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ORIGINAL

Growth ability in simian cells of monkey cell-tropic HIV-1 is greatly affected by downstream region of the *vif* gene

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Abstract : To obtain monkey-tropic (mt) HIV-1 derivatives with distinct biological characteristics and to improve the viral growth property, we have generated several variants from a prototype mt HIV-1 designated NL-DT5R (X4-tropic). The prototype HIV-1 contains a portion of *gag* and entire *vif* genes from SIVmac in its genome. The two derivatives carrying 3' half-genomic region of the SF162 (R5-tropic) or 89.6 (dual-tropic) isolate displayed very retarded or no viral growth, respectively, in a simian cell line HSC-F. In contrast, the three clones containing a part of *env* gene (encoding the V1-V4 region) from SF162, YU-2 (R5-tropic) or 89.6 showed different growth kinetics in HSC-F cells, although they grew somewhat more poorly than the NL-DT5R. Comparison of various viral proteins potentially involved in the different biological properties has revealed that, while amino acid sequences of Tat, Rev, Vpr, Vpu and Nef are quite conserved among the clones, those in the surface (SU) region of Env are relatively heterologous. Our data described here have shown that the 3' half of viral genome other than *gag* and *vif* genes greatly affects the growth property of mt HIV-1 in simian cells. J. Med. Invest. 55 : 236-240, August, 2008

Keywords : *HIV-1, Gag, Env, Vif, monkey cell tropism*

INTRODUCTION

The narrow host range of human immunodeficiency virus type 1 (HIV-1) has been a major impediment for establishing animal models for studies of viral replication and pathogenesis *in vivo* (1). To overcome this difficulty, we have recently generated an mt HIV-1 designated NL-DT5R (1-3). The parental clone of NL-DT5R contains a 21-nucleotide simian immunodeficiency virus from rhesus monkeys (SIVmac) Gag capsid (CA) element, corresponding to the HIV-1 cyclophilin A-binding loop, and the entire *vif* gene (2). Long-term passage of the virus in simian HSC-F cells has resulted in the acquisition of two non-synonymous changes in the *env* gene, which did confer an improved replication potential on the virus. A molecular clone thus obtained from the infected HSC-F cells was named NL-DT5R (2) and used for subsequent monkey infection experiments as a prototype mt HIV-1 (3). From the results of these experiments, we have learned that NL-DT5R virus grew more poorly both *in vitro* and *in vivo* than a standard SIV designated SIVmac239, which induces the AIDS in monkeys and is widely used for model studies of HIV-1/AIDS. As an apparent result of its biological property, NL-DT5R was unable to induce AIDS in the animals (3). Moreover, NL-DT5R is tropic for cells

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expressing the CXCR4 (X4) molecule but not for CCR5 (R5). It is well known that R5 viruses are clinically more important than X4 viruses (1). Taken together, new mt HIV-1s with R5 tropism and pathogenic potential are absolutely required to develop tractable animal models for AIDS research.

As a first step towards this purpose, we have generated five new mt HIV-1s by recombinant DNA techniques in this study. The proviral clones used here included R5-tropic NF462 (4), R5-tropic YU-2 (5) and dual-tropic 89.6 (6). The 3' half of NL-DT5R genome or a sequence within the *env* gene was replaced with the corresponding region of the other viral clones to obtain biologically distinct viruses. We demonstrate here that the regions other than Gag-CA and Vif certainly contain the determinants on accelerated viral growth and severe cytopathic effects in simian cells.

MATERIALS AND METHODS

Cells

A human monolayer cell line 293T (7) was maintained in Eagles's minimal essential medium containing 10% heat-inactivated fetal bovine serum. A simian lymphocytic cell line HSC-F (8) was maintained in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum.

Transfection

Sub-confluent 293T cells in 90 mm dishes were transfected with 20 μg of proviral clones in Fig. 1 by the calcium-phosphate co-precipitation method as previously reported (9). On day 2 post-transfection, cell-free culture fluids were prepared for virus samples for infection experiments (9).

Infection

HSC-F cells (3×10^6) were infected with an equal amount of viruses (1 to 2×10^7 reverse transcriptase (RT) units) prepared from transfected 293T cells, and monitored for RT production at intervals as previously described (9). Infected HSC-F cells were cultured in the presence of recombinant human IL-2 (50 units/ml) during the observation period.

RT assay

Viral growth property was determined by monitoring RT activity of culture supernatants prepared from infected HSC-F cells. RT assay using ^{32}P-dTTP has been previously described (10).

DNA constructs

An mt infectious DNA clone of HIV-1 designated NL-DT5R has been previously described (2). Infectious DNA clones of HIV-1 designated NF462 (4), YU-2 (5) and 89.6 (6) have been previously described. Construction of proviral clones in Fig. 1 were carried out by routinely used recombinant DNA methods. Appropriate DNA fragments from NF462, YU-2 and 89.6, generated by digestion with the restriction enzymes in Fig. 1, were inserted into NL-DT5R to make new full-length clones.

Amino acid alignments

Amino acid sequences of various HIV-1 proteins were aligned by the GENETYX system (Version 7). GenBank accession nos. for NL4-3, NL-DT5R, SF 162, 89.6 and YU-2 are AF324493, AB266485, M

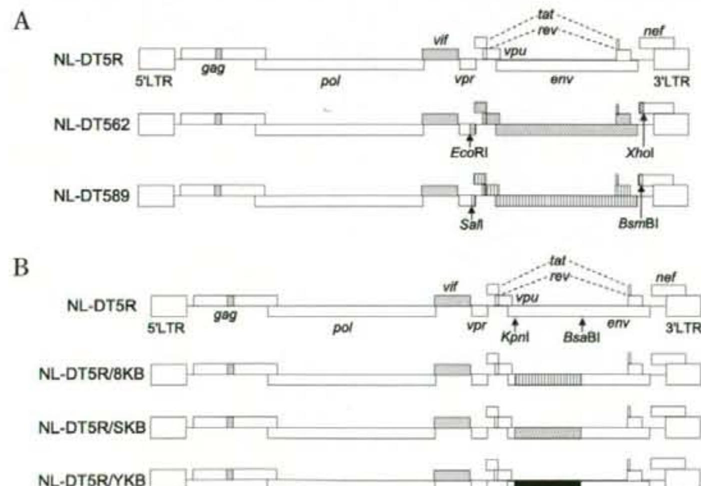


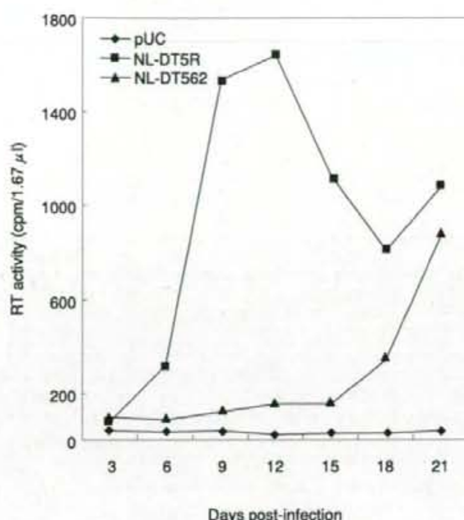
Fig. 1 Genome structure of various proviral clones used in this study. (A) Genomes of R5- and dual-tropic clones derived from NL-DT5R. The 3' half of the NL-DT5R genome was replaced with those of R5-tropic NF462 (4) and dual-tropic 89.6 (6) genomes at the sites indicated. White, grey, dotted and striped areas represent sequences from NL-DT5R (2), MA239 (SIVmac239) (13), NF462 and 89.6, respectively. LTR, long terminal repeat. (B) Genomes of *env* variants of NL-DT5R. Sequence within the *env* gene encompassing the V1-V4 region of NL-DT5R was substituted with those of 89.6, NF 462 and YU-2 (5) at the sites indicated. White, grey, striped, dotted and black areas represent sequences from NL-DT5R, MA239, 89.6, NF462 and YU-2, respectively. LTR, long terminal repeat.

65024, U39362 and M93258, respectively. NF462 clone carries the SF162 sequence in the backbone of NL4-3 genome (4).

RESULTS AND DISCUSSION

Our previous results have indicated that biologically significant mutations in the viral genome readily occur after long-term culture of infected cells (1, 2). Many of them were mapped to the *env* gene (2; our unpublished data). We, therefore, exchanged the 3' half of X4 tropic NL-DT5R genome with the corresponding regions of the other viral genomes to obtain biologically distinct and better-growing proviral clones (Fig. 1A). The exchanged sequences encompassing the *env* gene came from infectious molecular clones designated NF462 (4) and 89.6 (6), which are R5-tropic and dual-tropic, respectively. The replaced regions also contained *tat*, *rev*, *vpu*, and a part of *vpr* and *nef* genes (Fig. 1A).

To examine growth potentials in simian cells of new viral clones (NL-DT562 and NL-DT589 in Fig. 1A), they were transfected into 293T cells, and cell-free virus samples were prepared on day 2 post-transfection. Viruses obtained were then inoculated into HSC-F cells, and viral growth was monitored by RT assay. As shown in Fig. 2, the parental virus NL-DT5R readily established a spreading infection, which peaked on day 12 or 15 post-infection. In contrast, NL-DT562 grew much more poorly than NL-DT5R, and no virus growth was detected for NL-DT589. The infection experiments were repeated with similar results. Thus, it was clear that the 3' genomic region of the viruses harbors determinants on regulation of viral growth rate. We noticed that the slow-growing NL-DT562 is able to induce profound and severe cytopathic effects (mostly fusion-type) in HSC-F cells. Although NL-DT562 grew poorly, this property is quite evident and unique between the two growth-competent viruses.



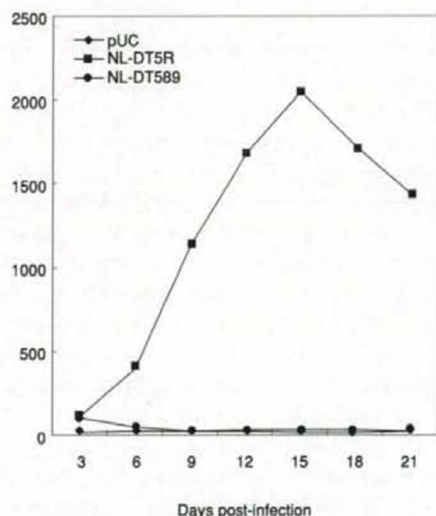


Fig. 2 Growth kinetics of various clones in simian HSC-F cells. Input cell-free viral samples were prepared from 293T cells transfected with the clones indicated, and an equivalent RT units were inoculated into HSC-F cells. Viral growth was monitored at intervals by RT activity in the culture supernatants. As a negative control, pUC19 was used.

In order to examine whether the growth property as described above is ascribed to the *env* gene, we next constructed three *env*-substitution variants as shown in Fig. 1B. In addition to the NF462 and 89.6 clones, another proviral clone YU-2, which is R5-tropic, was used to generate a variant. The substituted *env* sequence contained the regions of V1, V2, V3 and V4, which are important for X4/R5 tropism (V3 in particular). Cell-free virus samples derived from these proviral clones were prepared as described above, and inoculated into HSC-F cells. As shown in Fig. 3, while NL-DT5R grew best in the cells as above, all the other viruses did propagate. NL-DT5R/SKB virus consistently grew better than NL-DT5R/8KB and NL-DT5R/YKB in HSC-F cells. The data in Fig. 3 showed that the V1-V4 region (Fig. 1B) of *env* gene has in fact some sequences(s) controlling viral growth rate. However, when all the results in Figs. 2 and 3 were taken into consideration, it was concluded that region(s) other than V1-V4 affect very much the growth prop-

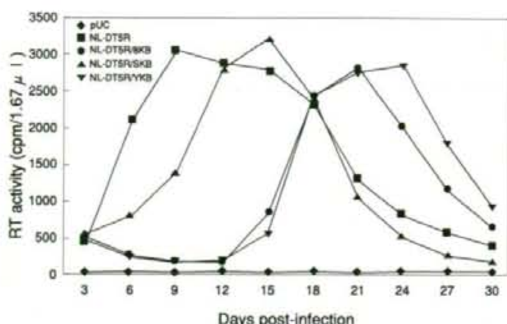


Fig. 3 Growth kinetics of various *env*-variants in simian HSC-F cells. Input cell-free viral samples were prepared from 293T cells transfected with the clones indicated, and an equivalent RT units were inoculated into HSC-F cells. Viral growth was monitored at intervals by RT activity in the culture supernatants. As a negative control, pUC19 was used.

erty of mt HIV-1.

We were interested in evaluating how different the amino acid sequences of various viral proteins are among the clones used. Amino acid alignments were made, and sequences were compared for Tat and Rev proteins (Fig. 4), for Vpr, Vpu and Nef proteins (Fig. 5) and for Env protein (Fig. 6). As is clear in the figures, all the proteins compared are different, to various degrees, with respect to the primary amino acid sequence. However, sequences of Tat, Rev, Vpr, Vpu, and Nef are quite conserved, especially those of functionally important domains of Tat and Rev (11). In addition, the accessory proteins except for Vif are known to affect relatively slightly the viral replication *in vitro* (11 ; our unpublished results). Furthermore, a *vpu*-minus mutant of SIV/HIV-1 chimeric virus, in fact, grew similarly well with wild-type virus in the HSC-F cells (12). As for Env, sequences are quite different, the N-terminal region in particular. More importantly, mutations in the *env* gene occurred quite readily

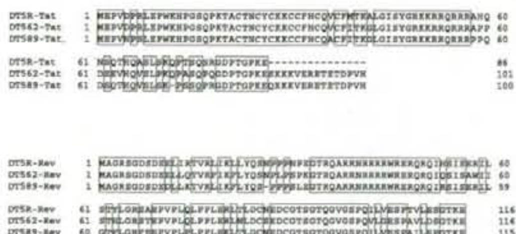


Fig. 4 Amino acid alignments of regulatory proteins Tat and Rev from NL-DT5R, -DT562 and -DT589. Identical amino acid residues are boxed.

Fig. 5 Amino acid alignments of accessory proteins Vpr, Vpu and Nef from NL-DT5R, -DT562 and -DT589. Identical amino acid residues are boxed. The sites used to construct NL-DT562 and and NL-DT589 are indicated (see Fig.1).

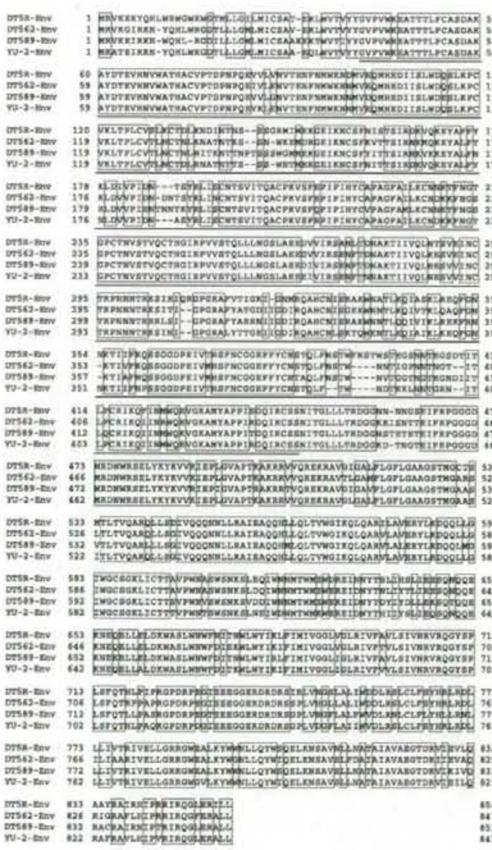


Fig. 6 Amino acid alignments of a structural protein Env from NL-DT5R, -DT562, -DT589 and YU-2. Identical amino acid residues are boxed. The substituted region is underlined (see Fig.1).

within cells, conferring augmented replicative properties on the virus (2, 10).

Taken all together, although it can not be excluded that some *cis*-acting elements in the viral genome may contribute to the growth potential of viruses, it is not unreasonable to assume that certain amino acid residues in Env are primarily important for the growth property of mt HIV-1 in simian cells. Consistent with this, we have readily found mutations in the *env* gene of cell-adapted mt HIV-1s (our unpublished observations). The identification of sequences in the *env* gene (and/or the other genes) responsible for altered virus growth phenotype, and the elucidation of the underlying mechanism need to be carried out.

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Role of HIV-1 Vpu protein for virus spread and pathogenesis

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Abstract

Vpu is an accessory viral protein almost unique to HIV-1 among primate immunodeficiency viruses, and has two major functions: degradation of the CD4 molecule in endoplasmic reticulum and enhancement of virion release from cells. Recent identification of a novel host restriction factor, tetherin, as a Vpu-antagonist suggests that Vpu contributes to virus spread by facilitating progeny virion production. This review focuses on the two distinct functions of Vpu and summarizes current knowledge on its virological role in the HIV-1 life cycle.
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Keywords: HIV-1; Vpu; Tetherin; Virus spread; Pathogenesis

1. Introduction

Host cells have evolved diverse defense mechanisms against pathogens. Confronting these, human immunodeficiency virus type 1 (HIV-1) can effectively subvert host factors, in various ways that impede virus replication and finally develop the AIDS and AIDS-related diseases in infected individuals. In addition to retroviral *gag*, *pol*, and *env* genes, the HIV-1 genome contains a unique set of accessory genes designated *vif*, *nef*, *vpr*, and *vpu*. Accessory proteins have been believed to disturb the host restriction machinery; they play essential functional roles for virus persistence, spread, and pathogenesis in natural target cells and/or in individuals by modulating and optimizing viral replication.

Among these accessory proteins, Vpu exists only in HIV-1, simian immunodeficiency virus (SIV) cpz, and SIVgsn, but not in the other primate immunodeficiency viruses such as SIVmac and HIV-2 [1]. Vpu is apparently multifunctional (Table 1) and distinct functions with/without cell-type dependency of Vpu have been described [2]. Although the conserved nature of the *vpu* gene in viruses of the HIV-1 family strongly suggests that Vpu has a positive effect on in vitro and/or in vivo viral replication, definitive evidence to fully support this proposal has not been reported until quite recently. In 2008, a novel host restriction factor, termed tetherin, that specifically inhibits virion release from cells and is counteracted by Vpu, has been discovered and demonstrated to be involved in the virus spread [3]. This finding has prompted us to extensively study Vpu again after a lengthy scientific hiatus. In this review, we give an outline of the functional activities of HIV-1 Vpu: CD4 degradation in the endoplasmic reticulum (ER) and enhancement of virion release from virus-producer cells. The virological significance and relevance of Vpu in the HIV-1 life cycle are also discussed.

2. Characteristics of HIV-1 Vpu

Vpu (NL4-3 clone) is an oligomeric, 81-amino acid type I membrane protein (16 kDa) that is translated from *vpu-env* bicistronic mRNA [4–6]. Vpu is expressed in the late stage of infection but is not a virion protein and found only in cells. Vpu consists of two major domains (Fig.1): an N-terminal hydrophobic membrane anchor domain (TM; transmembrane) (27 amino acid residues) that appears to form an ion channel

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Table 1
Functions of Vpu and their biological relevance

Function	Action	Biological relevance	Reference
(A) CD4 degradation	An adaptor of the $SCF^{\beta TrCP}$ complex	Maintenance of infectivity	[28,29]
		Efficient virion release	[30]
		Prevention of superinfection	[31]
		Release of Env precursor trapped	[18]
(B) Virion release enhancement	An antagonist of tetherin	Augmentation of virus spread	[3]
(C) Ion channel activity	Formation of ion-conductive pore	Virion release	[7,8]
(D) Apoptosis	A competitive inhibitor of TrCP	Not defined	[57]
(E) Down-regulation of MHC I and II	Not defined	Attenuation of immune response	[62,63]

Activities of Vpu and their favorable biological relevance for the HIV-1 life cycle are described. (A) Vpu acts as an adaptor of the $SCF^{\beta TrCP}$ complex and induces ubiquitination of CD4 in the ER, leading to CD4 degradation by the ubiquitin–proteasome pathway. (B) Vpu counteracts tetherin, which is a host restriction factor of retrovirus release, and enhances virion release. (C) Vpu is able to form ion-conductive pores and appears to have ion channel activity selective for monovalent cations. (D) The stable association of Vpu with TrCP causes the proteasomal degradation of IκB, resulting in the inhibition of NF-κB activation and subsequent NF-κB-dependent expression of anti-apoptotic Bcl-2 family proteins such as Bcl-x1 and A1/Bfl-1 or TNF-R complex proteins (e.g. TRAF1). (E) Vpu down-regulates surface MHC I and MHC II molecules by an unknown mechanism.

selective for monovalent cations [7,8] and a cytoplasmic domain (54 residues) that contains a pair of serine residues (at positions 52 and 56) constitutively phosphorylated by casein kinase II [9]. The phosphorylation of two serine residues in the cytoplasmic domain is critical for CD4 degradation in the ER (see Section 3.1). The mutations within the TM domain retain the activity of CD4 down-regulation but lose the ability to enhance virion release [10–12]. Thus, two separable biological functions of Vpu appear to be conducted by distinct domains: the cytoplasmic domain is involved in CD4 degradation in ER and the TM domain plays a role in virion release enhancement.

3. Functional role of Vpu in the HIV-1 life cycle

3.1. Vpu-mediated degradation of CD4 in ER

Although HIV-1 utilizes CD4 as a receptor for entry into target cells, three viral proteins, that is, Nef, Env and Vpu, efficiently down-regulate CD4 during the virus infection process. Nef down-regulates cell surface expression of CD4 by accelerating clathrin-mediated endocytosis and targeting it to lysosomes for degradation [13–15]. Env precursor gp160 forms a complex with newly synthesized CD4 in the ER. This interaction has contradictory effects. Gp160 inhibits cell surface expression of CD4 but gp160 itself is also trapped by CD4 in the ER and its maturation and trafficking is blocked [16,17].

Vpu mediates CD4 degradation in the ER, probably to free CD4-trapped gp160 and allow it to resume maturation, trafficking, and proper Env-incorporation into virions [18]. Vpu-mediated CD4 degradation proceeds in a stepwise fashion (Fig. 2A). First, it is necessary for initiation of CD4 degradation that Vpu physically interacts with the cytoplasmic domain of CD4 in ER [19,20]. Phosphorylation of Ser52 and Ser56 of Vpu is required for CD4 degradation but not for CD4 binding [20,21]. Subsequent study identified a human beta transducine repeat containing protein (βTrCP) as a Vpu partner that is required for the connection of Vpu to CD4 to induce CD4 degradation [22]. Vpu interacts with the human F-box protein βTrCP depending on the phosphorylation of Ser52 and Ser56 within the $DS^PG\Phi XS^P$ βTrCP recognition motif (Fig. 2) in the Vpu cytoplasmic domain (Fig. 1) [22]. The βTrCP in E3 ubiquitin ligase complex (Skp1, Cullin1 and βTrCP) for the ubiquitin–proteasome pathway binds to a target protein [23,24]. Usually, the target protein recognized by βTrCP is ubiquitinated and degraded (Vpu in this case). For Vpu-mediated CD4 degradation, however, simultaneous binding of Vpu to both CD4 and βTrCP leads to ubiquitination of CD4 in trans and its subsequent degradation. Vpu itself somehow escapes degradation in this process [22]. After Vpu-mediated ubiquitination of CD4, CD4 is exported from the ER to a cytosolic proteasome for its degradation. The process of dislocation of CD4 and subsequent degradation has been studied by a reconstituted yeast system [25] and in human cells [26].

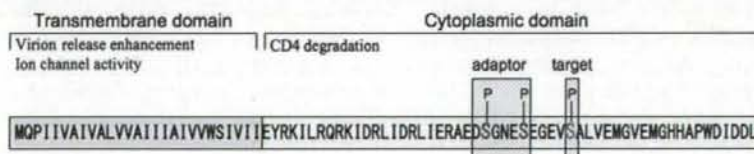

Fig. 1. Domain structure of HIV-1 (NL4-3) Vpu protein. Gray and white boxes represent transmembrane and cytoplasmic domains, respectively. Vpu functions critical for each domain are indicated. Serine residues at positions 52 (red), 56 (red), and 61 (blue) that are phosphorylated (P) are indicated. Two regions of Vpu as an adaptor of the E3 ligase complex (Skp1, Cullin1, and F-box protein βTrCP ($SCF^{\beta TrCP}$)) and as a target itself by the ubiquitin–proteasome pathway are highlighted. Consensus motif for βTrCP-binding is $DS^PG\Phi XS^P$ (S^P represents phosphorylated serine, Φ represents a hydrophobic residue, and X represents any residues) (see text). (For interpretation of the references to colour in figure legends, the reader is refered to the web version of this article).

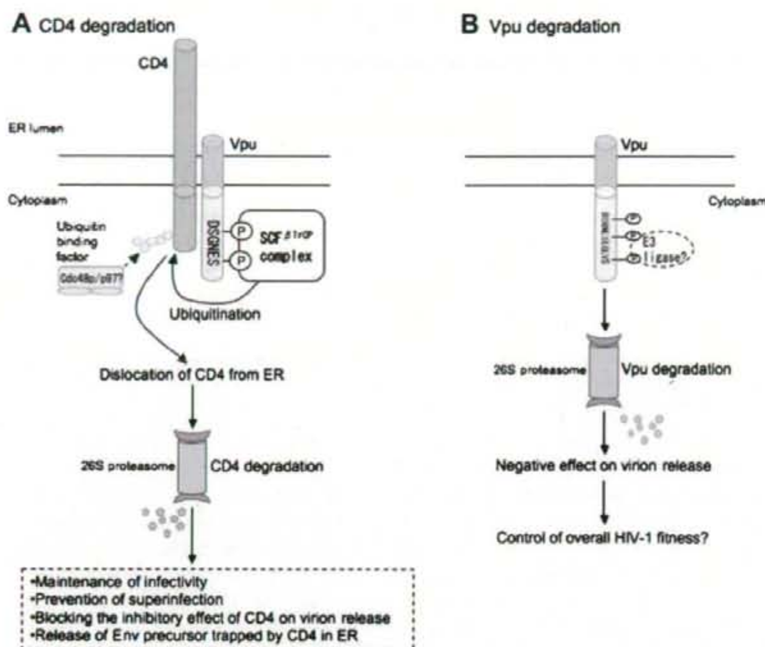

Fig. 2. Schematic representation of Vpu-mediated degradation. Degradation of CD4 in ER and of Vpu itself is illustrated in (A) and (B) as shown. Vpu acts as an adaptor or as a substrate for E3 ligase. (A) Vpu-mediated CD4 degradation is initiated by binding of Vpu to the CD4 cytoplasmic domain in the ER. Vpu is constitutively phosphorylated by casein kinase II at positions 52 and 56, two conserved serine residues. Phosphorylation of two serine residues is essential for interaction with the E3 ubiquitin ligase complex (Skp1, Cullin1, F-box protein βTrCP ($SCF^{βTrCP}$)). $SCF^{βTrCP}$ interacts with Vpu through the consensus motif $DS^PGΦXS^P$ (Fig. 1), which is required for binding to βTrCP, and ubiquitinates CD4 in trans. After Vpu-mediated ubiquitination of CD4, dislocation of CD4 from the ER membrane may be caused by a process involved in ubiquitin binding factor, the AAA ATPase Cdc48p/p97, resulting in CD4 degradation by 26S proteasomes. Vpu itself escapes degradation in this process. Vpu-mediated degradation of CD4 appears to have positive effects on the HIV-1 life cycle via various mechanisms as indicated. (B) Vpu is phosphorylated at the serine 61 residue, especially in cells arrested in early mitosis. This phosphorylation triggers the ubiquitination of Vpu itself by E3 ubiquitin ligase distinct from the βTrCP complex, leading to Vpu degradation. Although phosphorylation of Vpu at this site has a negative effect on virion release, regulation of Vpu turnover by this phosphorylation appears to play a role in controlling the balance between efficient HIV-1 virion release and viability of infected cells.

Vpu mediates CD4 degradation in the ER but Vpu itself is not degraded in this process. A recent study showed that Vpu degradation occurs in cells arrested in early mitosis (Fig. 2B) [27]. In this process, a novel phosphorylated serine residue at position 61 plays a role in recruiting unknown E3 ubiquitin ligase, distinct from the βTrCP complex, for degradation of Vpu by 26S proteasomes (Fig. 1). Ser61 is conserved among HIV-1 isolates and mutation of this residue increased virion release [27]. Thus, Vpu serves as an adaptor for CD4 degradation and as a target of the ubiquitin–proteasome pathway.

The biological relevance of CD4 degradation during HIV-1 infection is still unclear (Fig. 2A), although HIV-1 certainly down-regulates CD4 in the target cells. CD4 can be incorporated into nascent virions along with Env and viral infectivity to target cells consequentially would be reduced [28,29]. Virion release from cells may also be suppressed [30]. Furthermore, CD4 degradation may prevent superinfection of target cells with viruses [31]. Although down-modulation of CD4 appears to be advantageous for viral replication and virus spread as described above, more definitive answers to support this notion, not circumstantial evidence, are required.

3.2. Enhancement of virion release by Vpu

Early studies showed that virion release from cells infected with Vpu-deficient HIV-1 is reduced in a cell-type-dependent manner [32–35]. For example, Vpu is required for efficient virion release from human HeLa cells, monocyte-derived macrophages, and primary T cells (Vpu-dependent cells). In contrast, Vpu is unnecessary for normal virion production in human and simian cell lines such as 293T, HT1080, COS, CV-1 and Vero (Vpu-independent cells). In the late stage of Vpu-dependent cells infected with Vpu-deficient HIV-1, mature virions accumulate at the cell surface and in intracellular compartments, despite there being no effect on Gag processing or transport [36,37]. Recent studies have revealed that the accumulation of virions or Gag in intracellular vacuoles is caused by the internalization of nascent virions or Gag from the cell surface [35,38]. The observation that heterokaryons of HeLa and COS-7 exhibit the phenotype of HeLa has indicated that an inhibitor(s) for virion release exists in HeLa cells and that Vpu antagonizes the inhibitor [39]. Also, the inhibitor in HeLa cells appears to be responsible for the reduction of virion release and Vpu blocks the accumulation of nascent

virions in intracellular vacuoles and at the cell surface [35,38]. Enhancement of virion release by Vpu is effective not only for HIV-1 virions but also for diverse retroviruses (e.g. murine leukemia virus and HIV-2) despite the absence of the *vpu* gene in these viruses [37].

All the observations described above are consistent with the existence of a novel characteristic host restriction factor(s) for virion release. After a long incubation period, Neil and colleagues have finally identified an appropriate molecule designated tetherin that specifically inhibits virion release and is counteracted by Vpu [3]. Based on their finding that an inhibitory factor(s) for virion release is induced by interferon-α (IFN-α) treatment [40], they carried out microarray analyses of mRNA expressed in human cell lines treated with/without IFN-α, and revealed that CD317 (also called BST2 or HM1.24) is a tetherin candidate [3]. Confirmation that CD317 is tetherin has been obtained from the following results. First, CD317 is constitutively expressed in Vpu-dependent HeLa cells but not in Vpu-independent 293T and HT1080 cells without IFN-α treatment. Expression level of CD317 was induced in 293T and HT1080 cells and enhanced in Jurkat and primary CD4+ T cells by IFN-α treatment. Second, introduction of tetherin into 293T and HT1080 cells inhibited virion release of Vpu-deleted HIV-1 without affecting Gag expression or processing. Third, suppression of tetherin expression in HeLa cells by siRNA relieved Vpu-deleted HIV-1 virion release [3].

Needless to say, it is important to precisely elucidate the molecular basis for tetherin's antiviral activity and for counteraction by Vpu. CD317 is an integral membrane protein that has an N-terminal cytoplasmic tail followed by a TM domain, extracellular coiled-coil domain and a glycosyl phosphatidylinositol (GPI) anchor at the C-terminus (Fig. 3) [41]. CD317 localizes at the cell surface, probably in lipid rafts. The GPI anchor localizes within rafts but the TM domain resides outside the rafts. CD317 also resides in an intracellular pool and cycles between the intracellular pool and the cell surface. During this cycle, clathrin adaptor AP-2 interacts with the cytoplasmic tail of CD317 and triggers clathrin-mediated endocytosis [41]. This process may be associated with the internalization of nascent virions, even though it has been reported that internalization of HIV-1 Gag is clathrin-independent [42]. Vpu is present in the ER, golgi, endosomal structure and plasma membrane (Fig. 3) [36,43,44], and colocalizes with tetherin through the TM domain of Vpu in intracellular compartments [3]. It has been observed that Vpu down-regulates tetherin levels in HeLa cells [45]. We constructed a series of proviral mutant clones to functionally dissect HIV-1 Vpu. We found that production of progeny virions upon transfection, by cytoplasmic domain mutants containing mutations important for CD4 degradation as well as those of the TM domain, was significantly suppressed in HEp2 cells but not in 293T cells (unpublished data). It could be possible that Vpu interacts with tetherin through its TM domain, recruits ubiquitin ligase, and then degrades tetherin similarly to how it does CD4. A second possibility is that Vpu could relocate tetherin to late endosomes, which leads to protein degradation in lysosomes, while tetherin usually goes to early endosomes during cycles between the intracellular pool and the cell surface. A third possibility is as follows. Gag initially targets the plasma membrane, and then tetherin induces the internalization of Gag in Vpu-dependent cells [3,35]. In the absence of Vpu, Env is also internalized more extensively by AP-2/clathrin-mediated endocytosis, and accumulates in clathrin-coated endosomes [46,47]. Since the internalization of CD317 and Env appears to occur by association with AP-2, it could be possible that Vpu inhibits the tetherin-induced virion uptake by affecting the activity of AP-2. It has been reported that Nef down-regulates cell surface CD4 by interaction with AP-2 and subsequent clathrin-mediated endocytosis [48]. Vpu may also promote the endocytosis of tetherin itself or block tetherin-induced internalization of nascent virions by the association with AP-2.

Interaction of tetherin and Vpu would significantly affect HIV-1 biology. Tetherin could be the last barrier among various antiviral factors in cells to interfere with dissemination of the virus. Vpu promotes virus spread by overcoming the host restriction imposed by tetherin. It has already been reported by in vitro studies that virion release of HIV-1 is inhibited by IFN-α treatment even in the presence of Vpu [49,50]. It is interesting to know the in vivo situation: how and to what extent tetherin is induced in response to IFN-α and how it works to inhibit virion release. It should also be clarified how Vpu determines the timing to overcome tetherin-block and thereby promotes virus spread to a large extent. There are two modes of HIV-1 infection, that is, cell-free transmission and cell-to-cell spread through virological synapses [51], filopodial bridge [52], and/or membrane nanotubes [53]. Transmission via cell-to-cell spread occurs 100–1000-fold [53] or 18,000-fold [54] more efficiently than cell-free infection. Even in the cell-to-cell spread, HIV-1 virions are budded and released from infected cells [55]. Since cell-to-cell spread is empirically more important for viral propagation than cell-free transmission, subverting tetherin-induced inhibition by Vpu may impact on virus spread and viral pathogenesis.

3.3. Other functions of Vpu

It has been speculated that oligomeric Vpu forms ion-conductive pores based on structural similarity with the influenza virus M2 ion channel protein [56]. In fact, ion channel activity of Vpu has been demonstrated by two independent experiments [7,8]. Although it has been shown that mutations within the TM domain of Vpu which is required for the formation of ion-conductive pore decrease virion release [8], involvement of ion channel activity in virion release has not been directly determined.

It has been reported that Vpu induces apoptosis in infected cells [2,57]. Vpu stably binds to TrCP, which mediates IκB degradation, and thereby interferes with subsequent NF-κB activation. Inhibition of NF-κB activation by Vpu results in the block of NF-κB-dependent expression of anti-apoptotic Bcl-2 family proteins or TNF-R complex proteins [2,57], leading to caspase-3 activation and consequent cell death. In addition to

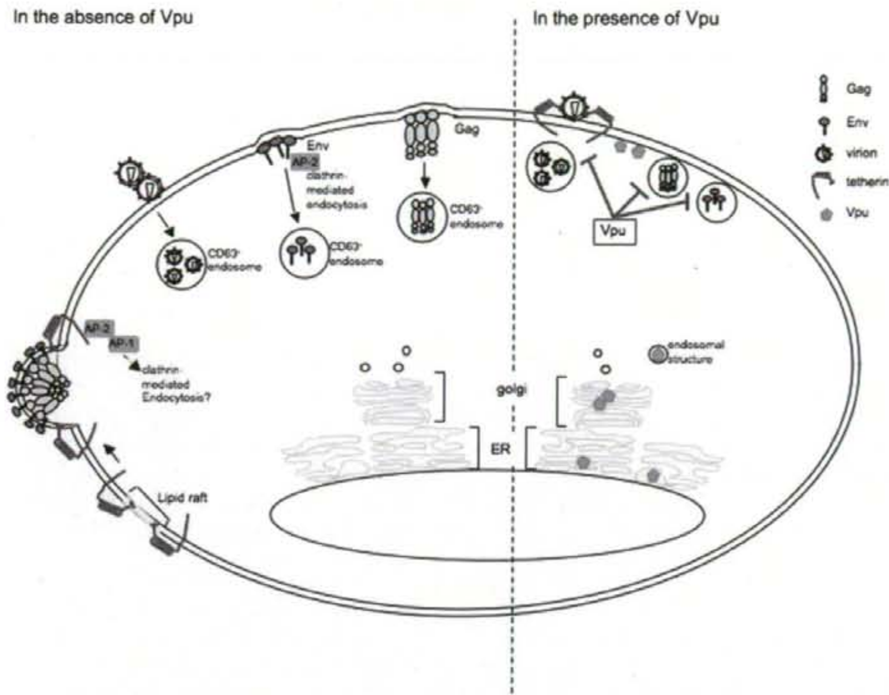

Fig. 3. Effects of HIV-1 Vpu on virion release and on localization of Gag and Env in Vpu-dependent cells. Gag is initially targeted to the plasma membrane. In the absence of Vpu, Gag is internalized and accumulated into $CD63^+$ endosomes. While Env has been known to be internalized rapidly from the plasma membrane by clathrin adaptor AP-2-mediated endocytosis, accumulation of Env within clathrin-coated endosomes seems to occur more extensively in the absence of Vpu. Vpu-deficient HIV-1 virions are tethered on the cell surface and such virions, then, are internalized and accumulated in $CD63^+$ endosomes. Tetherin specifically inhibits HIV-1 virion release in the absence of Vpu. It also induces the internalization of Gag by endocytosis. It appears to be responsible for tethering virions at the cell surface and for internalizing virions into intracellular compartments. Tetherin (CD317) has been shown to shuttle between the trans-golgi network and cell surface by clathrin-mediated endocytosis through interaction of its cytoplasmic tail with AP-2 adaptor. It is possible that tetherin-mediated endocytosis involved in internalization of Vpu-deficient nascent HIV-1 virions is clathrin-dependent.

Vpu, it has been shown that the other HIV-1 proteins such as Nef, Tat, Vpr, and Env induce apoptosis in infected cells and uninfected cells [58]. Recent studies have suggested that the immune activation state induced by HIV-1 infection, which may facilitate HIV-1 replication and impair CD4+ T cell function, causes the loss of CD4+ T cells by activation-induced cell death rather than infection [59–61]. It remains totally unknown whether the apoptosis induced by Vpu (and/or by the other viral proteins) in infected cells is associated with HIV-1 pathogenesis.

Vpu appears to down-regulate major histocompatibility complex (MHC) class I [62] and MHC II [63] on the surface of infected cells by an unknown mechanism. Since the reduction of either MHC I or MHC II results in the decrease of antigen presentation, this may lead to attenuating host immune response and contribute to viral persistence [62,63].

4. Vpu and virus spread/pathogenesis

The role of Vpu for pathogenesis in vivo has been genetically analyzed by using the SIV–HIV chimeric virus (SHIV)/macaque model. Mutations used were those in scattered regions of the TM domain and of Ser52 and Ser56 (phosphorylation sites in the cytoplasmic domain). Substitution mutation of subtype B Vpu with subtype C Vpu, which shows poor capability for CD4 degradation and is predominantly located at the cell surface rather than the ER/golgi compartment, was also used. These mutations were introduced into SHIV proviral clones for genetic analysis [64–68]. Monkeys infected with the altered SHIVs thus constructed were found to have a decreased level of viral loads in plasma and to show no loss or gradual decline of circulating CD4+ T cells. Consistently, poor viral replication in lymphoid tissues as well as non-lymphoid tissues was observed. Based on these results, one can claim that Vpu plays a role for HIV-1 pathogenesis probably by promoting virus spread through blocking the inhibitory effect of CD4 on virion release and/or by counteraction against tetherin.

SHIVs are genetically and virologically different from HIV-1 in a number of important points. For example, SHIVs show rapid disease progression compared with that of SIV in macaques (1–3 years) and HIV-1 in humans (10 years). In order to establish an animal model that would more closely reflect HIV-1 pathogenesis, we generated a monkey tropic

HIV-1 proviral clone designated NL-DT5R, which carries >90% of the HIV-1 genome [69]. Since NL-DT5R did not persist in monkeys for a long time [70], new versions of NL-DT5R have been constructed. These new viruses exhibit robust replication in monkey cells similar to that of SIVmac (our unpublished data). We believe that the HIV-1/macaque model system would shed light on a genuine role of Vpu for HIV-1 pathogenesis in vivo.

5. Conclusion

Vpu is a multifunctional protein [2] having distinct activities of CD4 degradation and virion release enhancement (Table 1). Vpu can contribute to efficient virus spread as a result of both functions. Vpu-mediated CD4 degradation facilitates virion assembly by liberating Env precursor gp160 trapped with newly synthesized CD4 in the ER and maintains virion release and the infectivity of nascent virions. Ability of Vpu to counteract tetherin, which is a virion release inhibitor imposed by host cells in response to an innate antiviral factor IFN-α, of course, enhances virion release. Thus, the lack of intact Vpu impacts on pathogenesis through the reduction of virus spread caused by poor viral replication in lymphoid tissues, and the decline of CD4+ T cell loss as shown in an SHIV/macaque model.

Can these results answer the following two questions? Why does HIV-1 carry the *vpu* gene and what is the specific role of Vpu, if any, for HIV-1 pathogenesis? Although activities of Vpu have been described and the mechanism for these has been defined in vitro, it is necessary to understand the function of Vpu in vivo. This may lead to establishing strategies to limit virus spread and accompanying pathogenicity.

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Original article

Identification of amino acid residues in HIV-1 Vif critical for binding and exclusion of APOBEC3G/F

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Abstract

To define a region(s) in human immunodeficiency virus type 1 (HIV-1) Vif that involves binding to its target APOBEC3G (A3G), we have generated a series of site-specific proviral *vif* mutants. Of 30 mutants examined, 15 did not grow at all or grew more poorly than wild-type virus in non-permissive cells. Eight clones with N-terminal mutations located outside of the HCCH motif and BC-box, which are known to be directly crucial for the degradation of A3G, were chosen from these growth-defective mutants and mainly analyzed in detail for functional activity of their mutant Vif proteins. By single-cycle replication and immunoprecipitation/immunoblotting analyses, mutants designated W21A, S32A, W38A, Y40A, and H43A were demonstrated to hardly or poorly bind to and neutralize A3G. Upon transfection, these mutants produced progeny virions containing much more A3G than wild-type clone. Interestingly, while mutants designated E76A and W79A acted normally to inactivate A3G, they were found to exhibit a Vif-defective phenotype against A3F. Another unique mutant designated Y69A incompetent against both of A3G/F was also identified. Our results here have indicated that at least two distinct regions in the N-terminal half of HIV-1 Vif are critical for binding and exclusion of A3G/F.

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Keywords: HIV-1; Vif; APOBEC3G; APOBEC3F

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) genome encodes Vif protein that counters the retroviral inhibitory effect by several members of apolioprotein B mRNA-editing enzyme-catalytic polypeptide (APOBEC) family of cytidine deaminases (for review, see [1]). APOBEC3G and APOBEC3F (A3G/F) are potent inhibitors of a wide range of retroviruses. In the absence of Vif, A3G/F are incorporated into HIV-1 virions and deaminate cytidine to uracil in the minus-strand viral DNA in the new target cells. Excessive C-to-U editing leads to the generation of hypermutated sequences that contain multiple guanosine to adenosine transitions in their plus strands and are genetically compromised. Moreover, APOBEC3 proteins may also exert antiviral activity independently of their cytidine deaminase activity. HIV-1 Vif antagonize A3G/F by inducing their degradation via ubiquitin–proteasome pathway. Mechanisms independent of proteasomal degradation may also contribute to the anti-A3G/F effect of Vif.

HIV-1 Vif contains a BC-box motif that is critical for association with Cul5-ElonginB-ElonginC and A3G ubiquitination [2,3]. The conserved BC-box is crucial for binding to ElonginC [3]. An HCCH domain, located upstream of the BC-box, is important for selective recruitment of Cul5 [4–6]. In contrast, a functional region(s) of Vif involving in binding to A3G/F and their exclusion from virions is poorly defined [7–9]. Although some amino acids (aa) in the N-terminal region of Vif were demonstrated, by mutational analyses, to be critical for interaction with A3G/F, whether A3G/F are incorporated into mutant virions is not directly analyzed, and remains to be examined. On the other hand, the N-terminal region is rationally thought to be important for the unique species-tropism of HIV-1 [10,11].

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In this study, we have newly constructed a series of site-specific point mutants of the HIV-1 (NL clone) *vif* gene encoding 192 aa, and examined their biological (multi- and single-cycle replication) and biochemical (immunoprecipitation/Western blotting) characteristics. Our results here have indicated that N-terminal aa (nos. 21, 32, 38, 40 and 43) and central aa (nos. 76 and 79) in Vif are critical for association with A3G/F and for their exclusion from virions, respectively. We also have showed that aa no. 69 in Vif is important for binding and exclusion of A3G/F.

2. Materials and methods

2.1. Plasmid construction

Twenty-six proviral *vif*-mutant clones of HIV-1 were newly constructed from wild-type (WT) pNL4-3 [12] by the QuikChange site-directed mutagenesis kit (Stratagene) as previously described [13]. Oligonucleotides used to introduce mutations into pNL4-3 are shown in Table 1. Construction and characterization of the mutants designated C114A, F115A, R132A and C133A have been previously described [14]. As a negative control clone (ΔVif), pNL-Nd [14] was used. A Flag-tagged human A3G-expression vector has been described previously [10]. Construction of Flag-tagged human A3F-expression vector was performed as follows. Human A3F was amplified by RT–PCR using mRNA from a lymphocyte cell line H9 with forward (5′-GCT CTA GAA TGA ATC ACT TCA GAA ACA CAG-3′) and reverse (5′-ACG CGT CGA CCT CGA GAA TCT CCT GCA GCT TGC-3′) primers containing *Xba*I and *Sal*I sites, respectively. The reactions were 95 °C for 1 min, 63 °C for 1 min, 72 °C for 2.5 min for 10 cycles; 95 °C for 1 min, 65 °C for 1 min, 72 °C for 2.5 min for 20 cycles. The PCR product was then cloned into pcDNA3.1-Flag, an expression vector containing the FLAG-tag sequence (Invitrogen). Amino acid sequence of human A3F from H9 cells differed from the GenBank database (accession number AAH38808) only by a single aa (Y345D).

2.2. Cell culture, transfection, and virion preparation

293T and MAGI cells were maintained in Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). H9 cells were cultured in RPMI1640 medium supplemented with 10% FBS. Plasmid DNA transfection into 293T cells was carried out by the calcium-phosphate co-precipitation method [12]. To make virion preparations, culture supernatants were harvested at 48 h post-transfection, filtered through 0.45-μm filters, and viral particles were concentrated by ultracentrifugation through 25% sucrose for 2 h at 80,000 × *g* using SW41 rotor as previously described [15].

2.3. Single- and multi-cycle replication assays

To determine single-cycle replication of mutants, viral samples were prepared from 293T cells co-transfected with each proviral clone and an expression vector of A3G or A3F at 48 h post-transfection. Single-cycle replication was then determined by the MAGI assay [16]. To determine growth kinetics of the mutants, viruses were prepared from 293T cells transfected with each proviral clone, and inoculated into H9 cells. Viral growth was monitored by the reverse transcriptase (RT) assay of the culture supernatants of infected H9 cells at intervals as previously described [17].

2.4. Western immunoblot analysis

Cell and virion fractions were prepared from 293T cells transfected with proviral clones, and lysed in a lysis buffer (1% Nonidet P-40, 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA and 1% protease inhibitor cocktail (Sigma)). The cell and virion lysates were then resolved on SDS–PAGE, followed by electrophoretic transfer to polyvinylidene fluoride membranes. The membranes were treated with anti-FLAG (Sigma), anti-Vif (NIH AIDS Research and References Reagent Program) or anti-p24 [15] antibody, and visualized by the ECL plus Western blotting detection system (Amersham Pharmacia Biotech Inc.).

2.5. Immunoprecipitation

Transfected 293T cells were lysed in the lysis buffer as above, mixed with anti-FLAG M2 agarose (Sigma), and incubated at 4 °C for 3 h. The reaction mixture was then washed three times with TBS buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl) and eluted by the addition of 3× FLAG peptides (Sigma). After centrifugation, the supernatants were analyzed by immunoblotting.

3. Results

3.1. Generation and characterization of Vif mutants

To determine whether each of highly conserved aa residue in HIV-1 Vif is required for viral infectivity, we generated a series of substitution mutants from WT pNL4-3 including 26 new clones (Fig. 1). Target residues were not chosen from C-terminal 19 aa because they are unnecessary for Vif function [18]. Mutant virus stocks were prepared from transfected 293T cells, and inoculated into H9 cells non-permissive for ΔVif virus. Out of 30 mutants in Fig. 1, 12 clones (W21A, S32A, W38A, Y40A, Y69A, W79A, H108A, C114A, F115A, R132A, C133A and H139A) did not grow at all similarly with the ΔVif virus. In addition, H43A, E76A and D104A mutants displayed retarded growth kinetics compared with WT virus. The other mutant viruses were found to be similarly infectious for H9 cells with WT virus. In total, aa residues which are critically required for viral replication in non-permissive H9 cells were located at scattered sites except for the downstream region of the BC box (Fig. 1), indicating that N-terminal and central portions of Vif are important for its function. The HCCH motif and BC box in Vif have been reported to be essential for association with Cul5 and ElonginC, respectively, but not for interaction with A3G [3]. Therefore, to determine a functional domain(s) for binding to A3G, we mainly examined the property of the mutants with

Table 1
Oligonucleotides used to construct HIV-1 *vif* mutants in this study

Clones	Primers
E2A	5′-GATCATCAGGGATTATGGCAAACAGATGGCAGGTG 5′-CACCTGCCATCTGTTTGCCATAATCCCTGATGATC
W11L	5′-GGCAGGTGATGATTGTGTTGCAAGTAGACAGGATG 5′-CATCCTGTCTACTTGCAACACAATCATCACCTGCC
W21A	5′-GACAGGATGAGGATTAACACAGCGAAAAGATTAGTAAAACACC 5′-GGTGTTTTACTAATCTTTTCGCTGTGTTAATCCTCATCCTGTC
L24A	5′-GAGGATTAACACATGGAAAAGAGCCGTAAAACACCATATG 5′-CARARGGTGTTTTACGGCTCTTTTCCATGTGTTAATCCTC
H28A	5′-GGAAAAGATTAGTAAAACACGCCATGTATATTTCAAGCAAAGC 5′-GCTTTCCTTGAAATATACATGGCGTGTTTTACTAATCTTTTCC
S32A	5′-CACCATATGTATATTGCAAGGAAAGCTAAGGACTG 5′-CAGTCCTTAGCTTTCCTTGCAATATACATATGGTG
W38A	5′-CAAGGAAAGCTAAGGACGCGTTTTATAGACATCAC 5′-GTGATGTCTATAAAACGCGTCCTTAGCTTTCCTTG
Y40A	5′-GCTAAGGACTGGTTTGCTAGACATCACTATGAAAG 5′-CTTTCATAGTGATGTCTAGCAAACCAGTCCTTAGC
H43A	5′-GCTAAGGACTGGTTTTATAGACATGCCTATGAAAGTACTAATCC 5′-GGATTAGTACTTTCATAGGCATGTCTATAAAACCAGTCCTTAGC
S53A	5′-CTAATCCAAAAATAAGTGCCGAAGTACACATCCC 5′-GGGATGTGTACTTCGGCACTTATTTTTGGATTAG
P58A	5′-GTTCAGAAGTACACATCGCACTAGGGGATGCTAAATTAG 5′-CTAATTTAGCATCCCCTAGTGCGATGTGTACTTCTGAAC
Y69A	5′-CTAAATTAGTAATAACAACAGCCTGGGGTCTGCATACAG 5′-CTGTATGCAGACCCCAGGCTGTTGTTATTACTAATTTAG
E76A	5′-GGGTCTGCATACAGGAGCAAGAGACTGGCATTTGG 5′-CCAAATGCCAGTCTCTTGCTCCTGTATGCAGACCC
W79A	5′-CAGGAGAAAGAGACGCGCATTTGGGTCAGGGAGTC 5′-GACTCCCTGACCCAAATGCGCGTCTCTTTCTCCTG
S86A	5′-CATTTGGGTCAGGGAGTCGCCATAGAATGGAGGAAAAAG 5′-CTTTTTCCTCCATTCTATGGCGACTCCCTGACCCAAATG
Y94A	5′-GGAGGAAAAAGAGAGCTAGCACACAAGTAGACCC 5′-GGGTCTACTTGTGTGCTAGCTCTCTTTTTCCTCC
T96A	5′-GAAAAAGAGATATAGCGCACAAGTAGACCCTGACC 5′-GGTCAGGGTCTACTTGTGCGCTATATCTCTTTTTC
D104A	5′-GACCCTGACCTAGCAGCCCAACTAATTCATCTGC 5′-GCAGATGAATTAGTTGGGCTGCTAGGTCAGGGTC
H108A	5′-CCTAGCAGACCAACTAATTGCCCTGCACTATTTTGATTGTTTTTC 5′-GAAAAACAATCAAAATAGTGCAGGGCAATTAGTTGGTCTGCTAGG
Y111A	5′-GACCAACTAATTCATCTGCACGCCTTTGATTGTTTTTCAGAATC 5′-GATTCTGAAAAACAATCAAAGGCGTGCAGATGAATTAGTTGGTC
S118A	5′-CTATTTTGATTGTTTTTCAGAAGCCGCTATAAGAAATACC 5′-GGTATTTCTTATAGCGGCTTCTGAAAAACAATCAAAATAG
H139A	5′-GAATATCAAGCAGGAGCTAACAAGGTAGGATCTC 5′-GAGATCCTACCTTGTTAGCTCCTGCTTGATATTC
L148A	5′-GGTAGGATCTCTACAGTACGCGGCACTAGCAGCATTAATAAAAC 5′-GTTTTATTAATGCTGCTAGTGCCGCGTACTGTAGAGATCCTACC
P162A	5′-CAAAACAGATAAAGCCAGCTTTGCCTAGTGTTAGG 5′-CCTAACACTAGGCAAAGCTGGCTTTATCTGTTTTG
S165A	5′-GATAAAGCCACCTTTGCCTGCTGTTAGGAAACTGACAGAGG 5′-CCTCTGTCAGTTTCCTAACAGCAGGCAAAGGTGGCTTTATC
E171A	5′-GTTAGGAAACTGACAGCGGACAGATGGAACAAGC 5′-GCTTGTTCCATCTGTCCGCTGTCAGTTTCCTAAC

Nucleotides for mutations are underlined.

mutations in N-terminal half (W21A, S32A, W38A, Y40A, H43A, Y69A, E76A and W79A) in the following analyses.

3.2. N-terminal region (aa 21–43) of HIV-1 Vif is important for suppression of A3G

Next, we examined the effect of N-terminal mutations in HIV-1 Vif on the suppression of A3G activity. As clearly seen in the result of single-cycle replication assay in the presence of A3G (Fig. 2A), while growth-defective mutant clones (W21A, S32A, W38A, Y40A and H43A in Fig. 1) were unable to completely counter A3G, growth-competent positive controls (L24A and H28A) behaved like WT. The mobility-shift observed for the H28A Vif would probably be related to its structure. The leaky mutant H43A still retained the ability to suppress A3G in this assay. These data were consistent

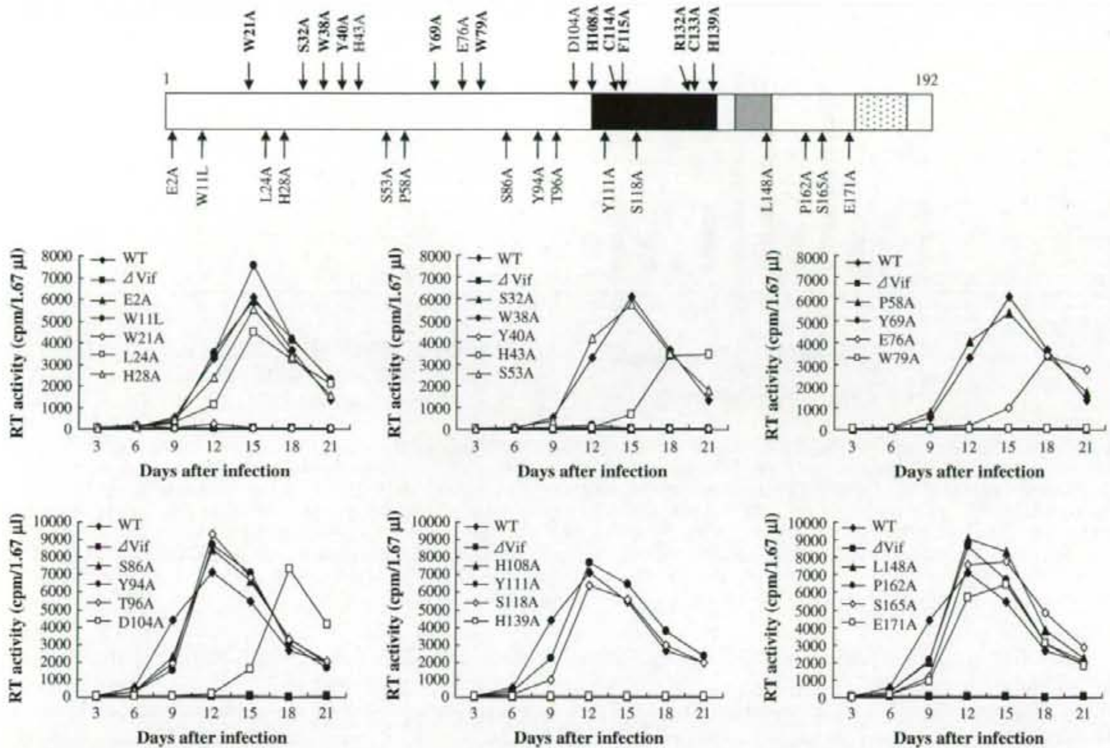

Fig. 1. Growth kinetics of various *vif* mutants in non-permissive H9 cells. H9 cells (1×10^6) were infected with an equivalent RT units (5×10^6) of cell-free viruses prepared from transfected 293T cells, and virus replication was monitored at intervals by RT production in the culture supernatants. The experiment was repeated with similar results. Locations of mutations in HIV-1 Vif (NL clone) and the mutant designations are indicated at the top. Mutants above and below the bar indicate the phenotypes of growth-defective and normal growth, respectively. Mutants in bold lines are growth-incompetent at all in H9 cells like ΔVif virus. Characterization of the mutants designated C114A, F115A, R132A and C133A has been reported previously [14]. Black, gray and dotted areas in the bar are the HCCH domain, BC-box and Basic domain, respectively.

with a conclusion that the impairment of multi-cycle replication of the N-terminal mutants (Fig. 1) is due to their inability to suppress A3G activity. We therefore analyzed the potentials of these mutants to control the level of A3G in virus-producing cells and in virions. As shown in Fig. 2B, WT Vif reduced somewhat the level of A3G in cells compared with the mutants, and no A3G was detected in WT virions. In contrast, none of the mutants examined here effectively excluded A3G from virions. Interaction of various Vif proteins with A3G was evaluated by co-immunoprecipitation analysis, as shown in Fig. 2C. A mutant control H108A and WT was associated with A3G but any of the other mutants was not. These data strongly suggest that the N-terminal region in Vif is required for its binding to A3G.

3.3. Glu76 and Trp79 of HIV-1 Vif are important for suppression of A3F but not for that of A3G

We further examined the effect of central region mutations (aa 69–79) in Vif on the suppression of A3G activity. As shown in Fig. 3A, although the three mutants studied here were all growth-defective in H9 cells (Y69A, E76A and W79A in Fig. 1), only the Y69A was unable to suppress A3G activity showing single-cycle replication similar to that of ΔVif clone. Thus, the expression level of A3G in virus-producing cells was monitored for the mutants (Fig. 3B). WT Vif reduced the expression of A3G relative to that by various mutants as described above. Among the mutants, only W79A was noticed to slightly reduce A3G expression. Interaction of the mutant proteins with A3G was then evaluated by the co-immunoprecipitation assay as described above. As shown in Fig. 3C, while Y69A did not bind to A3G, E76A and W79A mutants did as efficiently as WT. When the amount of A3G in virions was monitored, interestingly, only Y69A was found to be defective for exclusion of A3G among the three mutants (Fig. 3D). These results indicated that the growth-defective nature of E76A and W79A in H9 cells was not due to their defective anti-A3G activity. Based on this finding, we examined the ability of E76A and W79A mutants to act against A3F. As shown in Fig. 4A, single-cycle replication of the mutants in the presence of A3F was similarly inefficient with ΔVif mutant. The expression level in cells of A3F in the presence of mutant Vif proteins was similarly higher than that by WT

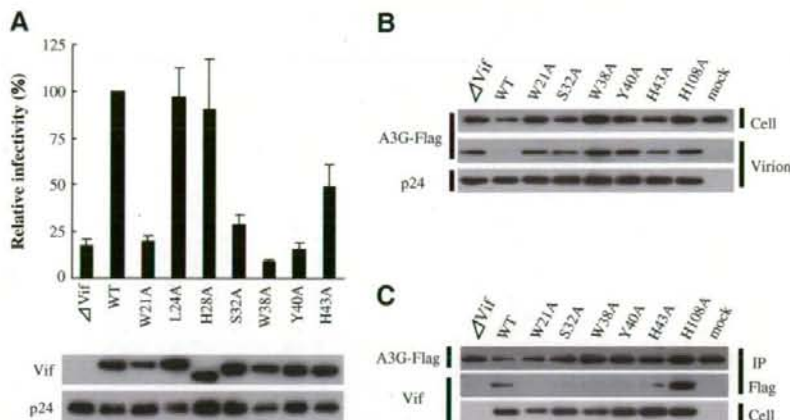

Fig. 2. Activity of various *vif* mutants (W21A, S32A, W38A, Y40A and H43A) against A3G. (A) Effect of A3G on the single-cycle replication of the mutants. Viral samples were prepared from 293T cells co-transfected with each proviral clone and an A3G-expression vector, and their infectivity was determined in MAGI cells. Expression levels of Vif and Gag-p24 as monitored by immunoblotting of lysates from transfected 293T cells are also shown at the bottom. (B) Level of A3G in virus-producing cells and in progeny virions. 293T cells were transfected with the mutant clones indicated, and cell/virion lysates for immunoblotting analysis were prepared 2 days later. H108A was used as a control (see Fig. 1). (C) Ability of WT and mutant Vif proteins to interact with A3G. Lysates of 293T cells co-transfected with each proviral clone and an A3G-expression vector were prepared, and used for immunoprecipitation (IP) of A3G-Flag by anti-Flag-M2 agarose. The interaction of various Vif proteins and A3G was then analyzed by immunoblotting using anti-Flag or anti-Vif anti-serum. Cell lysates were examined by immunoblotting analysis with anti-Vif anti-serum. The experiment in this figure was repeated with similar results.

(Fig. 4B). Interaction of mutants with A3F was then evaluated by the co-immunoprecipitation analysis as shown in Fig. 4C. Whereas WT Vif efficiently bound to A3F, Y69A and W79A (Fig. 4C, lane 5) clearly lost the ability. Even the leaky mutant E76A (Fig. 1) was very defective for binding activity. As shown in Fig. 4D, we finally analyzed the encapsidation of A3F into mutant virions. As expected, while the WT clone effectively excluded A3F from virions,

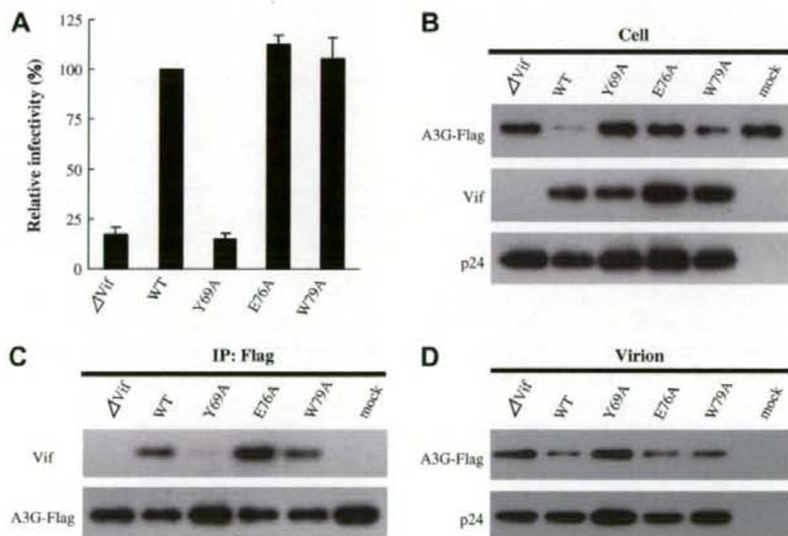

Fig. 3. Activity of *vif* mutants (Y69A, E76A and W79A) against A3G. (A) Effect of A3G on the single-cycle replication of the mutants. Viral samples were prepared from 293T cells co-transfected with each proviral clone and an A3G-expression vector, and their infectivity was determined in MAGI cells. (B) Expression level of A3G in the presence of WT or mutant Vif proteins. Samples were prepared from 293T cells transfected with each proviral clone, and analyzed by immunoblotting using antibodies indicated. (C) Ability of WT and mutant Vif proteins to interact with A3G. Lysates of 293T cells co-transfected with each proviral clone and an A3G-expression vector were prepared, and used for immunoprecipitation (IP) of A3G-Flag by anti-Flag-M2 agarose. The interaction of various Vif proteins and A3G was then analyzed by immunoblotting using anti-Flag or anti-Vif anti-serum. (D) Packaging of A3G into mutant virions. Virions prepared from 293T cells co-transfected with each proviral clone and an A3G-expression vector were examined by immunoblotting analysis using antibodies indicated. The experiment in this figure was repeated with similar results.

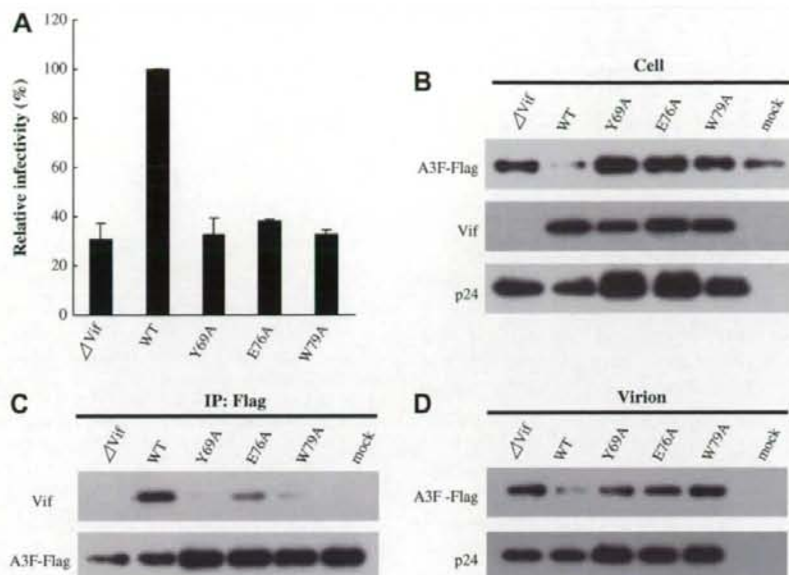

Fig. 4. Activity of *vif* mutants (Y69A, E76A and W79A) against A3F. (A) Effect of A3F on the single-cycle replication of the mutants. Viral samples were prepared from 293T cells co-transfected with each proviral clone and an A3F-expression vector, and their infectivity was determined in MAGI cells. (B) Expression level of A3F in the presence of WT or mutant Vif proteins. Samples were prepared from 293T cells transfected with each proviral clone, and analyzed by immunoblotting using antibodies indicated. (C) Ability of WT and mutant Vif proteins to interact with A3F. Lysates of 293T cells co-transfected with each proviral clone and an A3F-expression vector were prepared, and used for immunoprecipitation (IP) of A3F-Flag by anti-Flag-M2 agarose. The interaction of various Vif proteins and A3F was then analyzed by immunoblotting using anti-Flag or anti-Vif anti-serum. (D) Packaging of A3F into mutant virions. Virions prepared from 293T cells co-transfected with each proviral clone and an A3F-expression vector were examined by immunoblotting analysis using antibodies indicated. The experiment in this figure was repeated with similar results.

none of the mutants did so. These data strongly suggested that the central region in Vif is required for its binding to A3F.

4. Discussion

In this report, we have performed a systematic molecular genetic study on the A3G/F-binding function of HIV-1 Vif (NL clone). The data obtained have indicated that N-terminal aa (nos. 21, 32, 38, 40 and 43) and central aa (nos. 76 and 79) in Vif are critical for its binding to A3G/F and finally for their exclusion from virions, respectively. We also have demonstrated that aa no. 69 in Vif is important for binding and exclusion of both A3 proteins. Our results here have confirmed and significantly extended the previous reports [7–9]. Taken together, all the available data can be summarized as shown in Fig. 5. As is clear in the figure, several distinct regions in the N-terminal half of HIV-1 Vif are critical for its binding to anti-retroviral innate factors A3G/F. We showed that the N-terminal region (aa 21–38) is necessary for suppression of A3G activity through binding (Fig. 2), in addition to the conclusion previously published [8,9]. We also showed for

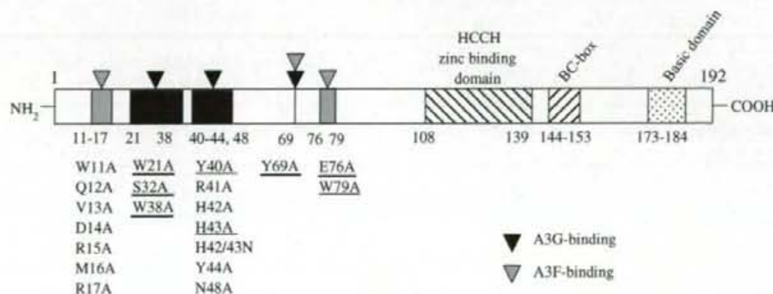

Fig. 5. Regions of HIV-1 Vif responsible for binding to A3G/F. Based on the results previously published [7–9] and presented in this study, amino acid residues critical for the binding activity of Vif are summarized. Mutations that abolish or diminish the activity are shown. The eight mutations identified in this study are underlined (bold underlines, newly identified).