

Defining HIV-1 Vif residues that interact with CBF β by site-directed mutagenesis

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ABSTRACT

Vif is essential for HIV-1 replication in T cells and macrophages. Vif recruits a host ubiquitin ligase complex to promote proteasomal degradation of the APOBEC3 restriction factors by poly-ubiquitination. The cellular transcription cofactor CBF β is required for Vif function by stabilizing the Vif protein and promoting recruitment of a cellular Cullin5-RING ubiquitin ligase complex. Interaction between Vif and CBF β is a promising therapeutic target, but little is known about the interfacial residues. We now demonstrate that Vif conserved residues E88/W89 are crucial for CBF β binding. Substitution of E88/W89 to alanines impaired binding to CBF β , degradation of APOBEC3, and virus infectivity in the presence of APOBEC3 in single-cycle infection. In spreading infection, NL4-3 with Vif E88A/W89A mutation replicated comparably to wild-type virus in permissive CEM-SS cells, but not in multiple APOBEC3 expressing non-permissive CEM cells. These results support a model in which HIV-1 Vif residues E88/W89 may participate in binding CBF β .

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Introduction

HIV-1 Vif is one of six viral accessory proteins and it is essential for the viral replication in T cells and macrophages (Gabuzda et al., 1992, 1994). Vif recruits host proteins, cullin 5 (CUL5), RING-box protein 2 (RBX2), elongin C (ELOC) and elongin B (ELOB), and forms a ubiquitin ligase complex that promotes poly-ubiquitination and proteasomal degradation of the APOBEC3 (A3) retrovirus restriction factors (Jäger et al., 2011; Marin et al., 2003; Sheehy et al., 2003; Shirakawa et al., 2006; Stopak et al., 2003; Yu et al., 2003). A3s are DNA cytosine deaminases that convert cytosines to uracils in single-stranded DNA (Chelico et al., 2006; Harris et al., 2003). In the absence of the Vif protein, at least two A3 family members, A3F and A3G, are efficiently incorporated into budding virions, where they deaminate cytosines in newly reverse-transcribed minus-strand virus DNA in target cells, leading to guanine to adenine hypermutation of the virus genome (Harris et al., 2003; Hultquist et al., 2011b; Sheehy et al., 2002; Zhang et al., 2003).

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Amino acid sequences of HIV-1 Vif vary among viral strains, but more than ten regions of residues are conserved (Dang et al., 2010), and several conserved motifs have been shown to interact with host proteins. The BC-box motif ¹⁴⁴SLQYLA¹⁴⁹ binds to ELOC (Mehle et al., 2004; Yu et al., 2004), and the HCCH motif ¹⁰⁸Hx₅Cx₁₇₋₁₈Cx₃₋₅H¹³⁹ binds to CUL5 (Luo et al., 2005; Mehle et al., 2006). Furthermore, the N-terminal half of Vif contains distinct regions involved in the Vif-A3 protein-protein interactions; ¹¹WQxDRMR¹⁷ and ⁷⁶ExxW⁷⁹ motifs are involved in neutralization of A3F (He et al., 2008; Russell and Pathak, 2007), whereas ⁴⁰YRHHY⁴⁴ motif is involved in neutralization of A3G (Russell and Pathak, 2007); ²²KSLVK²⁶ and ⁵⁵VxIPLx4-5Lxqpx2YWxL⁷² motifs were reported to be involved in neutralization of both A3F and A3G (Chen et al., 2009; Dang et al., 2009; He et al., 2008; Pery et al., 2009), although there exists a report that K26 is required for neutralization of A3G, but not of A3F (Albin et al., 2010).

Recently, the transcription factor core binding factor- β (CBF β) has been shown to be involved in the Vif ubiquitin ligase complex (Jäger et al., 2011; Zhang et al., 2011), and critical for its function by stabilizing the Vif protein in cells (Jäger et al., 2011), and enabling the recruitment of CUL5 (Zhang et al., 2011). There are two isoforms of CBF β , and both isoforms stabilize Vif protein, enhance A3 degradation, and increase virion infectivity (Hultquist et al., 2011a). Thus, the interaction between Vif and CBF β is a promising

therapeutic target, but little is known about the interfacial amino acids. Hultquist et al. reported that surface F68 residue of CBF β is involved in binding and stabilizing Vif (Hultquist et al., 2012). Zhang et al. reported that W21 and W38 residues of Vif are required for binding to CBF β by co-immunoprecipitation experiments (Zhang et al., 2011; Kim et al., 2013). Furthermore, Kim et al. recently suggested that L64 and I66 residues are involved in binding to CBF β by co-purification in *Escherichia coli* (Kim et al., 2013).

Because a previous study indicated that a hydrophilic region ⁸⁸EWRRKR⁹³ is essential for Vif expression and HIV-1 replication (Fujita et al., 2003), we hypothesized that conserved residues E88 and W89 in this region may be required for CBF β binding. In this study, we generated amino acid substitution mutants of these residues as well as W21 and W38, and simultaneously analyzed both binding to CBF β and Vif-mediated degradation of A3F and A3G. We show that the conserved residues E88 and W89 of HIV-1 Vif are directly involved in CBF β binding and Vif-mediated degradation of A3F and A3G.

Results

The conserved residue W89 of HIV-1 Vif is required for the interaction with CBF β

To test our hypothesis that conserved residues E88 and W89 may be required for CBF β binding, we generated nine Vif amino acid substitution mutants, D14A/R15A, W21A, W38A, Y40A, Y69A, G84D, E88A, W89A, and E88A/W89A (Fig. 1A). W21 and W38 were reported to be involved in CBF β binding (Zhang et al., 2011), D14 and R15 for APOBEC3F binding (Russell and Pathak, 2007), Y40 for A3G binding (Russell and Pathak, 2007), Y69 and G84 for both A3F and A3G binding (Dang et al., 2010; Pery et al., 2009). All of these residues are highly conserved, suggesting that they could be involved in CBF β binding region of Vif. We first performed co-immunoprecipitation

experiments in 293T cells by over-expression of Vif with C-terminal myc tag. Vif is a relatively unstable protein with a short half-life and it is degraded by the cellular proteasome (Dussart et al., 2004; Fujita et al., 2004; Mehle et al., 2004). Mouse double minute 2 homolog (MDM2) is the E3 ubiquitin ligase which targets Vif for degradation (Izumi et al., 2009). Because it has been reported that Vif degradation is accelerated in the absence of CBF β , and that treatment with the proteasome inhibitor MG132 reverses this effect (Jäger et al., 2011), we used MG132 to minimize proteasomal proteolysis of Vif in cell culture and immunoprecipitation experiments. Although we transfected with the same amount of plasmid DNA, expression levels of E88A, W38A, D14/R15A, Y40A, Y69A and G84D were comparable to wild-type Vif, but those of W89A, E88/W89A and W21A mutants were obviously impaired (Fig. S1, 2nd top panel). These modest expression levels of these mutants can be simply explained by misfolding, but an alternative explanation is due to loss of CBF β binding. The amounts of immunoprecipitated Vif protein showed a smaller variation, compared to expression levels (Fig. S1, bottom panel). More importantly, endogenous CBF β co-precipitated with wild-type Vif, D14A/R15A, Y40A, G84D, and E88A, but not with W21A, W89A, or E88A/W89A (Fig. S1, 3rd top panel). W38A and Y69A showed intermediate results (Fig. S1, lanes 7 and 10, 3rd top panel). To exclude the possibility that low expression of W21A, W89A and E88A/W89A mutants caused the low amount of co-immunoprecipitated CBF β , we compensated by increasing the amount of transfected plasmid DNA, and performed an additional round of co-immunoprecipitation experiments. Although expression levels of W21A, W89A and E88A/W89A mutants were now higher than wild-type Vif, endogenous CBF β did not co-precipitate with these mutants (Fig. 1B). We next examined whether CUL5 co-precipitated with Vif mutants by immunoblotting with some of the samples of Fig. 1B, because CBF β binding has been reported to be required for Vif to interact with CUL5 (Zhang et al., 2011). Endogenous CUL5 appeared to co-precipitate with wild-type Vif, but not with W21A, W38A, W89A, or E88A/W89A (Fig. 1C, 2nd bottom panel). All

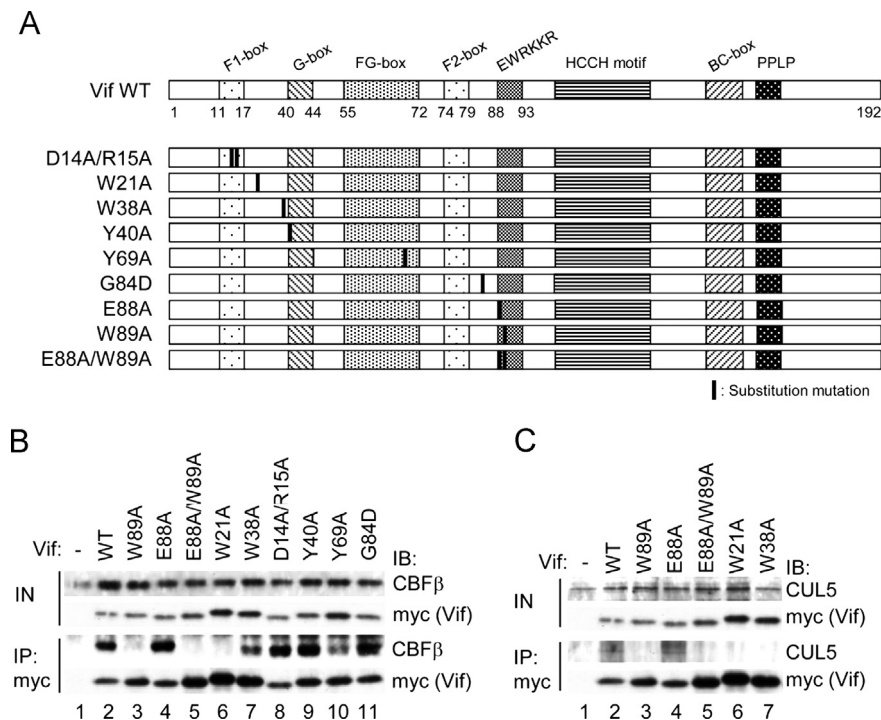


Fig. 1. Vif substitution mutants and binding capacity to CBF β . (A) Schematic of Vif conserved motifs and nine substitution mutants generated. The residues substituted are indicated by bold lines. (B) Co-immunoprecipitation of endogenous CBF β with Vif. Lysates of 293T cells transiently expressing myc-tagged Vif wild-type or mutant were immunoprecipitated by anti-myc serum. Samples were analyzed by immunoblotting with anti-CBF β and anti-myc sera. (C) Co-immunoprecipitation of endogenous CUL5 with Vif. Samples from (B) were also analyzed with anti-CUL5 serum. Panels for Vif were re-produced from (B).

of these mutants of Vif bound to ELOB (Fig. S2), suggesting that they are not entirely misfolded proteins, although previous reports indicated that fragments of the Vif BC box is sufficient for binding to ELOB and ELOC (Bergeron et al., 2010; Wolfe et al., 2010). Altogether, these results suggest that W89 is involved in CBF β binding, as well as W21 and W38.

The substitution of E88/W89 to alanines impairs Vif-mediated degradation of both A3F and A3G proteins

To examine whether the loss of binding to CBF β causes impaired Vif-mediated degradation of A3 proteins, we next performed a series of co-transfection and immunoblot experiments. 293T cells were co-transfected with expression vectors for A3G with myc tag and Vif wild-type or mutant, and protein levels of A3G were analyzed by immunoblotting. To obtain comparable expression levels of each of the Vif mutants, we again adjusted the

amount of plasmid DNA transfected. Co-transfection of wild-type Vif reduced APOBEC3G levels, but E88A/W89A, W21A or W38A did not (Fig. 2A, lanes 1, 2 and 7–12). E88A or W89A alone reduced A3G levels close to wild-type Vif (Fig. 2A, lanes 3–6). Because W21 and W38 are close to A3G binding residues of Vif, ⁴⁰YRHHY⁴⁴, the loss of A3G degradation by W21A or W38A might be caused by loss of A3G binding. To exclude this possibility, we next performed co-transfection experiments with Vif and A3F expression vectors and similar results were obtained (Fig. 2B). These results suggest that the substitution of E88/W89 as well as W21 and W38 to alanines leads to an impairment of Vif-mediated degradation of both A3F and A3G due to the loss of binding to CBF β , not to A3F or A3G.

The substitution of E88/W89 to alanines impairs the ability of Vif to counteract the restriction by both A3F and A3G

To examine the ability of Vif mutants to counteract the restriction of HIV-1 by A3 proteins, we next performed single cycle infection experiments using luciferase-reporter viruses. The Vif mutations were introduced into pNL4-3 Δ Env-Luc, and transfected into 293T cells with co-transfection of VSV-G expression plasmid in the presence or absence of co-transfection of A3G expression plasmid. Virus-containing supernatant was harvested and infectivity was measured by challenging to fresh 293T cells and assaying luciferase activity. Viruses with Vif mutations showed comparable infectivity in the absence of A3G, as expected (Fig. 3A). In the presence of A3G, virus with E88A mutation showed comparable infectivity to wild-type, but virus with W21A, W38A or E88A/W89A showed deeply impaired infectivity close to that of Δ Vif (Fig. 3A). Virus with W89A showed intermediate and obviously impaired infectivity in the presence of A3G (Fig. 3A). We also performed these assays with A3F instead of A3G, and obtained similar results (Fig. 3B). These data indicate that E88/W89 residues as well as W21 and W38 are involved in Vif activity to counteract both A3F and A3G, suggesting these residues are involved in CBF β binding, not A3F or A3G binding.

The substitution of E88/W89 to alanines impairs HIV-1 replication in non-permissive CEM cells

Finally, we performed spreading infection experiments in both permissive CEM-SS and multiple A3-expressing CEM cells. We introduced Vif mutations into NL4-3, a replication-competent

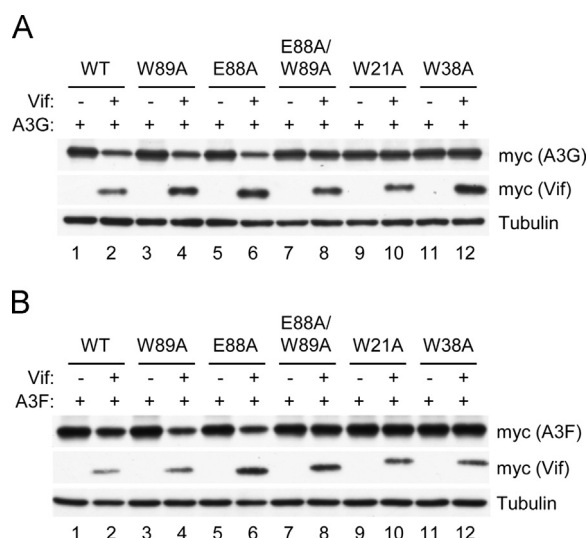


Fig. 2. Degradation of A3F and A3G by Vif. (A) Degradation of A3G. HEK293T cells were co-transfected with expression vectors for myc-tagged A3G and Vif wild-type or mutant, and protein levels of A3G and Vif were analyzed by immunoblotting with anti-myc serum and anti-tubulin antibody for loading control. The same amount of the A3G expression plasmid was transfected in each sample. (B) Degradation of A3F. Similar experiments to (A), but an A3F expression vector was used instead of that of A3G.

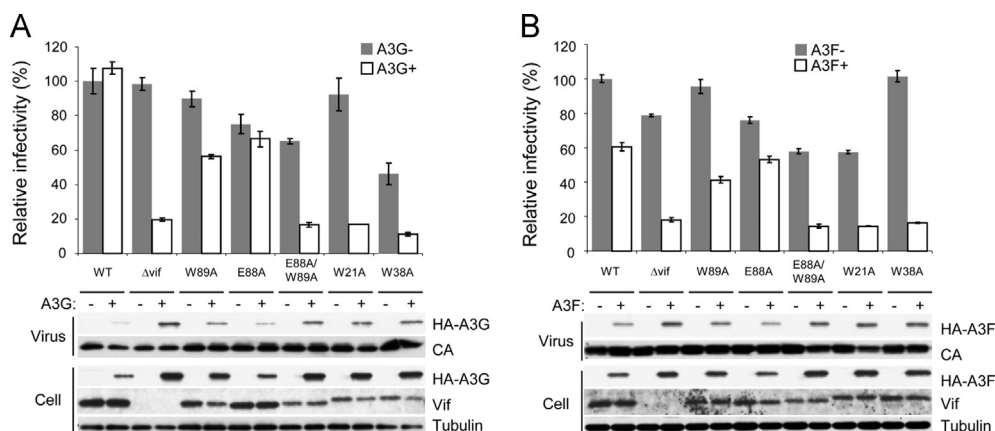


Fig. 3. Single-cycle infection experiments with VSV-G pseudo-typed viruses with vif mutations. (A) Counteracting abilities of Vif mutants against the restriction by A3G. 293T cells were transfected with pNL43/ Δ Env-Luc with vif mutation, together with pVSV-G, in the presence or absence of pcDNA3/HA-A3G. pNL43/ Δ Env-Luc without vif mutation and pNL43/ Δ Env Δ vif-Luc were also used for control. Virus-containing supernatant was challenged to fresh 293T cells and infectivity was determined by luminometer. Values were normalized to that of the virus without vif mutation in the absence of A3G. Average and standard errors of 3 independent experiments are shown. Levels of A3G in cells and virions and Vif in cells were also analyzed by immunoblotting. (B) Counteracting abilities of Vif mutants against the restriction by A3F. Similar experiments to (A), but pcDNA3/HA-A3F was used instead of the A3G plasmid.

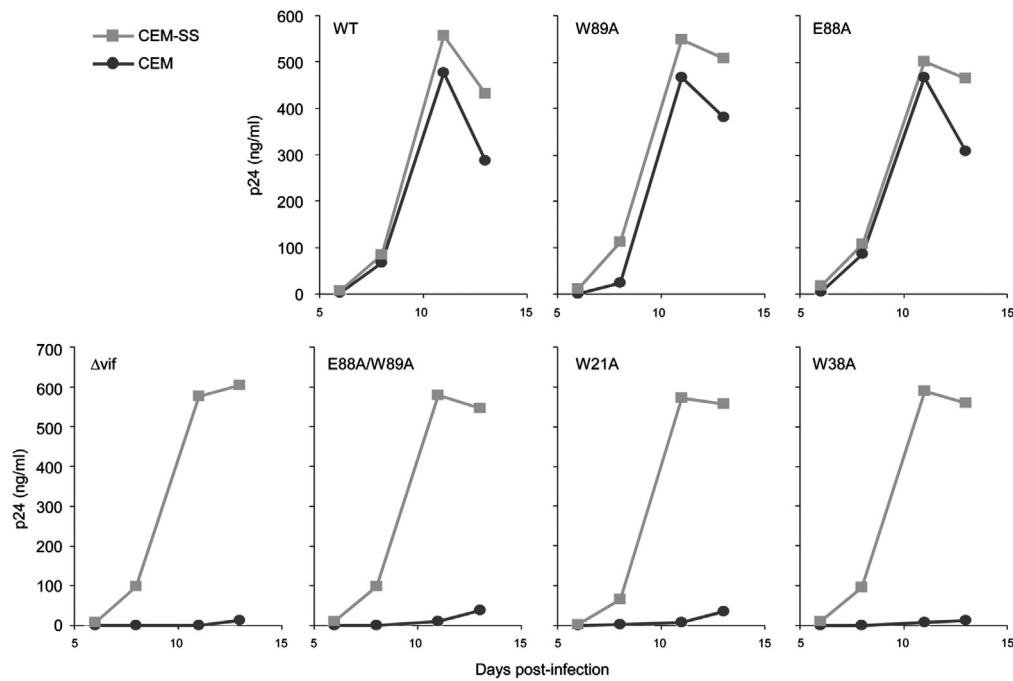


Fig. 4. Spreading infection of NL4-3 with vif mutations. The viruses were produced in 293T cells and challenged to permissive CEM-SS cells and nonpermissive CEM cells at MOI of 0.005. Culture supernatant was collected periodically and p24 levels were determined by ELISA.

molecular clone, and analyzed whether the substitution mutation impairs virus replication with spreading infection assays. Virus with E88A or W89A showed comparable replication profiles to wild-type virus in both CEM-SS and CEM cells. Virus with Δ Vif, E88A/W89A, W21A, or W38A showed indistinguishable replication profiles to wild type in CEM-SS cells, as expected, whereas in CEM cells, showed deeply impaired replication profiles (Fig. 4). These results indicate that the residues E88/W89 as well as W21 and W38 are critical for Vif to counteract multiple A3s, suggesting that these residues are directly involved in CBF β binding.

Discussion

In this study, we report that HIV-1 Vif residues E88 and W89 are involved in CBF β binding, therefore in rendering Vif capable of stable expression and inducing ubiquitination of both A3F and A3G proteins. Fujita et al. reported that deletion or substitution of these residues impairs steady-state levels of Vif protein and virus replication in nonpermissive H9 cells and monocyte-derived macrophages (Fujita et al., 2003). We confirmed lower expression levels of substitution mutants using both Vif expression vectors and molecular clones of HIV-1. We also confirmed inefficient replication of virus with the mutation using another nonpermissive T cell line, CEM cells. These observations may be all explained by the loss of CBF β binding.

Our results suggest that the single amino acid substitution mutant W89A does not bind to CBF β , however this mutant is capable of degradation of A3F and A3G, and supporting replication of the virus in non-permissive CEM cells. This modest conflict may be due to the differences in experimental settings; co-immunoprecipitation experiments test the interactions *in vitro* in complex total cell lysates, and degradation experiments and infectivity experiments test Vif functionality in living cells.

Zhang et al. reported that Vif residues W21 and W38 are involved in binding to CBF β , but functional correlation of these residues was not described (Zhang et al., 2011). We confirmed that these residues are critical for CBF β binding, and further

demonstrated that these residues are critical for degradation of both A3F and A3G, counteracting restriction by both A3F and A3G, and replication in nonpermissive, multiple A3-expressing cells.

Jäger et al. reported that CBF β functions to up-regulate steady-state level of Vif protein by using cells with CBF β knock-down (Jäger et al., 2011). We observed that the levels of Vif mutants that do not bind to CBF β were obviously lower than that of wild-type Vif. Our observations confirmed and support the Jäger's report by the experiments with different settings.

Several E3 ligases including CUL5, NEDD4, and AIP4, have been reported to induce Vif ubiquitination, although biological implications have not been well defined (Dussart et al., 2004; Mehle et al., 2004). We previously reported that MDM2 targets for Vif as an E3 ligase to induce its ubiquitination and proteasomal degradation, and that the N-terminal region of Vif (residues 4–22) is required for MDM2 binding (Izumi et al., 2009). Because one of the CBF β binding residues of Vif, W21, is located close to MDM2 binding region, the loss of CBF β binding might facilitate ubiquitination and degradation of Vif by MDM2. Further investigations will be required to test this possibility.

We provide here the evidence indicating that residues W21, W38, E88 and W89 of HIV-1 Vif are involved in binding surface to CBF β . Further studies on the Vif-CBF β co-crystal structure will be the key to understanding Vif-CBF β interaction surfaces, and to pharmaceutical applications of these pieces of information for patients with HIV-1 infection.

Materials and methods

Plasmid construction

C-terminally myc-tagged Vif expression plasmid, pDON-Vif-myc, was generated by amplifying NL4-3 vif coding sequence with primers ATA GGA TCC ATG GAA AAC AGA TG G CAG GTG GCA GGT CAT G and CGC GTC GAC CTA CAG ATC CTC TTC AGA GAT GAG TTT CTG CTC GTA GTG TCC ATT CAT TGT ATG GCT CCC, and inserting it into pDON-AI (Takara) at BamH I/Sal I sites. Expression plasmids for

Vif mutants were generated by a PCR-based method with properly mutated primers. HA-tagged expression plasmids for A3F and A3G were previously described (Shirakawa et al., 2006). N-terminally myc-tagged expression plasmids for A3F, pcDNA3-myc-A3F, was generated by amplifying coding sequences of human A3F with primers CTA GCT AGC ATG GAG CAG AAA CTC ATC TCT GAA GAG GAT CTG ATG AAG CCT CAC TTC AGA AAC ACA GTG G and GGG GTA CCT CAC TCG AGA ATC TCC TGC AGC TTG CTG, and inserting it into pcDNA3.1 (Invitrogen) at Nhe I/Kpn I sites. C-terminally myc-tagged expression plasmids for A3G, pcDNA3-A3G-myc, was generated by amplifying coding sequences of human A3G with primers ATA CTC GAG AAT GAA GCC TAC TTC AGA AAC ACA GTG and GGG GTA CCC TAC AGA TCC TCT TCA GAG ATG AGT TTC TGC TCG CAG TTT TCC TGA TTC TGG AGA ATG GC, and inserting it into pcDNA3.1 (Invitrogen) at Xho I/Kpn I sites. The luciferase-reporter HIV-1 plasmids for single-cycle infection, pNL43/ΔEnv-Luc and pNL43/ΔEnvΔvif-Luc were previously described (Shindo et al., 2003). Mutations in vif region of pNL43/ΔEnv-Luc and pNL4-3 were introduced by a PCR-based method using internal restriction sites, MSC I at position 4553, and EcoR I at position 5743.

Cell culture and transfection

293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and penicillin, streptomycin and glutamine (PSG). CEM and CEM-SS cells were maintained in RPMI1640 medium supplemented with 10% FBS and PSG. 293T cells on 6-well plates were transfected with about 1 μg of plasmid DNA in total using X-tremegene HP DNA transfection reagent (Roche) according to manufacturer's instruction.

Immunoblotting

Primary antibodies for immunoblotting against Vif, A3G and p24^{Gag} were obtained from the NIH AIDS Research and Reference Reagent Program. Rabbit anti-CBFβ serum and mouse anti-Cul5 and anti-HA antibodies were purchased from Santa Cruz. Rabbit anti-myc serum was purchased from Sigma. Mouse anti-tubulin antibody was purchased from Covance. HRP-conjugated secondary antibodies against mouse and rabbit were purchased from GE Healthcare. We used a standard chemiluminescence protocol for immunoblotting with PVDF membrane (Millipore).

Immunoprecipitation

For co-immunoprecipitation to test interaction of Vif mutant to CBFβ, 293T cells were transfected with pDON-Vif-myc or its derivative mutant, treated with MG132 at concentration of 2.5 μM for 16 h, and lysed with co-IP buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM MgCl₂) supplemented with protease inhibitor cocktail (Nacalai) and MG132. After centrifugation at 20,000 × g for 10 min, supernatant was mixed with 2 μg anti-myc rabbit serum (Sigma) for 1 h, and then mixed with 20 μl protein A sepharose (Pharmacia) for 1 h. Beads were washed with co-IP buffer 3 times, and bound protein was eluted with 1 × SDS sample buffer. Samples were analyzed by immunoblotting as described above.

Single-cycle infection

Luciferase encoding HIV-1 particles were produced by transiently transfecting 293T cells at 50% confluency using 0.8 μg pNL43/ΔEnv-Luc or derivative mutant, 0.2 μg pVSV-G and 0.02 μg pcDNA3/HA-A3F, pcDNA3/HA-A3G, or empty vector. After 48 h, virus-containing supernatants were harvested through PVDF filter with 0.45 μm pores (Millipore), and challenged to fresh 293T cells. After 48 h, cells were lysed with Passive lysis buffer (Promega) and luciferase

activity was determined by luminometer (2030 Arvo X, Perkin Elmer) using Luciferase Assay System (Promega). Sample preparation of producer cells and virus for immunoblotting was performed as described (Haché et al., 2008).

Spreading infection

293T cells were transfected with NL4-3 molecular clone or derivative mutant, and virus-containing supernatant was harvested through PVDF filters with 0.45 μm pores (Millipore) after 2-day incubation. CEM-SS and CEM cells were inoculated with the supernatant at MOI of 0.005. The culture supernatants were harvested periodically, and analyzed for p24 by an HIV-1 p24 antigen ELISA kit (Zeptometrix).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.virol.2013.11.004>.

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