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The regulation of primate immunodeficiency virus infectivity by Vif is cell species restricted: a role for Vif in determining virus host range and cross-species transmission

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The primate immunodeficiency virus Vif proteins are essential for replication in appropriate cultured cell systems and, presumably, for the establishment of productive infections *in vivo*. We describe experiments that define patterns of complementation between human and simian immunodeficiency virus (HIV and SIV) Vif proteins and address the determinants that underlie functional specificity. Using human cells as virus producers, it was found that the HIV-1 Vif protein could modulate the infectivity of HIV-1 itself, HIV-2 and SIV isolated from African green monkeys (SIV_{AGM}). In contrast, the Vif proteins of SIV_{AGM} and SIV isolated from Sykes' monkeys (SIV_{SYK}) were inactive for all HIV and SIV substrates in human cells even though, at least for the SIV_{AGM} protein, robust activity could be demonstrated in cognate African green monkey cells. These observations suggest that species-specific interactions between Vif and virus-producing cells, as opposed to between Vif and virus components, may govern the functional consequences of Vif expression in terms of inducing virion infectivity. The finding that the replication of murine leukemia virus could also be stimulated by HIV-1 Vif expression in human cells further supported this notion. We speculate that species restrictions to Vif function may have contributed to primate immunodeficiency virus zoonosis.

Keywords: HIV/SIV/Vif/viral infection/zoonosis

Introduction

The Vif protein of human immunodeficiency virus type 1 (HIV-1) is essential for virus replication in cultured human T cells and, in all likelihood, for pathogenic infections *in vivo* (Gabuzda *et al.*, 1992, 1994; von Schwedler *et al.*, 1993; Wieland *et al.*, 1994; Courcoul *et al.*, 1995; Simon and Malim, 1996). Virus particles expressed in the absence of a functional Vif protein are able to bind to and penetrate susceptible cells but are severely debilitated in their capacity to establish proviruses (Sova and Volsky, 1993; von Schwedler *et al.*, 1993; Simon and Malim, 1996). Consistent with this, it has been proposed that the defect in *vif*-deficient infections may be manifested as the post-entry instability of viral nucleoprotein complexes (Goncalves *et al.*, 1996; Simon and Malim, 1996). Significantly, challenges with *vif*-deficient viruses cannot be rescued back to productivity by the presence of Vif in the target cells (Gabuzda *et al.*, 1992; von Schwedler *et al.*, 1993). It is believed, therefore, that Vif functions during the late stages of the virus life cycle to confer infectivity on progeny virions. Possibilities for the affected step(s) would therefore include assembly, budding, maturation or some combination thereof.

Analyses of virus particles expressed in the presence or absence of Vif have indicated that Vif's effect, or effects, on the physical properties of virions may be subtle in nature. In particular, even though electron microscopy analyses have indicated that the nucleocapsid cores of *vif*-deficient virions frequently possess aberrant morphologies (Höglund *et al.*, 1994; Borman *et al.*, 1995; Bouyac *et al.*, 1997), several groups have reported that Vif does not exert a measurable quantitative or qualitative effect on the virus-encoded components of particles (von Schwedler *et al.*, 1993; Fouchier *et al.*, 1996; Ochsenbauer *et al.*, 1997). The one known exception to this is the Vif protein itself, which is associated with the viral core and is present at between 10 and 100 copies per virion (Liu *et al.*, 1995; Camaur and Trono, 1996; Fouchier *et al.*, 1996; Karczewski and Strebel, 1996); this compares with the ~2500 molecules of Gag that are incorporated into each virus particle (Arthur *et al.*, 1992). However, because the cell-associated levels of Vif in infected T cells are much higher and approach those of the Gag proteins (Camaur and Trono, 1996; Fouchier *et al.*, 1996), the relevance of virion incorporation for function remains uncertain. Interestingly, *in situ* analyses of HIV-1-infected cells have indicated that much of the intracellular Vif co-localizes with Gag (Simon *et al.*, 1997). Since the HIV-1 Gag and Gag–Pol polyproteins are targeted to the plasma membrane for virus assembly and subsequent budding (Göttlinger *et al.*, 1989; Wang and Barklis, 1993; Yuan *et al.*, 1993; Hunter, 1994; Spearman *et al.*, 1994; Zhou *et al.*, 1994), this region is presumably a major site of co-localization. Importantly, biochemical fractionations of T cells have also shown that substantial quantities of Vif co-purify with the plasma membrane in the absence of other viral proteins (Simon *et al.*, 1997). Taken together, these localization experiments suggest not only that Vif is appropriately positioned to modulate late steps in the viral life cycle but also that targeting to this site is mediated through direct interactions with host cell factors.

A second line of evidence that points towards there being an intimate relationship between Vif function and cellular factors devolves from the observed replication phenotypes of *vif*-deficient viruses in cultured T cells.

Specifically, whereas primary T lymphocytes isolated from peripheral blood and cell lines such as H9 and HUT78 require the presence of Vif for productive HIV-1 replication (non-permissive cells), other cell lines, for example C8166 and CEM-SS, display no such reliance (permissive cells) (Fisher *et al.*, 1987; Fan and Peden, 1992; Gabuzda *et al.*, 1992; Sakai *et al.*, 1993; Sova and Volsky, 1993; von Schwedler *et al.*, 1993; Borman *et al.*, 1995; Simon *et al.*, 1995). Accordingly, it is presumed either that permissive cells harbor an activity that substitutes for Vif or, alternatively, that non-permissive cells possess an inhibitory activity that is suppressed by Vif.

Importantly, HIV-1 is not the only retrovirus to carry a *vif* gene. All lentiviruses of primate hosts (also known as the primate immunodeficiency viruses) and all but one isolated from non-primate hosts—the exception being equine infectious anemia virus (EIAV)—possess *vif* genes (Oberste and Gonda, 1992; Tomonaga *et al.*, 1992; Gibbs *et al.*, 1994; Reddy *et al.*, 1995; Simon *et al.*, 1995; Harmache *et al.*, 1996). In contrast, members of the oncovirus and spumavirus subfamilies of retroviruses do not have *vif* gene equivalents. Previous work has demonstrated that the Vif proteins of HIV-2 and simian immunodeficiency virus from macaques (SIV_{MAC}) can functionally substitute for HIV-1 Vif in terms of virus replication in human T cells, but that the Vif proteins of three non-primate lentiviruses—visna virus and the feline and bovine immunodeficiency viruses—cannot (Reddy *et al.*, 1995; Simon *et al.*, 1995). The two most plausible reasons for selectivity with respect to which combinations of cells, viruses and Vif proteins reveal the Vif effect are: first, that a given Vif protein is only functional in cells of a limited number of animal species, or second, that a given Vif only confers infectivity on certain viruses. In molecular terms, the former possibility would suggest that Vif interacts with cellular factors whereas the latter would be more consistent with Vif interacting with viral factors.

To gain insight into the parameters that underlie the specificity of Vif action, we have been focusing on the Vif proteins of the primate lentiviruses. To date, phylogenetic analyses of these viruses have defined five major approximately equidistant lineages (Sharp *et al.*, 1994); these are HIV-1/SIV_{CPZ} (CPZ, chimpanzee), HIV-2/SIV_{SM}/SIV_{MAC} (SM, sooty mangabey), SIV_{AGM} (AGM, African green monkey,) SIV_{SYK} (SYK, Sykes' monkey) and SIV_{MND} (MND, mandrill). Here, we present evidence that Vif function is determined at the level of the cell rather than the virus. In particular, the HIV-1 Vif protein is able to modulate the infectivity of HIVs, SIVs and a type-C oncoretrovirus, murine leukemia virus (MLV), in human cells whereas the Vif proteins of both SIV_{AGM} and SIV_{SYK} are inactive in human cells irrespective of virus substrate. These results are consistent with a model for Vif function in which virus-expressing cells are modified in a species-specific manner that facilitates the production of infectious retrovirus particles.

Results

The initial objective of these studies was to elucidate the patterns of transcomplementation between primate lentivirus Vif proteins as a step towards determining the mechanism by which this protein family determines virus infectivity. To enable us to make measurements of infectivity in as straightforward and rapid a manner as possible, we have developed the experimental strategy illustrated in Figure 1. There are two critical components of this system. One, the cells that are employed as virus producers are non-permissive in that *vif*-deficient viruses expressed by such cells are relatively non-infectious. Specifically, H9 cells and peripheral blood mononuclear cells (PBMCs) can each be used for this purpose as DNAs introduced by transfection display Vif-mediated inductions of infectivity of 10- to 20-fold (Fouchier *et al.*, 1996). However, H9 cells are preferred over PBMCs for this purpose as DNAs are more readily introduced into these cells by electroporation (data not shown). Two, the T cells that are used as targets of infection harbor the chloramphenicol acetyltransferase (*CAT*) gene under the transcriptional control of the HIV-1 long terminal repeat (LTR) promoter element (Felber and Pavlakis, 1988; Fouchier *et al.*, 1996; Simon and Malim, 1996). The extent of infection is therefore measured as the induction of CAT expression since viral Tat proteins expressed by newly formed proviruses (as the result of productive infection) transcriptionally transactivate the resident LTR. It is of note that previous analyses have demonstrated that the HIV-1 LTR is sensitive to transactivation by a variety of HIV and SIV Tat proteins (Sakuragi *et al.*, 1991). To render the indicator cells susceptible to infection by HIVs and SIVs that utilize different co-receptors for viral entry, the C8166/HIV-CAT cell line (which expresses CD4 and CXCR4) was modified further to express CCR5 and is termed C8166-CCR5/HIV-CAT (Fouchier *et al.*, 1997). A typical experiment therefore consists of transiently transfecting H9 cells with a *vif*-deficient (or wild-type) provirus expression vector together with a Vif expression vector, normalizing virus stocks according to supernatant reverse transcriptase activity, challenging LTR-CAT indicator cells and determining infection as the accumulation of CAT within 1–2 days.

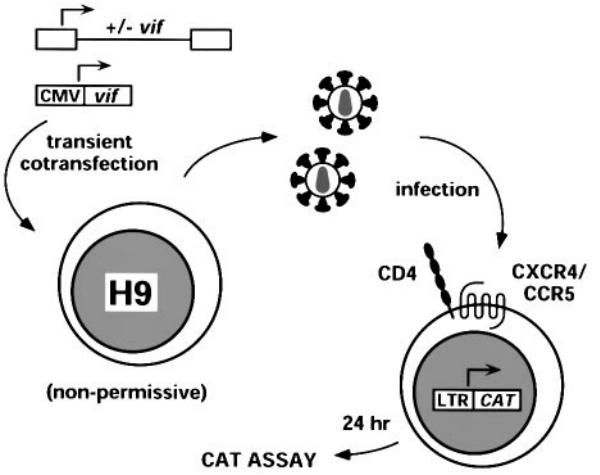

Fig. 1. Quantitative assay for measuring the Vif-mediated regulation of lentivirus infectivity. Refer to text for details.

The Vif proteins of SIV_{AGM} and SIV_{SYK} do not complement vif-deficient HIV-1 or HIV-2

To determine which primate immunodeficiency virus Vif proteins can functionally substitute for the Vif proteins of HIV-1 and HIV-2, a series of virus preparations were

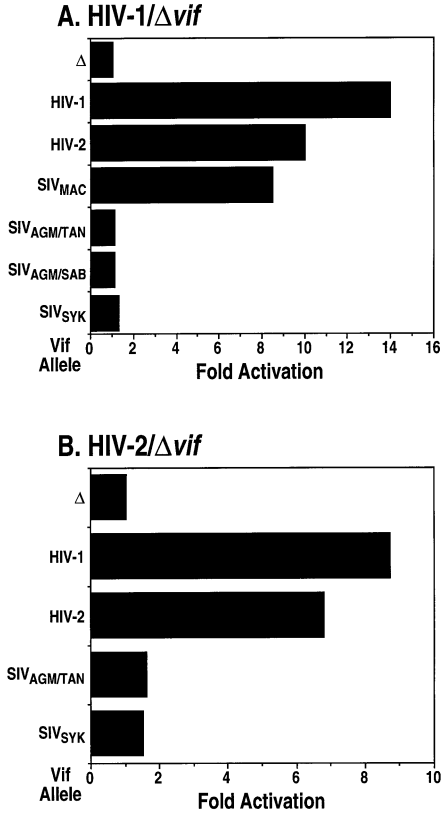

Fig. 2. Control of HIV-1 (A) and HIV-2 (B) infectivity in human T cells by primate lentivirus Vif proteins. (**A**) H9 cells were transiently transfected with pIIIB/Δ*vif* and each of the indicated pgVif:T7 expression vectors or the negative control vector pgΔVif. Viruses were normalized according to supernatant reverse transcriptase activity and the levels of infectivity determined using C8166/HIV-CAT indicator cells. The results are the average of three independent experiments and are shown as fold increases in infectivity relative to the ΔVif control (infectivity value of 1) following correction for background CAT activity (mock infections). (**B**) H9 cells were transfected as in (A) except that the provirus expression vector was pROD10/Δ*vif*.

generated by transfection of human H9 T cells. Specifically pIIIB/Δ*vif* (HIV-1/Δ*vif*) or pROD10/Δ*vif* (HIV-2/Δ*vif*) were co-transfected with pgVif:T7/HIV-1 or derivative vectors that expressed either a disrupted HIV-1 *vif* gene or the *vif* genes of HIV-2, SIV_{MAC}, $SIV_{AGM/TAN}$, $SIV_{AGM/SAB}$ or SIV_{SYK}. Resulting HIV-1 (Figure 2A) and HIV-2 (Figure 2B) stocks were harvested and their infectivities determined using C8166/HIV-CAT cells as targets. As expected, the HIV-1 Vif protein efficiently restored infectivity to HIV-1/Δ*vif* (~14-fold induction, Figure 2A). Also, and consistent with earlier results obtained in spreading infection assays (Reddy *et al.*, 1995; Simon *et al.*, 1995), the Vif proteins of HIV-2 (~10-fold induction) and SIV_{MAC} (~9-fold induction) both rescued infectivity efficiently. In contrast, no reproducible increases in infectivity over the background values for HIV-1/Δ*vif* were obtained with the Vif proteins of SIVs isolated from African green monkeys ($SIV_{AGM/TAN}$ and $SIV_{AGM/SAB}$) or Sykes' monkeys (SIV_{SYK}). The patterns of complementation of HIV-2/Δ*vif* by the various Vif proteins were essentially the same (Figure 2B). Thus, the HIV-1 and cognate HIV-2 Vif proteins conferred infectivity on HIV-2/Δ*vif* (~9- and ~7-fold inductions, respectively) whereas the Vifs of $SIV_{AGM/TAN}$ and SIV_{SYK} did not. Importantly, all the pgVif vectors expressed Vif proteins of the expected mass, and the addition of the epitope tag to the carboxy-terminus of each Vif was found not to affect biological activity in terms of the induction of virion infectivity (data not shown).

$SIV_{AGM/TAN}$ Vif regulates $SIV_{AGM/TAN}$ infectivity in African green monkey cells but not in human cells

The finding that the Vif proteins of $SIV_{AGM/TAN}$, $SIV_{AGM/SAB}$ and SIV_{SYK} did not functionally substitute for HIV-1 Vif but that those of HIV-2 and SIV_{MAC} did was unexpected given that all of these SIV and HIV-2 proteins share between 26 and 29% amino acid sequence identity with the Vif protein of HIV-1. Similarly, the complementation of HIV-2/Δ*vif* by HIV-1 Vif but not by the Vifs of $SIV_{AGM/TAN}$ or SIV_{SYK} was not anticipated as these three proteins each share 29–37% identity with the HIV-2 sequence. We hypothesized that there were four potential (non-mutually exclusive) explanations for these results. One, the Vif proteins of $SIV_{AGM/TAN}$, $SIV_{AGM/SAB}$ and SIV_{SYK} may not serve the same function in the life cycles of their parental viruses as do the Vifs of HIV-1, HIV-2 and SIV_{MAC}. Two, these particular alleles may be non-functional or inactive. Three, these Vif proteins may not be capable of modulating virus infectivity in cells of human origin. Four, these proteins may not be able to interface with the virion components of HIV-1 or HIV-2 in the manner required for the induction of infectivity.

To address these possibilities, we focused on $SIV_{AGM/TAN}$ and the activity of its Vif protein. To make this experiment as relevant as possible to virus growth *in vivo*, virus stocks were produced using PBMCs obtained from African green monkeys. We reasoned that the use of these cells for $SIV_{AGM/TAN}$ expression would be akin to the use of human PBMCs (or H9 cells) for HIV-1 Vif analysis, particularly as we are unaware of any immortalized African green monkey lymphoid cell lines. Therefore, by examining $SIV_{AGM/TAN}$ Vif function in PBMCs from both African green monkeys and humans, we anticipated being able to draw conclusions regarding functionality and host cell specificity. Because initial attempts at transfection of African green monkey PBMCs were unsuccessful, an alternative strategy for expressing wild-type and *vif*-deficient proviruses in PBMCs was developed. Specifically, high titer preparations of wild-type and Δ*vif* viruses that were pseudotyped with the glycoprotein of vesicular stomatitis virus (VSV G) were generated by transfection of 293T cells (a permissive cell line) and used for initial challenges of PBMCs. Since infections of this type are competent with respect to provirus establishment, these cultures served as transient producers of virus particles once the initial inocula had been removed.

Wild-type and *vif*-deficient $SIV_{AGM/TAN}$ stocks were therefore prepared from PBMCs obtained from African green monkeys and humans; as controls, wild-type and Δ*vif* HIV-1s were generated in parallel from the human PBMCs. All six virus preparations were normalized for reverse transcriptase activity and their respective infectivities examined using C8166-CCR5/HIV-CAT cells as targets (Figure 3). As expected, HIV-1 expressed in the presence of its intact *vif* gene was markedly more infectious (~90-fold in this experiment) than the Δ*vif* counterpart. It

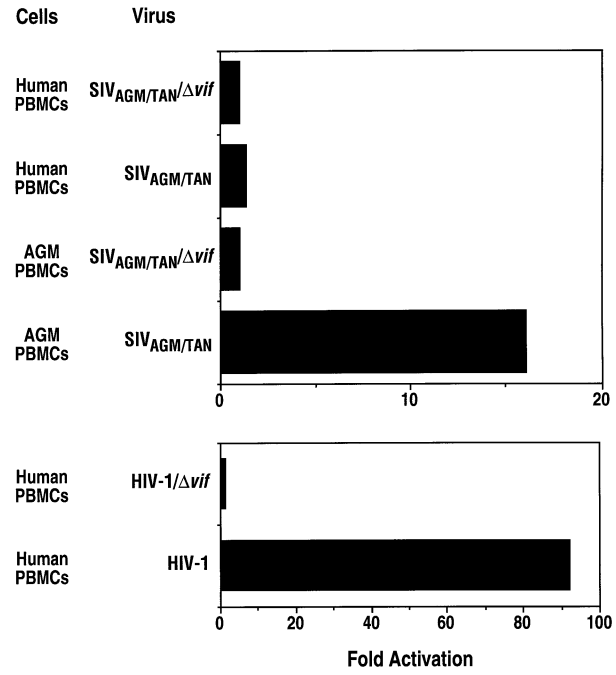

Fig. 3. SIV_{AGM} Vif function is cell species restricted. Human and African green monkey PBMC cultures were used to express wild-type or *vif*-deficient HIV-1 and $SIV_{AGM/TAN}$ as indicated following initial infection with VSV G pseudotyped viruses that had been produced in 293T cells. All ensuing viruses were normalized for reverse transcriptase activity and the relative virus infectivities determined as in Figure 2 using C8166-CCR5/HIV-CAT indicator cells. Note that the infectivities of $SIV_{AGM/TAN}$/Δ*vif* from human and African green monkey cells were equivalent. The average results of two independent experiments are shown.

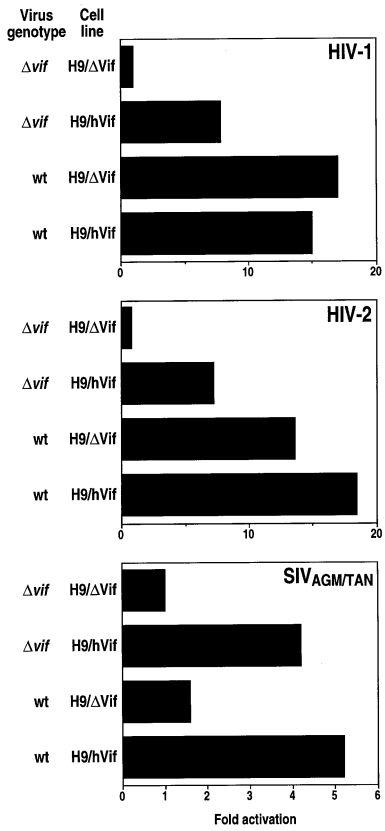

Fig. 4. HIV-1 Vif increases the infectivity of SIV_{AGM} in human T cells. Wild-type and *vif*-deficient stocks of HIV-1, HIV-2 and $SIV_{AGM/TAN}$ were generated by transient transfection of H9/hVif or H9/ΔVif cells. Each stock within each set of four viruses (two wild-type and two *vif*-deficient) was normalized for reverse transcriptase activity and their relative infectivities determined as in Figure 2 using C8166-CCR5/HIV-CAT cells. The average results of three independent experiments are shown.

is of note that this increase in infectivity was reminiscent of the ~50-fold effect described previously for analogous experiments that employed H9 cells as virus producers (Fouchier *et al.*, 1996; Simon and Malim, 1996). A similar result was observed when $SIV_{AGM/TAN}$ was produced from cognate African green monkey PBMCs, namely that Vif increased virus infectivity by ~16-fold. In sharp contrast, when this same pair of $SIV_{AGM/TAN}$s was expressed using human PBMCs, the infectivities were not only essentially the same as each other and but were also equivalent to *vif*-deficient $SIV_{AGM/TAN}$ derived from the African green monkey PBMCs.

A number of important inferences can be made from these observations. First, not only is $SIV_{AGM/TAN}$ Vif a functional protein, but, like the Vifs of HIV-1, HIV-2 and SIV_{MAC}, it is also a potent regulator of virion infectivity. Second, the $SIV_{AGM/TAN}$ Vif protein is inactive in human cells; this is true when either cognate $SIV_{AGM/TAN}$ (Figure 3) or non-cognate HIV-1 or HIV-2 (Figure 2A and B) encoded virus components are present as potential substrates. This latter conclusion implies that if virally encoded factors contribute towards the specificity of Vif action, their interactions with Vif cannot, by themselves, be sufficient to determine Vif function. Accordingly, it seemed possible that the identity (or species) of the host cell, and therefore the ability of certain cellular factors to interact with Vif, could contribute to the functionality of a given Vif protein.

HIV-1 Vif expressed in human cells confers infectivity on $SIV_{AGM/TAN}$

To address the possibility that the species of the virus-producing cell, as opposed to the identity of the virus, can determine Vif protein function, we evaluated whether the Vif protein of HIV-1—a virus of humans—is capable of regulating the infectivity of $SIV_{AGM/TAN}$ in human cells. Because we have found that reducing the number of different plasmids that are present in electroporation cocktails results in improved levels of co-expression in transfected H9 cells, we used cell lines that stably expressed Vif, instead of unmodified H9 cells (Figures 1 and 2), for the preparation of virus stocks in this experiment; this resulted in the pgVif vectors being eliminated from each transfection.

H9 cell lines that stably expressed the *vif* gene of HIV-1 or its non-functional Δ*vif* derivative were constructed using retrovirus-mediated gene transfer. Wild-type or *vif*-deficient provirus expression vectors for HIV-1, HIV-2 and $SIV_{AGM/TAN}$ were transfected into these two cell lines and the infectivity of the resulting virus stocks determined using C8166-CCR5/HIV-CAT cells (Figure 4). The infectivities of these three sets of viruses segregated into two clear categories. For HIV-1 and HIV-2, the results were entirely as anticipated in that the wild-type viruses (Vif-expressing) were infectious irrespective of cell origin,

and the genotypically *vif*-deficient viruses had relatively low levels of infectivity unless produced from the complementing H9/hVif cells. These results are consistent with those obtained using co-transfections (Figure 2).

Interestingly, a very different result was obtained for $SIV_{AGM/TAN}$ (Figure 4). For viruses produced from H9/ΔVif cells, the levels of infectivity were similar whether or not the virus carried an intact *vif* gene; these findings illustrated, once again, that the Vif protein of $SIV_{AGM/TAN}$ does not function in human cells. The novel and surprising finding was, however, that the infectivities of wild-type and *vif*-deficient $SIV_{AGM/TAN}$ were significantly increased (~5-fold) when the viruses were produced by cells expressing the HIV-1 Vif protein. Thus, even though the $SIV_{AGM/TAN}$ Vif protein does not regulate the infectivity of its cognate virus in human cells, the Vif protein of the distantly related virus, HIV-1, does. These data not only suggest that interactions between a Vif protein and an infected cell may determine functional outcome in terms of inducing virus infectivity but also imply that these interactions may be governed by species-specific restrictions.

As discussed earlier, Vif is not required for HIV-1 infectivity in certain human T cell lines; such cells have been termed permissive and are exemplified by the CEM-SS line (Simon and Malim, 1996). In terms of supporting the replication of *vif*-deficient HIV-1, these cells are functionally equivalent to non-permissive cells that contain Vif. To assess whether permissive cells also impart infectivity on SIVs whose Vif proteins are inactive in human cells, the infectivities of $SIV_{AGM/TAN}$ and SIV_{SYK} produced in CEM-SS or H9 cells were determined. For both viruses, it was found that virus stocks produced in CEM-SS cells were ~10-fold more infectious than matched samples derived from H9 cells (data not shown). That this was true for both wild-type and Δ*vif* viruses indicated, once again, that the Vif proteins of these SIVs are non-functional in human cells. As expected for viruses whose Vif proteins are active in human cells, no such differences were noted for wild-type HIV-1 or HIV-2 expressed in CEM-SS versus H9 cells. It therefore appears that the mechanism(s) by which primate lentiviruses acquire infectivity, either as a result of Vif expression or by virtue of the character of the virus-producing cells, is conserved for viruses of human (HIV-1 and HIV-2) as well as non-human ($SIV_{AGM/TAN}$ and SIV_{SYK}) hosts.

HIV-1 Vif enhances the replication of the oncoretrovirus MLV in human cells

As a further test of the notion that Vif proteins may interact with, and thereby modify, cells in a manner that facilitates the expression of retrovirus particles with increased infectivity, we extended our analysis to the oncoretrovirus MLV. Importantly, and as noted earlier, members of this retrovirus subfamily do not encode equivalents of *vif*; interestingly, however, it has been demonstrated that it is possible for the Vif protein of HIV-1 to be incorporated into MLV virions (Camaur and Trono, 1996). As above, we evaluated the effect of the HIV-1 Vif protein on MLV infection in non-permissive (HUT78) as well as permissive (CEM-SS) human T cells; the rationale for this being that a *bona fide* Vif effect should not be observed in permissive cells. Two different input inocula of amphotropic MLV were therefore used to challenge HUT78 or CEM-SS cells that constitutively expressed either HIV-1 Vif or the corresponding Δ*vif* allele. Virus replication was then monitored over time as the accumulation of reverse transcriptase activity in the culture supernatants (Figure 5). In non-permissive cultures, the expression of HIV-1 Vif either accelerated (higher inoculum) or permitted (lower inoculum) virus replication and dissemination. In contrast, and concordant with the absence of any Vif effect on HIV or SIV infectivity in permissive cells, MLV grew equally well in CEM-SS cells whether Vif was present or not. These novel and unexpected results for MLV are, when taken together with those obtained with $SIV_{AGM/TAN}$ (Figure 4), consistent with the hypothesis that the interplay between cellular factors and Vif creates an environment that fosters the production of infectious retroviral particles.

Discussion

The critical contribution of the Vif family of proteins to virus infectivity and replication has been established for a variety of primate and non-primate lentiviruses. Although the precise mechanism by which Vif regulates infectivity remains to be defined, the prevailing view is that late steps of the virus life cycle (assembly, maturation and/or budding) are manipulated such that ensuing virions are able to complete reverse transcription and establish proviruses following penetration into susceptible cells (Gabuzda *et al.*, 1992; Sova and Volsky, 1993; von Schwedler *et al.*, 1993; Goncalves *et al.*, 1996; Simon and Malim, 1996). In an endeavor to understand Vif function in greater detail, we have been investigating the patterns of cross-complementation between the Vif proteins of divergent lentiviruses. It is likely that defining the basis for functional specificity in genetic terms will provide important clues for the design and interpretation of future genetic and biochemical studies.

In these analyses, we have focused our attention on the Vif proteins of four of the five major phylogenetic lineages of primate lentiviruses, namely, HIV-1/SIV_{CPZ}, HIV-2/SIV_{SM}/SIV_{MAC}, SIV_{AGM} and SIV_{SYK}. Inspection of the amino acid sequences of these proteins is uninformative with respect to predicting cross-complementation capabilities; each pair of Vif proteins among those of the $HIV\text{-}1_{HXB}$, $HIV\text{-}2_{ROD}$, $SIV_{AGM/TAN}$ and SIV_{SYK} isolates shares 26–37% sequence identity, yet some are functionally interchangeable whereas others are not. In particular, it was found that the Vif proteins of HIV-1, HIV-2 and SIV_{MAC} (a virus that is closely related to HIV-2) each increases the infectivity of *vif*-deficient HIV-1 or HIV-2 in human T cells (Figure 2). In contrast, no inductions of infectivity for either HIV-1 or HIV-2 were observed with the Vif proteins of SIV_{AGM} or SIV_{SYK} (Figure 2). Importantly, it was then found that the Vif proteins of SIV_{AGM} and SIV_{SYK} are also incapable of modulating the infectivity of their parental viruses when human cells are used for virus production (Figures 3 and 4 and data not shown). To our surprise, however, the infectivity of SIV_{AGM} was significantly increased in human cells by co-expression of the Vif protein of HIV-1 (Figure 4), a virus that naturally replicates effectively in human cells.

An important aspect of these data that warrants consider-

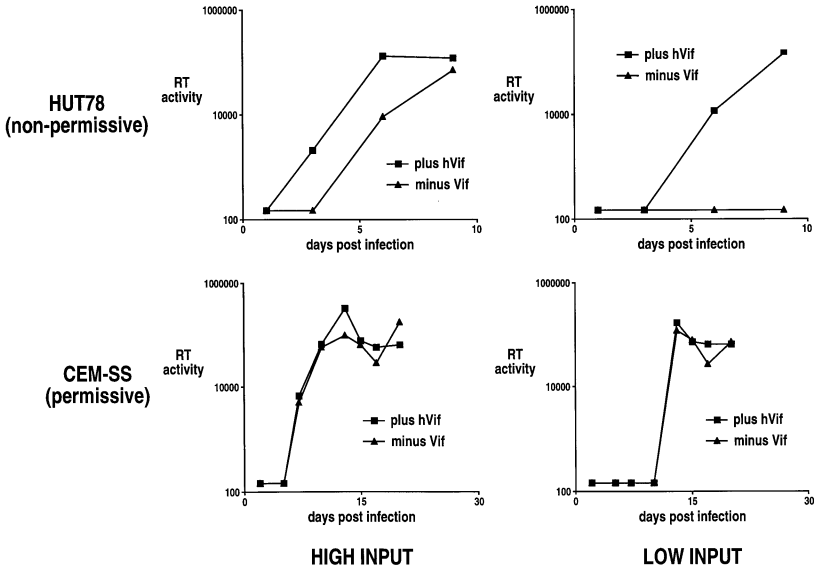

Fig. 5. Enhancement of MLV replication in human T cells by the HIV-1 Vif protein. HUT78 and CEM-SS cells that stably expressed HIV-1 Vif or a disrupted Δ*vif* allele were challenged with two inocula of amphotropic MLV and the cultures maintained in a proliferative state by passage every 2 or 3 days. Virus production, and hence replication, was measured as the accumulation of reverse transcriptase activity in the culture supernatants. Representative replication profiles are shown.

ation is the variations in the magnitude of Vif's effect on infectivity. In particular, the HIV-1 Vif protein increased the infectivity of $SIV_{AGM/TAN}$ by ~5-fold (Figure 4). We speculate that two factors can account for this seemingly low level of induction. First, proviruses that are expressed following transfection exhibit a reduced Vif effect when compared with proviruses expressed as a result of virus infection (Fouchier *et al.*, 1996); thus, for HIV-1, transient transfection reveals a 10- to 20-fold effect (Figures 2 and 4) whereas infection results in a 50- to 100-fold effect (Figure 3). Second, the expression of $SIV_{AGM/TAN}$ Vif enhances the infectivity of its parental virus by only ~16-fold in cognate PBMCs, a value well below that seen for HIV-1 in analogous experiments. (Figure 3). Accordingly, it is possible that the effects seen in Figure 4 for HIV-1 Vif with $SIV_{AGM/TAN}$ might represent the maximum that could be expected for this transfection-based experiment.

The most unexpected, and potentially instructive, observation that was made during the course of these experiments was that HIV-1 Vif significantly enhanced the replication (and by inference the infectivity) of MLV in human T cells (Figure 5). Because this effect, like that of the HIV-1 and HIV-2 Vif proteins on HIV and SIV infection, is mimicked by certain (permissive) cell lines, we favor the idea that the mechanism that underlies Vif-mediated increases in infectivity is conserved for all these retroviruses. Important future questions are, therefore, how do Vif proteins exert their influence on virus infectivity, and, what role does the virus-producing cell play in this process? We hypothesize that the restriction of Vif activity to cells of limited host species, exemplified here by SIV_{AGM} Vif being active in African green monkey cells but not in human cells (Figures 3 and 4), is indicative of interactions between host cell factors and Vif being critical to Vif function. The availability of such species-restricted, yet biologically active, Vif proteins will allow the importance of Vif–host factor binding events (for instance, as might be detected in co-immunoprecipitation or yeast two-hybrid experiments) to be assessed in the context of functional relevance. In addition, the future construction of chimeric *vif* genes (e.g. using HIV-1 or HIV-2 and SIV_{AGM}) should make it possible to map the region(s) of Vif that participate in such interactions.

Here, we have argued that specific interactions between Vif proteins and host factors underlie the Vif-induced acquisition of virus infectivity. Importantly, it remains possible that specific interactions between Vif and viral components are also involved. However, one would predict that the structure and/or sequence of such a putative interacting viral component would have to be conserved not only among the primate lentiviruses but also by MLV. It is noteworthy that lentiviruses and MLV assemble their nucleocapsids at the plasma membrane prior to budding (Bolognesi *et al.*, 1978; Gelderblom, 1991; Hunter, 1994). Because it has been demonstrated previously that a substantial fraction of intracellular HIV-1 Vif is specifically localized to this region of T cells (Simon *et al.*, 1997), it will be important to determine whether the infectivities of type-B and type-D retroviruses, such as mouse mammary tumor virus and Mason–Pfizer monkey virus, whose nucleocapsids assemble in the cytoplasm (Fine and Schochetman, 1978; Rhee and Hunter, 1990; Hunter, 1994), are also sensitive to modulation by Vif. It will also be of interest to examine whether Vif expression can modulate the infectivity of EIAV, the only known lentivirus that lacks a *vif* gene. The result of such an experiment might allow one to speculate on the possible adaptation of EIAV to growth without Vif.

The discovery that the replication of primate lentiviruses in cells of different species depends on the capacity of Vif to function in those cells may have important implications for the transmission of SIVs to humans and their subsequent evolution into HIVs. Current hypotheses posit that HIV-1 was established in humans on two separate occasions, with SIV_{CPZ} probably serving as the transmitted virus (Sharp *et al.*, 1995). This compares with HIV-2,

which appears to have entered the human population on multiple occasions as SIV_{SM}. Interestingly, no viruses of humans have been identified so far that would appear to be the counterparts of either SIV_{AGM} or SIV_{SYK}. Given that the natural animal hosts of these viruses, in particular African green monkeys (Sharp *et al.*, 1995; Soares *et al.*, 1997), are distributed abundantly and widely throughout Africa, are usually infected with SIV, and would presumably have frequently come into direct contact with humans, it seems probable that ample opportunity for zoonosis would have occurred. Accordingly, we propose that one factor that may have contributed to the lack of cross-species transmission of SIV_{AGM} and SIV_{SYK} into humans is the inability of their Vif proteins to function in human cells. Any exposures to humans that might have taken place would be predicted to have been self-limiting as they would have been effectively *vif*-deficient for the second round of infection. In contrast, human exposures to SIV_{SM} would, at least from the standpoint of Vif function, be expected to result in virus transmission as Vif proteins from this lentivirus lineage are active in human cells.

Coincidentally, a somewhat similar hypothesis was proposed recently following an analysis of the cell cycle arrest capabilities of primate lentivirus Vpr proteins (Stivahtis *et al.*, 1997). In these and other experiments, it was noted that the Vpr proteins of SIV_{AGM} and SIV_{SYK} are able to induce G2/M arrest in simian (African green monkey) cells but are inactive in human cells (Planelles *et al.*, 1996; Stivahtis *et al.*, 1997). Thus, two of the accessory proteins of primate immunodeficiency viruses—Vif and Vpr—are only able to function in cells of a limited number of species. It is feasible, therefore, that both proteins may contribute to the capacity of these viruses to spread between different host species.

Materials and methods

Molecular clones

The wild-type and *vif*-deficient (Δ*vif*) HIV-1 (pIIIB and pIIIB/Δ*vif*, respectively) as well as the wild-type HIV-2 (pROD10), $SIV_{AGM/TAN}$ (TAN, tantalus monkey), SIV_{AGM}/SAB (SAB, sabaeus monkey) and SIV_{SYK} proviral expression vectors have been described (Hirsch *et al.*, 1993; Jin *et al.*, 1994; Ryan-Graham and Peden, 1995; Simon *et al.*, 1995; Soares *et al.*, 1997). The *vif* gene of pROD10 was disrupted by Klenow fragment-mediated fill-in of the *Xba*I restriction site at position 5624. The SIV-expressing clones were rendered *vif*-deficient using the Quick Change site-directed mutagenesis kit as directed (Stratagene, La Jolla, CA); in each case, a frameshift mutation was introduced into *vif* by the insertion of two nucleotides (-GT-), at position 5298 for $SIV_{AGM/TAN}$ and at position 5308 for SIV_{SYK}. The Δ*vif* derivatives of HIV-2, $SIV_{AGM/TAN}$ and SIV_{SYK} therefore express truncated Vif proteins of 46, 27 and 25 amino acids, respectively. The replication-competent amphotropic MLV provirus clone pAM has been described (Miller *et al.*, 1985).

The Rev-regulated wild-type HIV-1 Vif expression vector pgVif/HIV-1 (Simon *et al.*, 1997) was modified by PCR-mediated mutagenesis such that the stop codon of *vif* was replaced by a *Bam*HI restriction site followed by the T7 epitope tag (Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly-STOP); this vector is termed pgVif:T7/HIV-1. A variant of pgVif/HIV-1 that carried a disrupted (Δ*vif*) *vif* gene (Simon *et al.*, 1995), pg/ΔVif, was generated by substitution of the *Xba*I–*Sal*I fragment with the analogous restriction fragment from pIIIB/Δ*vif*. Analogous vectors harboring the wild-type *vif* alleles of pROD10, SIV_{MAC239}, $SIV_{AGM/TAN}$, $SIV_{AGM/SAB}$ and SIV_{SYK} were constructed by PCR amplification of the relevant gene followed by substitution into pgVif/HIV-1 or pgVif:T7/HIV-1 as *Xba*I–*Sal*I or *Xba*I–*Bam*HI fragments, respectively. The exceptions were pgVif:T7/HIV-2 and pgVif:T7/$SIV_{AGM/SAB}$, where the substitutions were as *Eco*RI–*Bam*HI and *Xba*I–*Bgl*II fragments. All amplified *vif* genes were sequenced to confirm integrity.

The retrovirus vectors LN-M/hVif and LN-M/ΔhVif, which express wild-type HIV-1 Vif and its disrupted variant, respectively, have been described (Simon *et al.*, 1995) as have the expression vectors for HIV-1 Rev, pcRev (Malim *et al.*, 1988), vesicular stomatitis virus envelope, pHIT/G (Fouchier *et al.*, 1997), and MLV *gag* and *gag–pol*, pHIT60 (Soneoka *et al.*, 1995).

Cells and cell lines

The immortalized human cell lines H9, HUT78, CEM-SS and 293T, as well as the modified C8166/HIV-CAT and C8166-CCR5/HIV-CAT lines that each contain the *CAT* gene under the transcriptional control of the HIV-1 LTR promoter element, have been described (Simon *et al.*, 1995; Simon and Malim, 1996; Fouchier *et al.*, 1997). Standard retrovirus-mediated gene transfer using stocks generated in 293T cells by transfection with one of the LN-M vectors together with pHIT/G and pHIT60 was used to express the wild-type or disrupted HIV-1 *vif* genes stably in H9, HUT78 or CEM-SS cells (Fouchier *et al.*, 1997). Polyclonal cell populations were selected and maintained in complete RPMI 1640 medium supplemented with 10% fetal bovine serum and 1 mg/ml G418 (Gibco BRL Inc., Gaithersburg, MD).

Human PBMCs were isolated from healthy volunteer donors by venipuncture, purified, stimulated and maintained at ~1×10^6 cells/ml in interleukin-2 (IL-2)-containing medium as described (Simon and Malim, 1996). African green monkey PBMCs were obtained and cultured in the same manner using peripheral blood obtained from SIV-negative animals and supplied by the Tulane Regional Primate Center.

Production and quantitation of viruses

For the production of HIVs and SIVs from H9 and CEM-SS cells, or derivative lines, electroporation with provirus vectors, alone or together with Vif expression plasmids or pHIT/G (pseudotyping with VSV G was required for the experiments using SIV_{SYK} proviruses), was performed using 10×10^6 cells and 5–15 μg (total) of DNA. Viruses were harvested at 20 h, filtered through a 0.45 μm filter and the levels of reverse transcriptase activity determined for virus particles pelleted from 0.5 ml of supernatant by centrifugation at 17 000 *g* for 30 min at 4°C (Goff *et al.*, 1981); virus preparations were used for infections immediately after harvest. To express HIV-1 and $SIV_{AGM/TAN}$ from PBMCs, 100 mm monolayers of 293T cells were first transiently co-transfected with the provirus vector and pHIT/G. At 24 h, virus supernatants were collected and used to challenge 10×10^6 PBMCs (1 day after culture in IL-2-containing medium) at high multiplicity. After a further 3 h, input virus was eliminated by three washes in phosphate-buffered saline (PBS), the cells maintained for 3 days in IL-2-containing medium, washed three times with PBS and newly synthesized virions allowed to accumulate for 24 h prior to harvesting and quantitation. Amphotropic MLV was generated by transient transfection of 293T cells with pAM.

Infectivity assays

One-step quantitations of viral infectivity were performed using the C8166/HIV-CAT and C8166-CCR5/HIV-CAT indicator T cell lines. Viruses produced by various means were normalized for each independent experiment, according to reverse transcriptase levels, and used to challenge 0.5×10^6 cells. At 24–48 h post-infection, whole cell lysates were prepared and the levels of CAT activity determined as described (Simon and Malim, 1996; Fouchier *et al.*, 1997).

MLV replication was monitored by supernatant reverse transcriptase activity (Goff *et al.*, 1981) following initial challenge of 2×10^6 cells and subsequent maintenance of the cultures at a density of $0.5–2\times10^6$ cells/ml.

Western analysis

Total lysates prepared after co-transfection of 293T monolayers with pgVif:T7 vectors in the presence of pcRev were resolved on SDS–polyacrylamide gels and transferred to nitrocellulose. Vif proteins were detected by initial hybridization with a T7-Tag antibody (Novagen, Madison, WI) followed by secondary hybridization with a horseradish peroxidase-conjugated anti-mouse antibody raised in goats. Bound antibody was visualized by enhanced chemiluminescence and autoradiography.

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