these lysates quantified using Bradford reagent (Bio-Rad), which revealed that the
protein concentration of the two lysates was similar (data not shown). Based on the protein concentration of the lysates and the level of virus produced by these cells, we used approximately 10% more SHIV/NVif than HIV-1 lysate in our analyses of HIV-1 Vif expression. For these experiments, the lysates were probed with polyclonal anti-Vif antibody (NIH AIDS Research & Reference Reagent Program). The blot was then probed with a secondary peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia) and incubated with chemiluminescence solution (Perkin Elmer Life Sciences). Radiographic film (Eastman Kodak) was exposed to the membrane and developed. These experiments demonstrated that the level of expression of HIV-1 Vif from SHIV/NVif and HIV-1 Vif was comparable (Fig. 3). These data revealed that HIV-1 Vif was not overexpressed from the nef locus in SHIV/NVif and suggest that HIV-1 Vif most likely functions efficiently in rhesus monkey cells resulting in efficient SHIV/NVif replication in these cells (Fig. 2B).

HIV-1 and SIV Vif have limited homology and demonstrate species-specific activity (Simon et al., 1998) and therefore it has been speculated that HIV-1 Vif could determine the replication block for HIV-1 in rhesus monkey cells. It has been shown that HIV-1 Vif could substitute for SIVagm Vif in a single round of replication (Simon et al., 1998). The experiments described here establish that the HIV-1 Vif can interact not only with putative SIVmac239 factors but also with putative rhesus monkey factors to facilitate efficient primate lentivirus replication in rhesus monkey PBMC. One such factor would be the viral inhibitor CEM15/APOEC3G (Sheehy et al., 2002), the rhesus monkey homologue of which was apparently neutralized efficiently by HIV-1 Vif expressed from SHIV/NVif. These data strongly suggest that the block in HIV-1 replication in rhesus monkeys is not Vif determined, which could allow the development of a pathogenic rhesus monkey model to study HIV-1 Vif activity in vivo. Replication-competent SHIVs have now been established that contain RT, nef/U3, vpr, tat, rev, env and/or vif sequences. Thus, the LTR, Gag, protease and/or integrase most likely determine the block in HIV-1 replication in rhesus monkeys. A role for Gag in this block is supported by recent findings that the capsid subunit of Gag can dictate primate lentivirus species specificity and that the HIV-1 block in simian cells occurs post-entry but prior to reverse transcription (Munk et al., 2002; Owens et al., 2003).

Nef and Vif are normally expressed at distinct times in the virus life-cycle (Desrosiers, 2001; Freed & Martin, 2001). Nef is an early gene product derived from a multiply spliced message, whereas Vif is an intermediate gene product derived from a singly spliced message. Our experiments show that HIV-1 Vif, expressed from the nef locus, efficiently supported recombinant primate lentivirus replication. Therefore, Vif can be expressed as an early protein product without substantially inhibiting virus replication in human or rhesus monkey cells.

In its normal location, approximately one-third of the HIV-1 or SIV Vif open reading frame overlaps with pol (5′) and vpr or vpx (3′) sequences, respectively (Desrosiers, 2001; Freed & Martin, 2001). Thus, it would be difficult to mutate these overlapping sequences without altering additional HIV-1 or SIV gene sequences, which could have adverse effects on virus replication in cell culture that are not Vif related. Our data reveal that HIV-1 Vif can be expressed from the nef locus without substantially affecting virus replication, which we suspect would also apply to SIV Vif. This characteristic could facilitate structure/function studies of N- and C-terminal Vif residue sequences in cell culture in the context of full-length HIV-1 or SIV genomes without the potentially confounding effects of mutated overlapping viral open reading frames.

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