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JOURNAL OF VIROLOGY, Aug. 2008, p. 7752–7756 Vol. 82, No. 15
0022-538X/08/$08.00+0 doi:10.1128/JVI.01003-07

Vpx Is Critical for Reverse Transcription of the Human Immunodeficiency Virus Type 2 Genome in Macrophages[▽]

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Received 8 May 2007/Accepted 8 May 2008

The abilities of wild-type and *vpx*-defective human immunodeficiency virus type 2 (HIV-2) clones to synthesize viral DNA in human monocyte-derived macrophages (MDMs) and lymphocytic cells were comparatively and quantitatively evaluated. While the *vpx*-defective mutant directed the synthesis of viral DNA comparably to the wild-type virus and normally in lymphocytic cells, no appreciable viral DNA was detected in MDMs infected with the mutant. To substantiate this finding and to determine whether there is some specific region(s) in Vpx crucial for viral DNA synthesis in MDMs, we generated a series of site-specific point mutants of *vpx* and examined their phenotypes. The resultant five mutants, with no infectivity for MDMs, showed, without exception, the same defect as the *vpx*-defective mutant. Our results here clearly demonstrated that the entire Vpx protein is critical for reverse transcription of the HIV-2 genome in human MDMs.

Viruses of the human immunodeficiency virus type 2 (HIV-2) group carry a *vpx* gene that encodes virion-associated Vpx protein. Vpx is an accessory viral protein and is completely unnecessary and dispensable for virus replication in established cell lines and primary lymphocyte cells prepared from peripheral blood mononuclear cells (10, 21, 25). However, in human monocyte-derived macrophages (MDMs), the *vpx*-defective viruses do not grow at all (6, 20, 21, 25). Because Vpx is specifically incorporated into virions by association with Gag-p6 protein in significant quantities (1, 11, 12, 24), it has been believed that Vpx has a specific and early functional role at the Env-independent postentry replication step. In fact, there have been some articles directly addressing the early function of Vpx in the life cycle of HIV-2. Worthy of note, one report has shown that Vpx is dispensable for reverse transcription of the viral RNA genome but important for nuclear import of the viral preintegration complex in MDMs (6). But in that study (6), a unique simian immunodeficiency virus (SIV) isolated from the sooty mangabey (SIV_{SM}PBj1.9), which causes an acute fatal disease in pig-tailed monkeys (5), was used to determine the defect of *vpx* mutants in simian MDMs. Another paper has described results similar to those mentioned above, obtained for a U937 cell line growth arrested by mimosine treatment (20). Furthermore, in both studies (6, 20), the conclusions were based on the data obtained from rather qualitative PCR analysis. Therefore, quite surprisingly, virtually no studies focusing on the functional role of HIV-2 Vpx in human MDMs with clear and convincing data have been published yet. In this study, we have performed an extensive mutational functional analysis by quantitative assays of HIV-2 Vpx in human MDMs. We demonstrate here, in contrast to the previously published conclusions, that Vpx is critical for reverse transcription of the HIV-2 genome in human MDMs.

We first evaluated the extent of viral DNA synthesis by a *vpx*-defective mutant at the postentry step in human MDMs by using HSC-F cells (3, 4) as a cell control (21). The mutant used was derived from a well-characterized and widely distributed molecular clone (13, 14, 21) for easy scientific comparison. Infection of human MDMs by virus samples from 293T cells (17) transfected with proviral clones was very much inefficient and gave ambiguous data. To obtain reproducible quantitative results, we conducted the assay as follows. Virus samples (pseudotype viruses) were prepared from 293T cells cotransfected with an expression vector of the vesicular stomatitis virus G protein (pCMV-G) (23) and an *env*-defective proviral clone (pGL-Ns) (21) for the wild type (WT) or an *env*- and *vpx*-defective clone (pGL-Ns/St) (21) for the *vpx* mutant and inoculated into HSC-F and MDM cells. On day 2 postinfection, DNAs were prepared from these infected cells and subjected to real-time PCR analysis using appropriate primer pairs to detect the late reverse transcription product (U5/5′-end noncoding region) in the cytoplasm and the two-long terminal repeat (two-LTR) circle in the cell nucleus. As is clear in Fig. 1, a major replication defect in HSC-F cells of the *vpx*-defective mutant was noticed at the nuclear import process of viral DNA, in good agreement with our previous report (21). By contrast, the mutant was unable to synthesize viral DNA in MDMs, as judged by the absence of the late reverse transcription product, indicating that Vpx is crucial for reverse transcription of the viral RNA genome in a cell type-dependent manner. The same experiments were repeated, using MDMs from different individuals, with perfectly reproducible outcomes. These results prompted us to do a systemic mutational analysis of HIV-2 Vpx in MDMs to dissect its function in the virus replication cycle.

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▽ Published ahead of print on 21 May 2008.

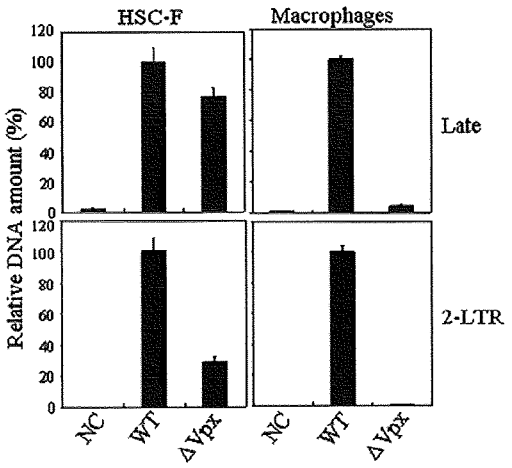

FIG. 1. Quantitative estimation of viral DNA synthesis in HSC-F and human MDM cells infected with the *vpx*-defective mutant. Cell-free virus samples (pseudotype viruses) were prepared from 293T cells cotransfected with 10 μg of pCMV-G and 10 μg of pGL-Ns (WT) or pGL-Ns/St (ΔVpx). For a negative control (NC), pGL-Ns (10 μg) and pUC19 (10 μg) were used for cotransfection. HSC-F cells (1×10^7) and confluent human MDMs, which had been prepared from peripheral blood mononuclear cells and cultured in each well of six-well tissue culture plates as previously described (7, 21) and were 95 to 97% CD68 positive and completely negative for CD3, were infected with equal amounts of these cell-free viruses (4×10^7 reverse transcriptase [RT] units [22] and 1.4 μg of Gag-p27, as determined by enzyme immunoassays of SIV Gag-p27 [Coulter, Miami, FL], for HSC-F and MDM cells, respectively) in the presence of DNase I (40 μg/ml), $MgCl_2$ (10 mM), EGTA (2 mM), and DEAE-dextran (5 μg/ml), as previously described (16). On day 2 postinfection, DNA was extracted from the infected cells and subjected to real-time PCR analysis using TaqMan probes (Applied Biosystems, Foster City, CA) to detect the late reverse transcription product (U5/5′-end noncoding region) in the cytoplasm and the two-LTR circle in the cell nucleus. The β-globin gene was amplified for normalization (19). The primers and probes used were as follows: for the late reverse transcription product (U5/5′-end noncoding region), 5′-TCCGCTTTGGGAATCCAA-3′ (forward primer), 5′-GGGCTTCTCAGTCCCTTTCAA-3′ (reverse primer), and 5′-FAM (6-carboxyfluorescein)-AAAATCCCTAGCAG GTTGGCGCCC-TAMRA (6-carboxytetramethylrhodamine)-3′ (probe); and for the two-LTR circular product (U5/U3 region), 5′-TCGCCGCCT GGTCATT-3′ (forward primer), 5′-CCCTACTGTAAAACATCCCA TCCA-3′ (reverse primer), and 5′-FAM-ACCCTGGTCTGTTAGGA CCCTTCCGC-TAMRA-3′ (probe). The reaction mixtures were heated at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

As shown in Fig. 2, 19 point mutations were introduced into scattered regions of WT *vpx* of an infectious HIV-2 molecular clone designated pGL-AN (13) by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). To determine the target amino acids for mutation, amino acids that are well conserved among various HIV-2 isolates were carefully selected. In addition, since the 5′ region of *vpx* encoding amino acids 1 to 58 of Vpx was overlapping with *vif*, care was taken not to change the amino acids of WT Vif. The mutants thus constructed were introduced into 293T cells, and all the mutants generated progeny virions at a normal level, with MAGI infectivity (15) comparable to that of the WT virus, as expected (data not shown). Various virus clones were then inoculated into human MDMs, and their growth properties were determined. As shown in Fig. 2, out of 19 mutants, 9 grew more poorly than the WT virus and 5 did not grow at all in MDMs. These results were confirmed in repeated experiments, using MDMs from different individuals. The mutations causing the noninfectious mutants (E15G, W24L, H39L, W49L, and Q76A) were not clustered, suggesting that there may be no specific regions or domains important for virus growth in MDMs.

There was a possibility that the damaged or noninfectious nature of the 14 mutants (Fig. 2) is due to the lack of incorporation of mutant Vpx proteins into virions. Initial attempts to detect the expression of Vpx in transfected 293T cells by Western immunoblotting were mostly unsuccessful, probably because the monoclonal and polyclonal antibodies against Vpx used for detection were insensitive. We therefore constructed a Vpx expression vector with a FLAG tag for the five noninfectious mutants and monitored the transfected 293T cells for mutant Vpx by anti-FLAG antibody. A Vpx protein level comparable to that for the WT clone was detected for each mutant (Fig. 3A). We then determined whether the mutant Vpx proteins were actually detectable in the progeny virions. We previously reported that HIV-1 virion-associated viral proteins can be examined after partial purification and concentration of the virions by ultracentrifugation (8). We applied the same method to monitor Vpx in HIV-2 virions. As controls for this experiment, we newly constructed two Gag-p6 site-specific mutants (designated p6/3AS and p6/2A) from pGL-AN, which have mutated amino acid sequences in Gag-p6 critical for the incorporation of Vpx into virions (1) (Fig. 3B). These two mutants were transfected into 293T cells, and 3 days later, virion samples for Western blot analysis were prepared as described above. As is clear in Fig. 3B, the incorporation of Vpx into virions was not detected at all for the two control mutants, as expected, indicating that the procedure used for HIV-1 can be applicable for the preparation of HIV-2 virions. Mutant virions prepared from transfected 293T cells by this method were then monitored for Vpx. As shown in Fig. 3C, the virions of the P4L (WT growth properties), P10L (intermediate growth properties [between those for WT and noninfectious viruses]), and E15G (noninfectious virus) mutants (Fig. 2) contained Vpx, like those of the WT virus. The presence of Vpx in virions of all the other mutants shown in Fig. 2 was also verified by this Western blot analysis. The percentages of specific virion incorporation of Vpx (Vpx/Gag-p27 ratio, as quantified by immunoblotting [Fig. 3C]) of the five noninfectious mutants (E15G, W24L, H39L, W49L, and Q76A) relative to the WT level were 195 ± 16, 193 ± 14, 28 ± 6, 56 ± 6, and 103 ± 19, respectively. Of note, mutant N33S, having a low level of virion-incorporated Vpx in this assay (14 ± 6), was still somewhat infectious for MDMs (Fig. 2).

We finally evaluated the abilities of the five point mutants noninfectious for MDMs (E15G, W24L, H39L, W49L, and Q76A) to synthesize viral DNA in infected human MDMs. MDMs were infected with the mutants (pseudotype viruses) as described above, and the infected cells were similarly analyzed by real-time PCR using two sets of primer pairs. As shown in Fig. 4, the reverse transcription processes at early and late phases of the five mutants were critically impaired, generating no significant quantities of viral DNA in the cell nucleus. The Q76A mutant appeared less attenuated for reverse transcrip-

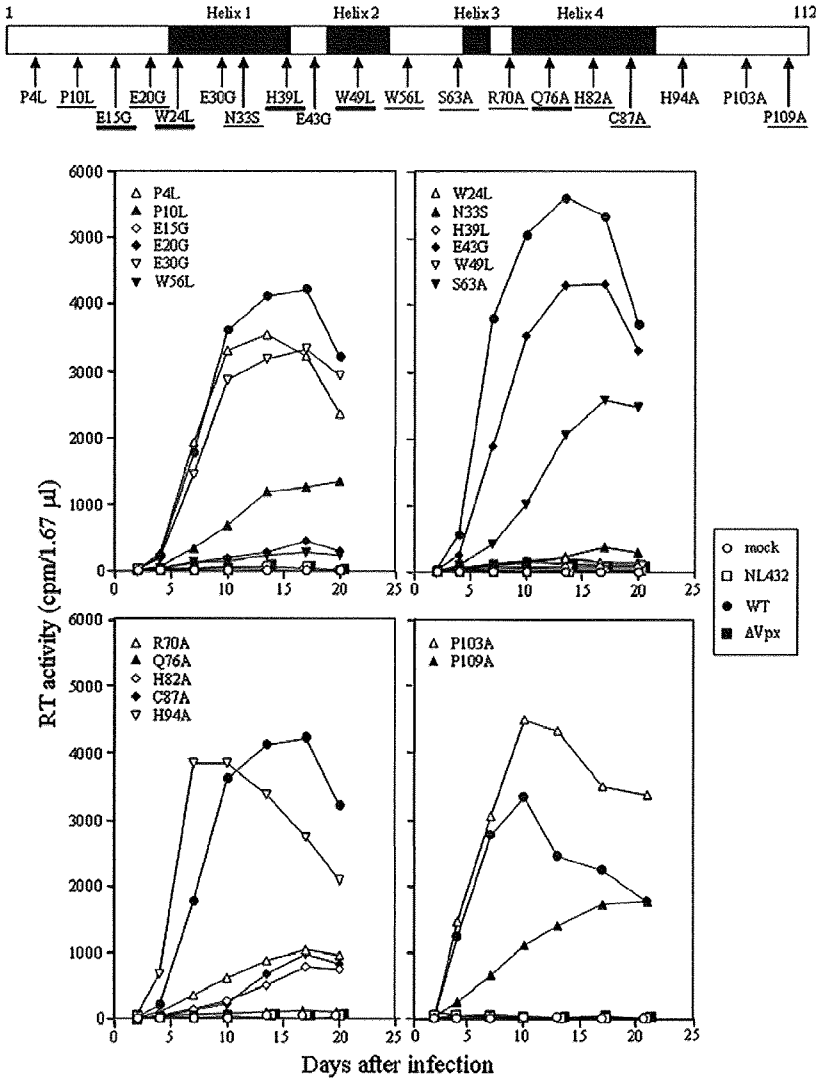

FIG. 2. Growth kinetics in human MDMs of various *vpx* point mutants. Confluent MDMs in each well of 24-well tissue culture plates prepared as described in the legend to Fig. 1 were infected with equivalent numbers of RT units of cell-free viruses (6×10^5) in the presence of DEAE-dextran (5 μg/ml), and viral replication was monitored at intervals by determining RT production in the culture supernatants (22). Input viruses were prepared from 293T cells transfected with 20 μg of pGL-AN, its *vpx* mutants, or the HIV-1 infectious clone pNL432 (2) as a negative control. At the top, the locations of the point mutations in pGL-AN Vpx, consisting of 112 amino acids (GenBank accession no. M30895) with four predicted helices (14), and the standard designations of the *vpx* mutants are indicated. The noninfectious and growth-defective mutants are indicated by bold and thin underlines, respectively. Mock, pUC19; WT, pGL-AN (13); ΔVpx, pGL-St (13).

tion than the other four mutants. The experiment for Fig. 4 was repeated extensively, with reproducible results.

Based on the results described above, we concluded that Vpx is crucially required for reverse transcription of the HIV-2 RNA genome in human MDMs. We also claim here, by our mutational analysis, that a specific region or domain(s) in Vpx may not be responsible for the Vpx activity shown in this report; rather, the entire structure of Vpx is important. Of the five mutations that completely abrogate viral infectivity in MDMs, H39L and W49L might affect the stability of mutant proteins and give the phenotype shown in Fig. 2 and 4. However, this was quite unlikely, because the mutant proteins were stably expressed in cells by a FLAG tag expression vector (Fig. 3). Furthermore, the N33S mutant, which contains a smaller amount of Vpx in virions than the H39L and W49L mutants, still retained viral infectivity. In any case, our main conclusion, that the overall structure of Vpx is crucial for reverse transcription of the HIV-2 genome in human MDMs, is unchanged. Whether inactive or defective mutants other than the five noninfectious mutants shown in Fig. 2 display the defect in nuclear import of viral DNA is another intriguing question to address, and this needs to be determined. Determination of the subcellular localizations of these mutant Vpx proteins could explain their biological differences, if there are any.

Our results described in this report are quite distinct from those previously published (6, 20) but not inconsistent. Clearly,

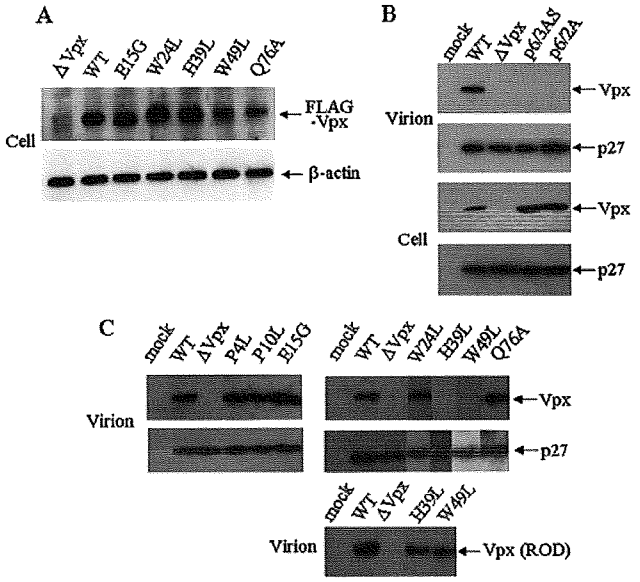

FIG. 3. Immunoblot analysis of mutant Vpx proteins in cells and virions. 293T cells were transfected with 10 μg of an expression vector of WT Vpx designated pME18Neo-Fvpx (14) and its mutants or 20 μg of WT proviral clone pGL-AN (13, 21) and its mutants, as previously described (2), and on day 2 or 3 posttransfection, cell or virion lysates were prepared as previously described (8, 22). The lysates normalized by β-actin or RT activity were then analyzed by Western immunoblotting (7, 8, 14) with the HIV-2 Vpx monoclonal antibody 6D2.6 (Vpx) (NIH AIDS Research and References Reagent Program, catalog no. 2710), an HIV-2 ROD Vpx polyclonal antibody (ROD) (catalog no. 2609), and an antiserum to SIV-p27 (p27) (NIBSC Centralised Facility for AIDS Reagent, repository reference no. ARP414). Commercially available monoclonal antibodies were used for detection of FLAG-Vpx (ANTI-FLAG M2; Sigma-Aldrich, St. Louis, MO) and β-actin (anti-β-actin clone AC-15; Sigma-Aldrich). The results obtained for the mutant Vpx proteins in cells, for the Vpx-defective-virion mutants as predicted by their Gag-p6 amino acid sequences (p6/3AS and p6/2A), and for the mutant Vpx proteins in virions are shown in panels A, B, and C, respectively. Gag-p6 mutants designated p6/3AS and p6/2A have A^{17}, S^{19}, A^{22}, and A^{23} (instead of D^{17}, A^{19}, L^{22}, and L^{23}, respectively) and A^{22} and A^{23} (instead of L^{22} and L^{23}, respectively) in the Gag-p6 amino acid sequence. Because the monoclonal antibody 6D2.6 did not react with the H39L and W49L mutant proteins, the polyclonal antibody was used to detect them as shown in panel C. Mock, pUC19.

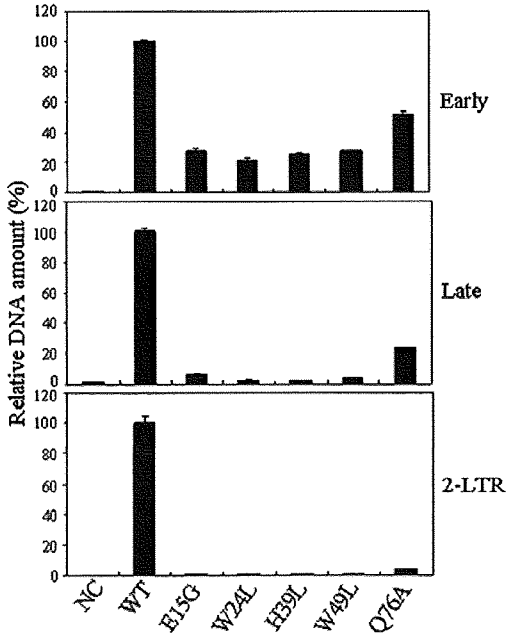

FIG. 4. Quantitative estimation of viral DNA synthesis in human MDMs infected with *vpx* point mutants. Pseudotype viruses were prepared by transfection and inoculated into human MDMs as described in the legend to Fig. 1. The procedures for real-time PCR analysis for Fig. 1 were also used, but the early reverse transcription product (R/U5 region) was additionally monitored here. The primers and probe for the early reverse transcription product (R/U5 region) were as follows: 5′-CAAGT TAAGTGTGTGTTCCCATCTCT-3′ (forward primer), 5′-CCAGGGT CTTGTTATTCAGATGAA-3′ (reverse primer), and 5′-FAM-CTAGT CGCCGCCTGGTCATTCGG-TAMRA-3′ (probe).

we and they have used different experimental systems, including different methods for infection, virus clones, cell types, and methods for analysis of viral DNAs. Therefore, the data obtained could be different. Interestingly, one report has described reproducible reductions in the abundances of reverse transcription products in MDMs infected with *vpx*-defective mutants (6). In agreement with this and our results here, it has recently been demonstrated that Vpx of SIV_{MAC} of the HIV-2 lineage plays an essential role for the reverse transcription process in human dendritic cells (9).

Determination of the molecular basis underlying the macrophage-specific requirement of Vpx for reverse transcription of the viral genome is virologically very important. In this regard, two recently published articles are quite provocative. Goujon et al. reported that Vpx may counteract a restriction factor present in human dendritic cells to escape the proteasome-mediated degradation pathway (9). Le Rouzic et al. showed that Vpx binds to DCAF1/VprBP, an adaptor molecule of the ubiquitin ligase complex (18). These findings have raised the possibility that there is a proteasome-dependent factor(s) in a certain cell type that suppresses reverse transcription. It is not unreasonable to assume that HIV-2 Vpx antagonizes such a factor, thus efficiently promoting viral replication. In addition, it has been well established that innate antiretroviral factors, such as TRIM5α and APOBEC3G/F, target the step of viral DNA synthesis. The association of HIV-2 Vpx with the reverse transcription process of the viral RNA genome needs to be biochemically proved to clarify the early events of HIV-2 replication precisely.

We thank Kazuko Yoshida for editorial assistance. We are indebted to the NIH AIDS Research and Reference Reagent Program (catalog no. 2609 and 2710) and the NIBSC Centralised Facility for AIDS Reagents (repository reference ARP414) for antibodies.

This work was supported in part by a Grant-in-Aid for Scientific Research (B) (18390140) from the Japan Society for the Promotion of Science (to A.A.) and by a Grant-in-Aid for Scientific Research on Priority Areas (19041051) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to A.A.).

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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

Received for publication April 30, 2008; accepted June 10, 2008.

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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

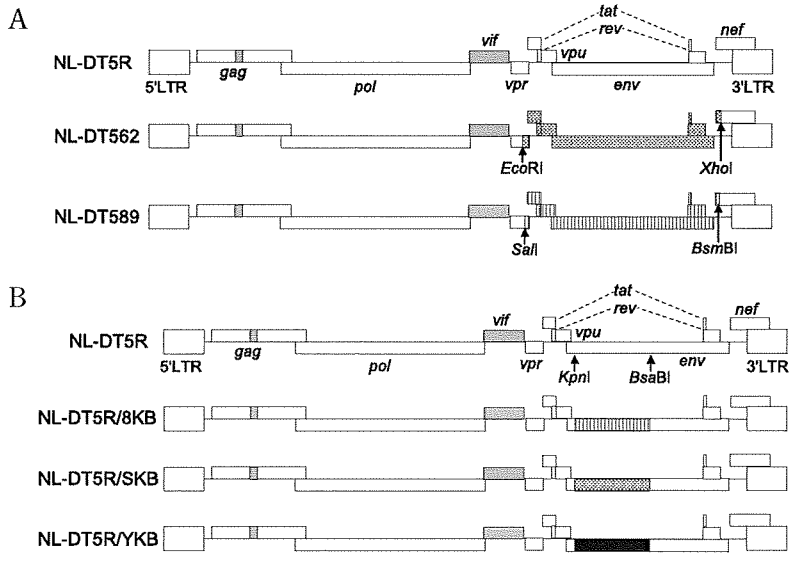

Fig. 1 Genome structure of various proviral clones used in this study. (A) Genomes of R5- and dual-tropic clones derived from NL-DT5R. The 3' half of the NL-DT5R genome was replaced with those of R5-tropic NF462 (4) and dual-tropic 89.6 (6) genomes at the sites indicated. White, grey, dotted and striped 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.

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

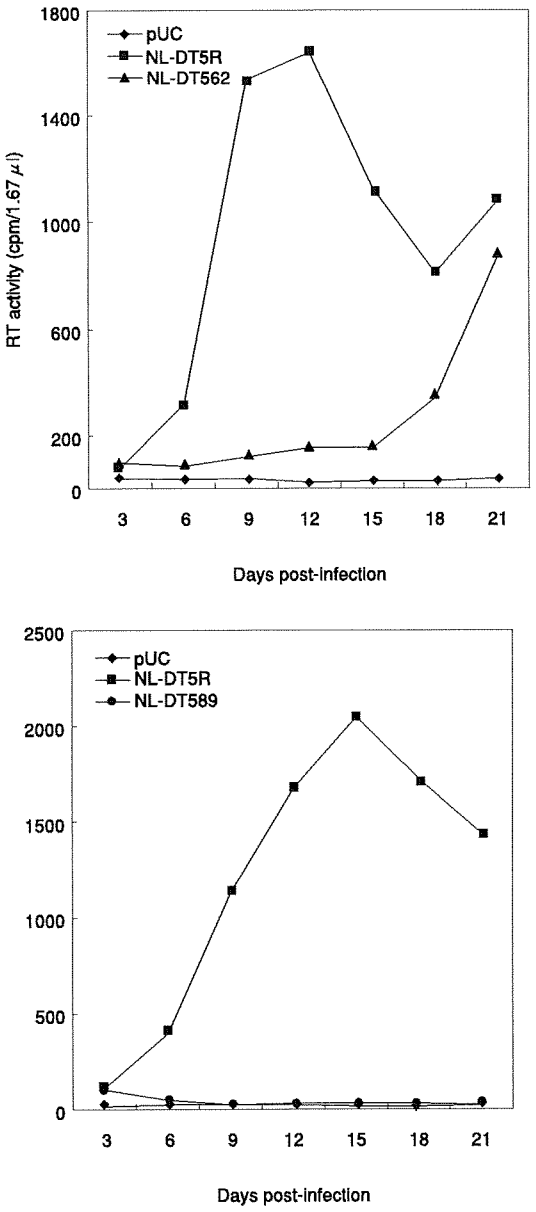

Fig. 2 Growth kinetics of various clones in simian HSC-F cells. Input cell-free viral samples were prepared from 293T cells transfected with the clones indicated, and an equivalent RT units were inoculated into HSC-F cells. Viral growth was monitored at intervals by RT activity in the culture supernatants.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

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-

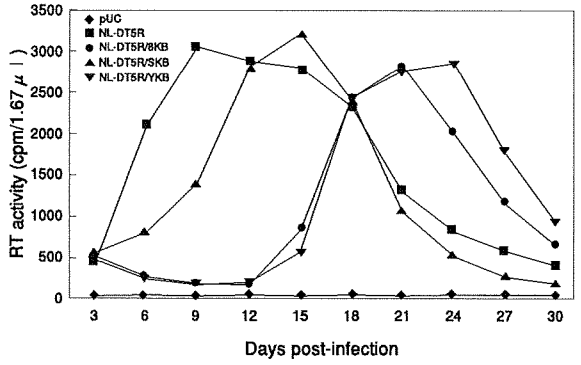

Fig. 3 Growth kinetics of various *env*-variants in simian HSC-F cells. Input cell-free viral samples were prepared from 293T cells transfected with the clones indicated, and an equivalent RT units were inoculated into HSC-F cells. Viral growth was monitored at intervals by RT activity in the culture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

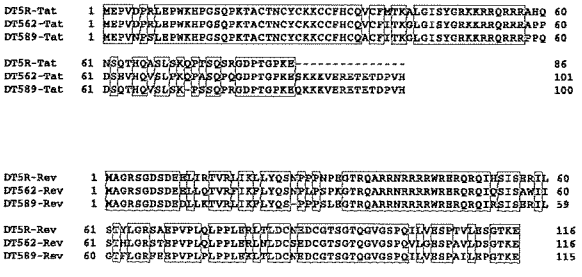

Fig. 4 Amino acid alignments of regulatory proteins Tat and Rev from NL-DT5R, -DT562 and -DT589. Identical amino acid residues are boxed.

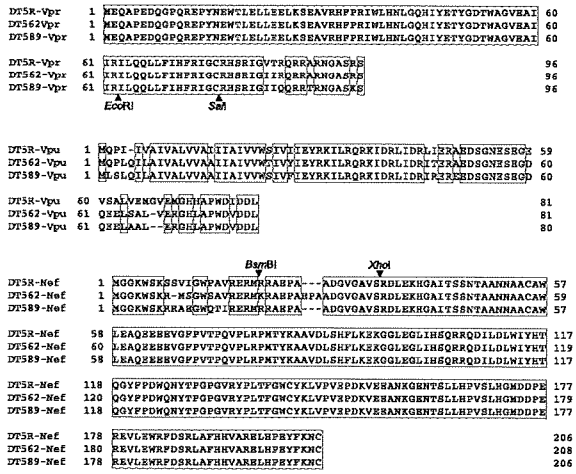

Fig. 5 Amino acid alignments of accessory proteins Vpr, Vpu and Nef from NL-DT5R, -DT562 and -DT589. Identical amino acid residues are boxed. The sites used to construct NL-DT562 and and NL-DT589 are indicated (see Fig.1).

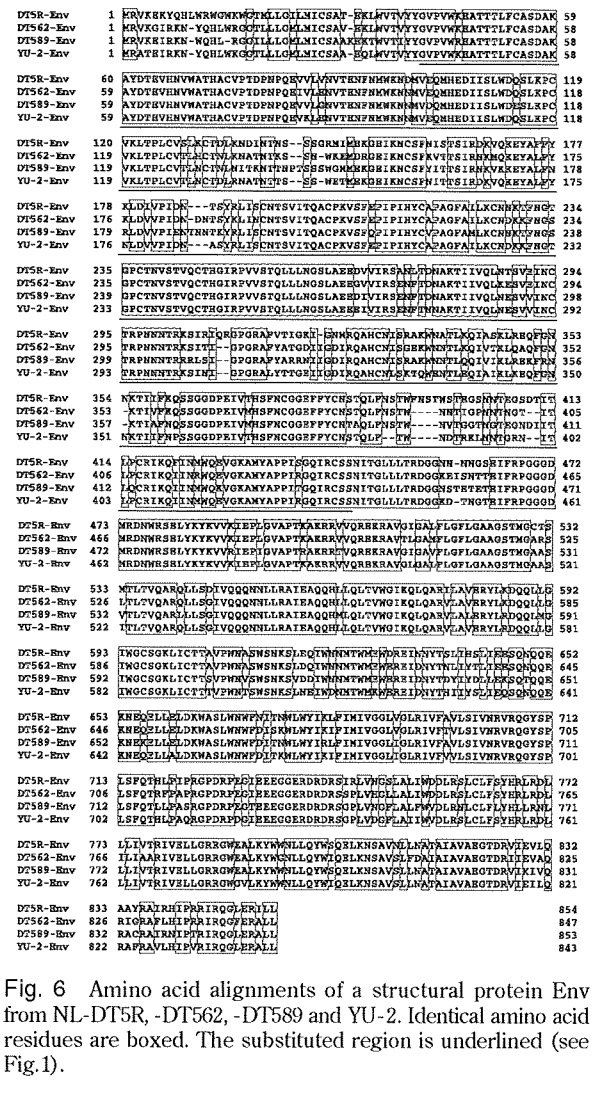

Fig. 6 Amino acid alignments of a structural protein Env from NL-DT5R, -DT562, -DT589 and YU-2. Identical amino acid residues are boxed. The substituted region is underlined (see Fig.1).

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.

ACKNOWLEDGMENTS

We thank Ms. Kazuko Yoshida for her excellent editorial assistance. We also thank Ms Yuri Takiguchi for technical help. We are indebted to Dr. Boonruang Khamsri for construction of some of proviral clones used in this study. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (19041051) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to A. A.), and by a Health Sciences Research Grant (Research on HIV/AIDS (2007-2009)) from the Ministry of Health, Labour and Welfare of Japan (to A. A.).

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

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Available online 10 July 2008

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.

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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doi:10.1016/j.micinf.2008.07.006

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-xl 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].

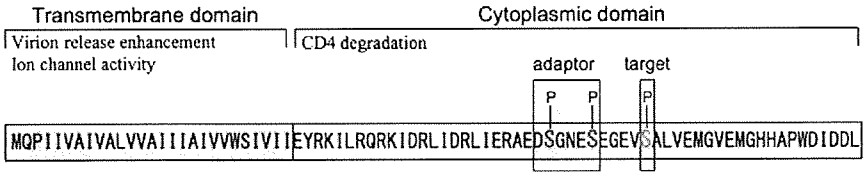

Fig. 1. Domain structure of HIV-1 (NL4-3) Vpu protein. Gray and white boxes represent transmembrane and cytoplasmic domains, respectively. Vpu functions critical for each domain are indicated. Serine residues at positions 52 (red), 56 (red), and 61 (blue) that are phosphorylated (P) are indicated. Two 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).

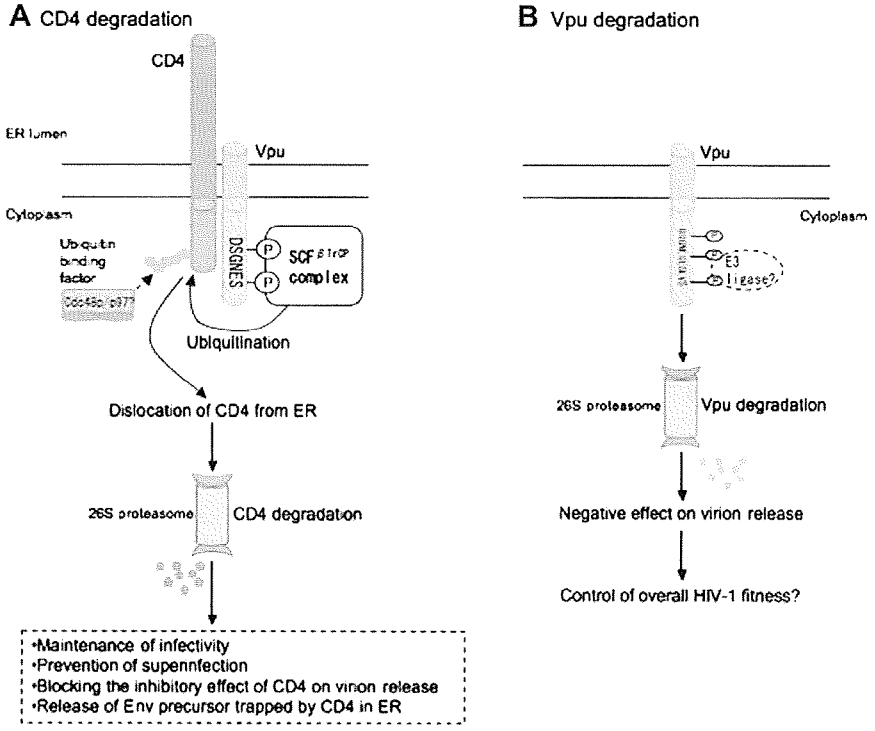

Fig. 2. Schematic representation of Vpu-mediated degradation. Degradation of CD4 in ER and of Vpu itself is illustrated in (A) and (B) as shown. Vpu acts as an adaptor or as a substrate for E3 ligase. (A) Vpu-mediated CD4 degradation is initiated by binding of Vpu to the CD4 cytoplasmic domain in the 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 ($SCF^{\beta TrCP}$)). $SCF^{\beta TrCP}$ interacts with Vpu through the consensus motif $DS^{P}G\Phi XS^{P}$ (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

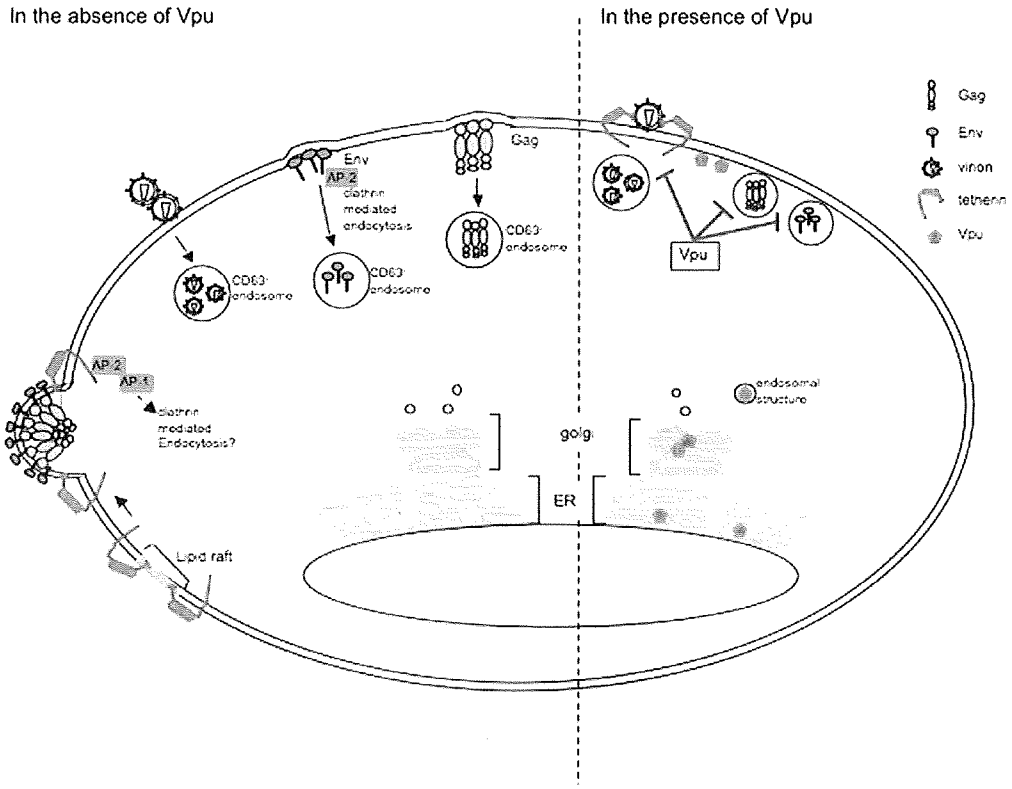

Fig. 3. Effects of HIV-1 Vpu on virion release and on localization of Gag and Env in Vpu-dependent cells. Gag is initially targeted to the plasma membrane. In the absence of Vpu, Gag is internalized and accumulated into $CD63^+$ endosomes. While Env has been known to be internalized rapidly from the 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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Microbes and Infection 10 (2008) 1142–1149

www.elsevier.com/locate/micinf

Original article

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

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Received 20 May 2008; accepted 9 June 2008
Available online 18 June 2008

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.

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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doi:10.1016/j.micinf.2008.06.003