

Effects of lysine to arginine mutations in HIV-1 Vif on its expression and viral infectivity

BOONRUANG KHAMRSRI^{1,2}, MIKAKO FUJITA¹, KAZUYA KAMADA¹, AHMAD PIROOZMAND¹, TOMOKI YAMASHITA¹, TSUNEO UCHIYAMA¹ and AKIO ADACHI¹

¹Department of Virology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770-8503, Japan; ²Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok 65000, Thailand

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Abstract. We previously demonstrated that the expression in cells of human immunodeficiency virus type 1 (HIV-1) Vif is maintained at low level by proteasome-degradation. We examined the contribution of 16 lysines present in Vif (NL432 clone), which is composed of 192 amino acids (aa), to its expression within cells and to viral infectivity for non-permissive cells. To this end, various lysine-arginine mutations were introduced into wild-type (wt) Vif, and the mutational effects were monitored by transfection experiments. When all the lysines were changed to arginines, the mutant Vif was expressed in cells at much higher level than wt and was much more stable. Both N-terminal (aa nos. 34 and 36) and C-terminal (aa nos. 179 and 181) lysines were found to be almost sufficient for wt property. Different from this observation, one of the lysines at aa nos. 22 and 26 was demonstrated to be essential for the virus to grow in non-permissive cells. Our results showed that there is no clear correlation between the expression level of HIV-1 Vif and viral infectivity.

Introduction

Vif is one of the human immunodeficiency virus type 1 (HIV-1) accessory proteins, and is conserved in all known primate immunodeficiency viruses (1). It is dispensable for the replication of HIV-1 in permissive cells like MT-4 (2) and M8166 (3), but is critical for the viral growth in non-permissive cells such as H9 (4) and peripheral blood mononuclear cells (5-9). Recent studies have shown that the non-permissive cells have a cytidine deaminase APOBEC3G

carrying anti-viral activity, and that the Vif counteracts the virion incorporation of APOBEC3G (10-19). The precise molecular mechanism for this activity of Vif, however, remains to be elucidated.

We have recently demonstrated that the expression of HIV-1 Vif is controlled uniquely to be at low level among accessory proteins by proteasome degradation (20,21). Virological significance of this degradation can be explained by the fact that a high expression level of Vif inhibits viral infectivity through modulating proteolytic processing of the Gag precursor at the p2/nucleocapsid processing site (22). However, the experiments were done in a quite artificial system; expression of a large amount of Vif by pNL-A1 (23) and examination of infectivity by a single-round replication assay.

In this study, we investigated the relationship between the Vif expression level within cells and viral multi-cycle infectivity for the non-permissive cells. For this purpose, we introduced a wide variety of lysine to arginine mutations into wild-type (wt) Vif, because it is well known that proteins are poly-ubiquitinated at their lysine residues or N-terminus to become a marker recognized by the proteasome, and that the polyubiquitinated proteins are then degraded (24); the lysine and arginine have similar physicochemical characteristics. We identified lysines in Vif which are important for the wt expression level within cells and for the viral multi-cycle infectivity in non-permissive cells.

Materials and methods

Cells. A lymphocytic cell line H9 (4) was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). A monolayer cell line 293T (25) was cultured in Eagle's minimal essential medium supplemented with 10% heat-inactivated FBS.

Transfection. For transfection of plasmid DNAs into adherent 293T cells, the calcium-phosphate coprecipitation technique (26) or the Lipofectamine Plus™ system (Invitrogen, Carlsbad, CA, USA) was used. For transfection of lymphocytic H9 cells, the electroporation method was used as previously described (26).

Correspondence to: Dr Akio Adachi, Department of Virology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima-shi, Tokushima 770-8503, Japan
E-mail: adachi@basic.med.tokushima-u.ac.jp

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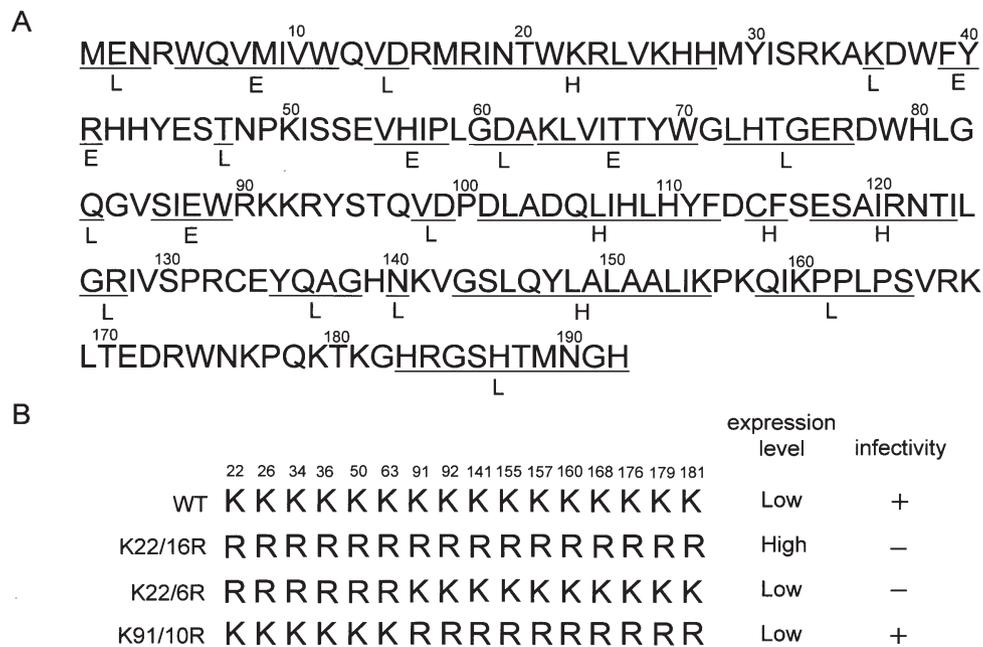


Figure 1. The lysine-arginine alterations in the first group of Vif mutants. Structure of the Vif of HIV-1 NL432 clone by the PredictProtein (<http://www.predictprotein.org/>) (A) and the alterations in this Vif (the first group mutants) (B) are indicated. L, E and H in (A) represent the loop, β -strand and α -helix structures, respectively. Data on the expression level in cells of the mutated Vif proteins and the infectivity for H9 cells of the mutants are also shown in (B) (see Figure 2).

Reverse transcription (RT) assay. Virus production in the culture supernatants of transfected H9 cells was monitored by RT assay as previously described (27).

Western immunoblot analysis. Transfected 293T cells were collected and solubilized by dissolving in PBS-Laemmli's sample buffer (1:1) for SDS-PAGE as previously described (20). Samples resolved by the SDS-PAGE were then electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). The membranes were treated with anti-FLAG antibody (Ab) (ANTI-FLAG M2 Monoclonal Ab, Sigma-Aldrich, St. Louis, MO, USA) and visualized using the ECL plus Western blot detection system (Amersham Biosciences, Buckinghamshire, UK).

Pulse/chase analysis. Transfected 293T cells were pulse-labelled with ^{35}S , and chased as previously described (20). Cells harvested were lysed with the CHAPS/DOC buffer as described previously (20), and the cell lysates were precipitated with a Vif-specific polyclonal antibody Vif93 (28). Wt and mutant Vif proteins were identified by SDS-PAGE followed by fluorography as previously described (20).

DNA constructs for infection experiments. An infectious proviral clone of HIV-1 designated pNL432 (26) was used as wt clone for infection experiments. Appropriate fragments of pNL432 were subcloned into pBluescript SK(+) (Stratagene, La Jolla, CA, USA) and mutations were introduced. The alterations of lysines into arginines were performed by the QuikChange site-directed mutagenesis kit (Stratagene). The mutated fragments were cloned back to wt to construct pNL-K22/6R, pNL-K91/10R, pNL-K22/2R, pNL-K34/2R, pNL-K50R, pNL-K63R, pNL-K22R and pNL-K26R. To make

pNL-K22/16R, appropriate fragments of pNL-K22/6R and pNL-K91/10R were used. Clone pNL-K34/14R was constructed from the pNL-K22/16R similarly as above. As a negative control, pNL-Nd (29) carrying a frame-shift mutation in *vif* was used.

DNA constructs for Western immunoblot and pulse/chase analyses. The pNL-A1S (21) was used to construct expression vectors for Western blot and pulse/chase analyses. To generate pNL-ASCF, a *Cla*I site and the FLAG sequence (in this order) were introduced just upstream of the stop codon of *vif* in pNL-A1S (21) by the QuikChange site-directed mutagenesis kit. The *vif* sequences of pNL432 and its mutants, pNL-K22/16R, pNL-K22/6R, pNL-K91/10R and pNL-K34/14R, were amplified by polymerase chain reaction (PCR) with *Sma*I at 5' and *Cla*I at 3' ends, respectively. The *Sma*I-*Cla*I fragment from the pNL-ASCF was replaced with these PCR-amplified sequences to construct pNL-ASCF-fWT, pNL-ASCF-fK22/16R, pNL-ASCF-fK22/6R, pNL-ASCF-fK91/10R and pNL-ASCF-fK34/14R. An appropriate fragment of pNL-ASCF-fK22/16R was subcloned into pBluescript SK(+), and mutations were introduced by the QuikChange site-directed mutagenesis kit. The mutated fragments were cloned back to pNL-ASCF-fK22/16R to construct pNL-ASCF-K22/2R/50/12R, pNL-ASCF-K22/6R/141/8R, pNL-ASCF-K22/9R/160/5R and pNL-ASCF-K22/14R. As an expression vector for luciferase, pGL3-Control Vector (Promega, Madison, WI, USA) was used.

Results

Expression and infectivity of various HIV-1 Vif mutants. We examined the importance of lysines present in Vif for its

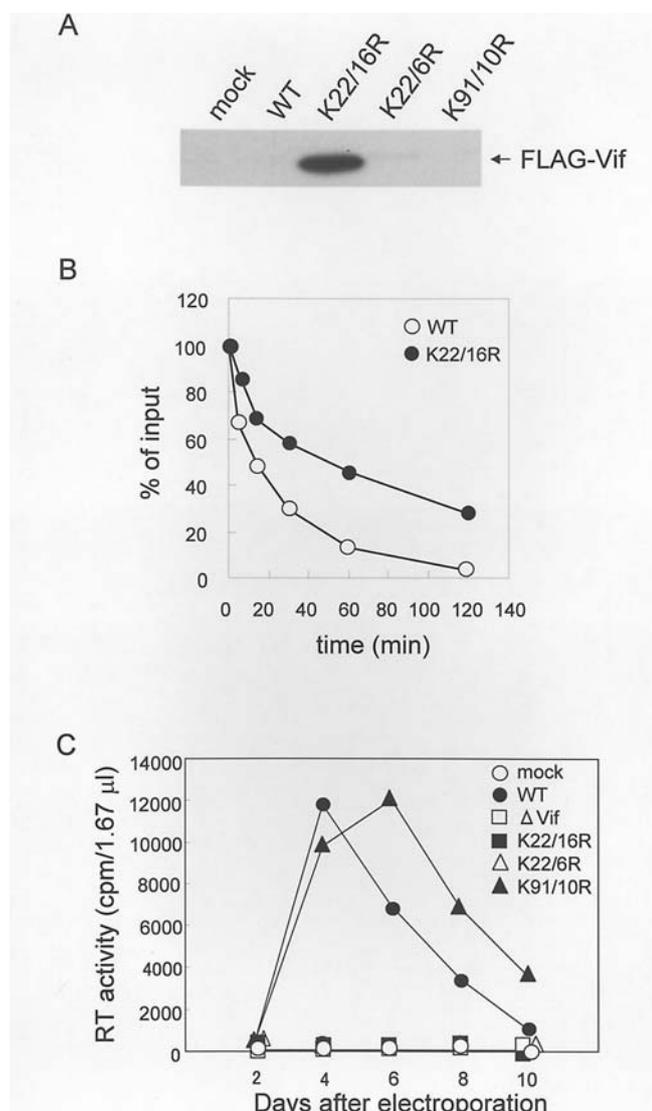


Figure 2. Characteristics of the first group of Vif mutants. (A) Expression level in cells of wt and mutant clones. Co-transfected 293T cells with 7.5 μ g of the subgenomic viral clones derived from pNL-ASCF-fWT and 2.5 μ g of an expression vector for luciferase, at 48-h post-transfection, were harvested for Western blot analysis and luciferase assay. The loading amount in each lane was normalized by the luciferase activity. mock, pUC19; WT, pNL-ASCF-fWT. (B) Degradation kinetics in 293T cells of the K22/16R mutant. Transfected 293T cells with 5 μ g of the subgenomic clones, and at 24-h post-transfection, were collected, 35 S-labeled, and chased for ≤ 120 min for immunoprecipitation analysis. WT, pNL-ASCF-fWT. (C) Growth kinetics in non-permissive cells of wt and mutant clones. H9 cells were transfected with 10 μ g of the full-length viral clones, and virus production in the culture supernatants was monitored by RT assay. mock, pUC19; WT, pNL432; Δ Vif, a frame-shift mutant pNL-Nd.

expression and viral infectivity. As the first group mutants for this study, clones K22/16, K22/6R and K91/10R, which carry all 16, N-terminal 6 and C-terminal 10 lysine-arginine exchanges, respectively, were constructed and characterized (Fig. 1). For a quantitative comparison, their expression to a high level was achieved by the subgenomic-type clone (21) and analyzed by Western immunoblotting using anti-FLAG antibody. As shown in Fig. 2A, the mutant K22/16R produced its Vif at an extremely high level relative to those of wt, K22/6R and K91/10R. We determined the stability of the K22/16R

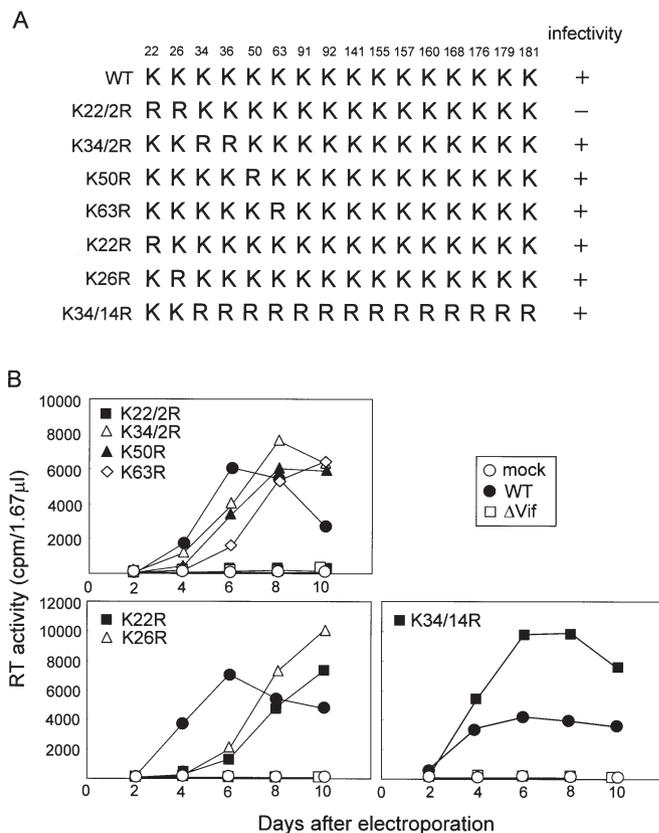


Figure 3. Identification of the lysine residue in Vif critical for viral infectivity. Location of the lysine to arginine alterations in the Vif of the mutants (A) and their growth kinetics in H9 cells (B) are shown. For determination of viral infectivity in H9 cells, cells were transfected with various full-length clones and monitored for virus production as above. Data in (B) are summarized in (A) as indicated. mock, pUC19; WT, pNL432; Δ Vif, a frame-shift mutant pNL-Nd.

Vif by the pulse/chase experiment as previously described (20). As shown in Fig. 2B, in a good agreement with the steady-state expression level, K22/16R was much more stable than wt. We next examined the infectivity of these mutants for non-permissive cells. H9 cells were electroporated with the full-length version of the mutant clones, and virus growth was monitored by RT assay (30). As shown in Fig. 2C, only the mutant K91/10R among the three mutants, which express a high or negligible level of Vif in cells (Fig. 2A), grew fairly well.

In total, our results described herein indicated that the lysines present in Vif were important for the stable expression of Vif, and that there is no clear negative or positive co-relationship between the expression level of Vif and infectivity of lysine-arginine mutants.

Lysine residues in Vif important for viral infectivity. To determine the lysine residue in Vif crucial for viral infectivity, we constructed another set of proviral mutant clones. One or two lysines located at the N-terminal region of Vif were changed to the arginine residue (K22/2R, K34/2R, K50R and K63R in Fig. 3A), and the resultant clones were examined for their growth kinetics in H9 cells as above. As shown in Fig. 3B, only the K22/2R were not infectious. Therefore, we

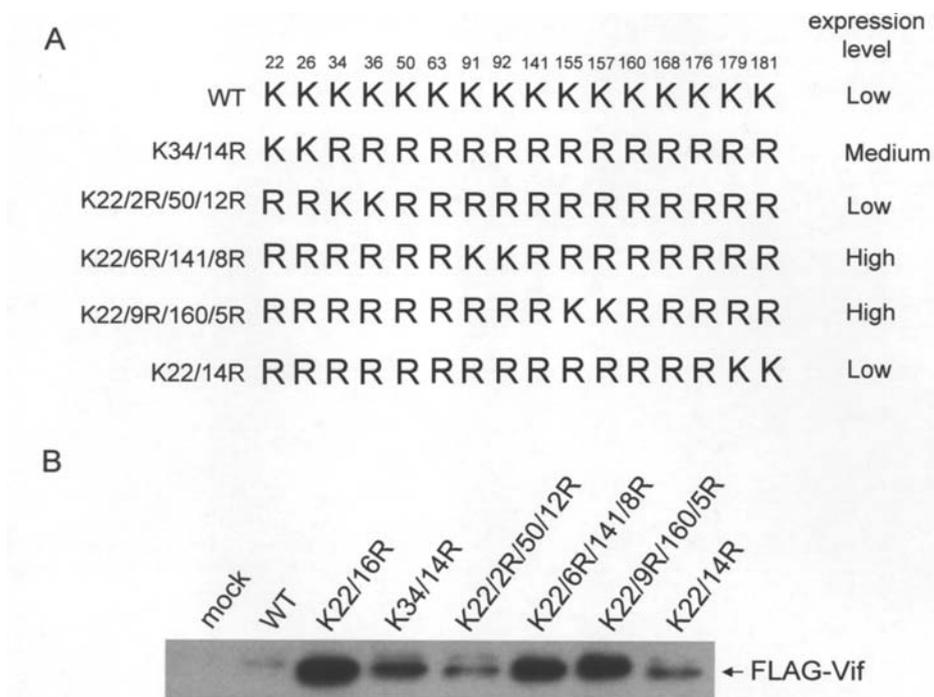


Figure 4. Identification of the lysine residue in Vif critical for the wt expression level within cells. Location of the lysine to arginine alterations in the Vif of the mutants (A) and their expression in the transfected 293T cells (B) are shown. For monitoring the expression level in 293T cells of the Vif, cells were transfected with various subgenomic clones and analyzed for their expression as above. Data in (B) are summarized in (A) as indicated. mock, pUC19; WT, pNL-ASCF-fWT.

constructed K22R, K26R and K34/14R (Fig. 3A) to determine whether one of the K²² and K²⁶ or both are essential for viral infectivity, and whether the lysines other than K²² and K²⁶ are critical for viral infectivity. The three additional mutants thus constructed were transfected into H9 cells, and their growth was examined. As shown in Fig. 3B, the three mutants grew to a comparable extent. All the mutants described above were confirmed to propagate in permissive M8166 cells (data not shown). Collectively, we concluded that either K²² or K²⁶ of Vif is critical for the productive infection of HIV-1 NL432 in non-permissive cells.

Lysine residues in Vif important for the expression level of Vif. To determine the lysine residue in Vif crucial for the Vif expression, we constructed a series of double lysine-arginine mutants other than the K34/14R, based on the subgenomic clone (21) (Fig. 4A). The mutants were then analyzed for their Vif expression by Western immunoblotting as described above. When the mutant K34/14R, which is infectious for H9 cells (Fig. 3B), was monitored for its Vif expression, it displayed a medium expression level between wt and the stable and non-infectious mutant K22/16R (Fig. 4B). This observation again indicated the absence of a detectable close relationship between viral infectivity and the Vif expression level. The data on the other mutants in Fig. 4B clearly showed that the K³⁴ and K³⁶ or the K¹⁷⁹ and K¹⁸¹ are responsible for the low expression level of Vif. While the mutants K22/6R/141/8R and K22/9R/160/5R produced a similarly high expression level to that of the K22/16R, the mutants K22/2R/50/12R and K22/14R expressed a low level of Vif quite similar to the wt clone. We also constructed a complete set of single lysine-arginine mutants through one

by one alterations to evaluate the contribution of each lysine to the Vif expression. All the single mutants constructed were found to produce Vif, upon transfection, at a level higher than that of the mutants K22/2R/50/12R and K22/14R (data not shown). These results strongly suggested that each lysine residue in Vif is less important than the combination of K³⁴ and K³⁶ or of K¹⁷⁹ and K¹⁸¹ for the wt expression level of Vif.

Discussion

In this study, we showed by a mutational analysis that the lysines in HIV-1 Vif are important for its steady-state expression in transfected 293T cells, and that the two lysines in Vif (K³⁴ and K³⁶ or K¹⁷⁹ and K¹⁸¹) are nearly sufficient for the expression property of the Vif (Figs. 2 and 4). We also demonstrated that either K²² or K²⁶ in Vif is critical for the replication of HIV-1 in H9 cells (Figs. 2 and 3). Thus, we did not find any clear co-relationship between the expression level in cells of Vif and viral infectivity for the non-permissive cells.

Together with the results previously published (20,22), it was reasonable to assume that the K³⁴ and K³⁶ or K¹⁷⁹ and K¹⁸¹ are important to maintain the low appropriate expression level of Vif by the proteasome-degradation, and thereby enable the virus to grow in non-permissive cells. However, our data herein on the mutants demonstrating that either K²² or K²⁶ is critical for viral infectivity do not support this prediction, and instead, the mechanism(s) and molecule(s) other than the proteasome-degradation and its associated factors which confer the infectivity on the virus should be considered. They would include 1) the structure of the lysine itself, 2) covalent modification(s) of the lysine and 3) interaction of the lysine

with some unknown factor(s). Further study is required to clarify the molecular basis for our observations on the Vif mutants.

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