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Original article

Expression of HIV-1 accessory protein Vif is controlled uniquely to be low and optimal by proteasome degradation

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Abstract

While the Vif protein of human immunodeficiency virus type 1 (HIV-1) is essential for viral replication in non-permissive cells, it is rapidly degraded intracellularly. We have previously suggested that the rapid turn-over of Vif is biologically meaningful to prevent detrimental effects of this protein at high expression levels. We now studied the mechanism of Vif degradation by examining the blocking effect of protease inhibitors in pulse/chase experiments and by monitoring the extent of Vif ubiquitination. The rapid turn-over of Vif could be blocked by proteasome inhibitors, and Vif was highly ubiquitinated. Cytoskeletal Vif was found to be more stable than soluble cytosolic Vif. These degradation characteristics of Vif were cell type-independent and observed in both non-permissive and permissive cells. Characterization of a series of *vif* deletion mutants showed that amino acids predicted to be important for formation of β-strand structures (amino acid nos. 63–70 and 86–89) were critical for maintaining a normal expression level of Vif and for viral infectivity. Finally, we performed comparative stability analysis of the four HIV-1 accessory proteins. Vif was unique in its short half-life and in the magnitude of the degradation. Taken together, we conclude that the proteasome degradation of HIV-1 Vif is a virologically important process and crucial for the function of Vif.
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Keywords: HIV-1; Vif; Proteasome degradation; Accessory proteins

1. Introduction

The Vif protein of HIV-1 is encoded by an accessory gene that is conserved in all known lentiviruses, except for equine infectious anemia virus [1], and is indispensable for viral replication in a certain type of cell (for review, see Refs. [2–5]). It acts during the stage of assembly, budding, or maturation to greatly augment the infectivity of progeny virions in a producer cell-dependent manner. Producer cells are, therefore, divided into permissive and non-permissive, and HIV-1 grown in non-permissive cells like primary human T cells and a restricted number of cell lines such as H9 and CEM is unable to replicate in any type of target cells in the absence of Vif. Many cell lines, such as 293T and HeLa, are classified as permissive cells. Previous studies suggested that Vif counteracts certain cellular anti-HIV-1 factor(s) present in non-permissive cells [6,7]. Indeed, the recent identification of APOBEC3G (CEM15) as a cellular inhibitor of HIV-1 replication [8] confirmed this speculation on the existence of an inhibitory factor in non-permissive cell types. APOBEC3G was subsequently found to deaminate dC to dU in the newly synthesized minus strand DNA of HIV-1, resulting in G to A hypermutation of the viral plus strand DNA [9–12]. However, the mechanism by which Vif overcomes the function of APOBEC3G remains unclear. Some reports show that Vif induces proteasome-dependent degradation of APOBEC3G [13–16] while others report an effect on APOBEC3G translation [17,18] or both [19]. The precise degradation profile of Vif itself remains to be determined.

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We have previously shown that Vif is rapidly decayed both in the absence and presence of APOBEC3G in transiently transfected HeLa cells, and that expression of Vif to an excessive level is inhibitory to viral replication [20]. In the current study we report that newly synthesized Vif is rapidly and similarly degraded in transiently transfected 293T, HeLa and H9 cells. We found that Vif present in detergent-resistant compartments was more stable than soluble cytosolic Vif. Treatment of 293T cells with proteasome inhibitors but not with inhibitors of lysosome degradation and the calpain system blocked the degradation of Vif. In fact, Vif was highly polyubiquitinated in the cells. We investigated the structural basis of stable expression of Vif by deletion analysis and found that amino acids in the predicted β-strand structures (amino acid nos. 63–70 and 86–89 in Vif) are critically important for a normal expression level of Vif and for viral infectivity. We further examined and compared the stability of all four HIV-1 accessory proteins. Rapid turn-over was demonstrated to be unique to Vif among the viral proteins tested. These results show that expression of Vif is controlled to be limited and optimal by proteasome degradation, and suggest that HIV-1 has apparently evolved to regulate Vif levels to suit its requirements for efficient replication and spread.

2. Materials and methods

2.1. Plasmids

The full-length molecular clone pNL432 was used for production of wild-type (wt) infectious virus [21]. Construction and characterization of proviral deletion mutants designated pNL-fE88*del*, -fW89*del*, -fR90*del*, -fK91*del*, -fK92*del*, and -fR93*del* have been previously described [22]. New proviral deletion mutants designated pNL-fL64*del*, -fV65*del*, -fI66*del*, and -fT67*del*, -fS86*del*, and -fI87*del* were constructed from pNL432 by the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) as previously described [22,23]. For efficient expression of wt Vif, the subgenomic expression vector pNL-A1 [24] was used. A pNL-A1 version of mutant Vif (E88*del*) expression vector designated pNLA1-E88*del* was constructed by insertion of the *Nde*I-*Eco*RI fragment of pNL-fE88*del* into the *Nde*I and *Eco*RI sites of pNL-A1. An HIV-1 Gag-p24 expression vector pSG-Gag (p24) cFLAG was constructed from pcDNA3 (Invitrogen Corp., Carlsbad, CA, USA) carrying FLAG (pcDNA3cFLAG) and pSG5 (Stratagene, La Jolla, CA, USA). The ubiquitin expression vector pcDNA3.1-HA-Ub constructed from pcDNA3.1 (Invitrogen, Corp., Carlsbad, CA, USA) [25] was used to monitor the ubiquitination of Vif.

2.2. Cells

H9 [26] cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). The 293T [27] and HeLa (ATCC CCL-2) cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FBS.

2.3. Transfection

For transfection of H9 cells, the electroporation method was used, as previously described [21]. For transfection of HeLa and 293T cells, the calcium-phosphate coprecipitation method [21] or the LipofectAMINE PLUS™ system (Invitrogen Corp., Carlsbad, CA, USA) was used.

2.4. Pulse/chase analysis of viral proteins

Transfected cells were metabolically labeled for 5 min with the Redivue Pro-mix L-[^{35}S] in vitro cell labeling mix system (Amersham Biosciences Corp., Piscataway, NJ, USA). After the labeling, cells were pelleted and suspended in complete RPMI containing all amino acids and 10% FBS. Cells were incubated for various times at 37 °C as indicated in the text. Cells were then pelleted and stored at –80 °C. Cell pellets were subsequently extracted with CHAPS buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 100 mM NaCl, and 0.5% (v/v) CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)) supplemented with 0.2% deoxycholate (DOC), incubated on ice for 5 min, vortexed, and pelleted for 3 min at 15000 × *g*. Proteins in the supernatant were designated sol (soluble fraction). Proteins present in the pellet were solubilized by dissolving in PBS–Laemmli's sample buffer (1:1) and heating at 95 °C for 30 min. The solution was then centrifuged for 3 min at 15000 × *g*. Proteins in the resultant supernatant were designated insol (insoluble fraction). These two fractions were used for immunoprecipitation with a specific antibody. Immunoprecipitated proteins were solubilized by boiling in the sample buffer and separated by SDS-PAGE. Radioactive bands were visualized by fluorography and quantitated by scanning.

2.5. Western immunoblot analysis of viral proteins

Transfected 293T cells were collected and solubilized by the CHAPS/DOC method as above or by dissolving directly into Laemmli's sample buffer. For some experiments, cells collected were suspended in PBS, subjected to two cycles of freeze–thaw, and pelleted for 3 min at 15000 × *g*. Proteins in the supernatant were designated sol-1. Proteins in the pellet were solubilized by the CHAPS/DOC and pelleted for 3 min at 15000 × *g*. The resultant supernatant was designated sol-2. Proteins present in the pellet were solubilized by dissolving in PBS–Laemmli's sample buffer (1:1) and heating at 95 °C for 30 min. The solution was then centrifuged for 3 min at 15000 × *g*. Proteins in the resultant supernatant were designated insol. Protein samples were resolved by SDS-PAGE followed by electrophoretic transfer to polyvinylidene fluoride membranes. The membranes were treated with specific antibodies and visualized with the ECL plus Western Blot-

ting Detection System (Amersham Biosciences UK Limited, Buckinghamshire, England), as previously described [22,23].

2.6. Immunoprecipitation/western blot analysis of Vif ubiquitination

The 293T cells were transfected with pNL-A1, pcDNA3.1-HA-Ub or pNL-A1 plus pcDNA3.1-HA-Ub, and cultured for 48 h. Transfected cells were cultured in the presence of MG-132 (50 μM) during the last incubation period, as indicated in the text. Cells were collected, lysed by TNE buffer (10 mM Tris–HCl pH 7.8, 0.15 M NaCl, 1 mM EDTA, 1% NP40, 10 μg/ml of aprotinin) and immunoprecipitated with a polyclonal anti-Vif antibody (no. 2221 of NIH AIDS Research and Reference Reagent Program Catalog). The precipitated proteins were analyzed by western blotting using a monoclonal anti-HA antibody HA.11 (BabCO, Berkeley, CA, USA).

2.7. Reverse transcriptase (RT) assay

Virion-associated RT activity was determined as previously described [28].

3. Results

3.1. Vif is rapidly degraded by proteasomes in a cell-type-independent manner

Recent work by us has indicated that soluble cytosolic Vif is rapidly degraded in HeLa cells both in the absence and presence of APOBEC3G [20]. Extensive analysis of subcellular localization of Vif has demonstrated the existence of a soluble and a cytoskeletal form, and to a much lesser extent, the presence of a detergent-extractable form of Vif [29]. We first asked whether insoluble cytoskeletal Vif is unstable and whether the rapid degradation of Vif is generally observed in various cell types. We also asked whether the degradation can be blocked by proteasome inhibitors [30,31]. To address these questions, we performed pulse/chase and western blot analyses in transiently transfected 293T cells (Fig. 1). Vif was expressed from the subgenomic vector pNL-A1 [24], which is known to produce a high level of Vif upon transfection. Transfected 293T cells were labeled for 5 min with [^{35}S]-methionine and [^{35}S]-cysteine, chased for up to 60 min in the absence or presence of MG-132, and used for cell fractionation studies, as described in Section 2. As shown in Fig. 1A and B, while cytosolic Vif was degraded rapidly, cytoskeletal Vif was quite stable and accumulated with time. The proteasome inhibitor MG-132 blocked significantly the degradation of cytosolic Vif. This blocking effect was not observed for inhibitors of lysosome degradation (Bafilomycin A1 and EST) and calpain system (calpeptin) (data not shown). Accumulation of cytoskeletal Vif in cells was con-

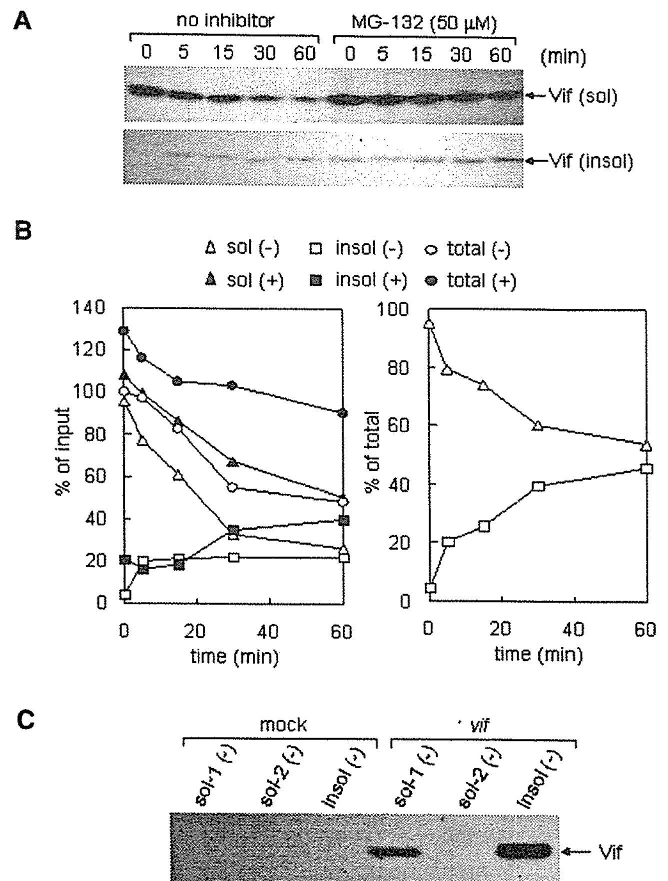

Fig. 1. Stability analysis of HIV-1 Vif in 293T cells. (A) Stability of Vif in the absence or presence of proteasome inhibitor MG-132. 293T cells were transfected with 5 μg of pNL-A1. Cells were collected 24 h after transfection, labeled for 5 min with [^{35}S]-methionine and [^{35}S]-cysteine, and chased for up to 60 min, as indicated above the lanes. Cells were incubated in the presence of MG132 (50 μM) during the labeling and chasing time where indicated. Cell lysates were prepared as described in Section 2 and precipitated with a Vif-specific polyclonal antibody Vif93 [29]. Vif proteins were identified by SDS-PAGE followed by fluorography; sol: soluble fraction; insol: insoluble fraction. (B) Degradation kinetics of Vif. Vif-specific bands in (A) were quantified by scanning, and relative values were plotted as a function of time; (–), absence of MG-132; (+), presence of MG-132; total: sol plus insol. (C) Steady-state expression of Vif. 293T cells were transfected with 5 μg of pNL-A1 (*vif*) or pUC19 (mock). Cells were harvested at 40 h post-transfection, and cell lysates were prepared as described in Section 2 for western blot analysis using an antibody Vif93. For sol-1, sol-2 and insol, see Section 2.

firmed by western blot analysis of transfected 293T cells at 40 h post-transfection (Fig. 1C). We extended these analyses to H9 and HeLa cells (Fig. 2). Transfected H9 and HeLa cells were pulse-labeled and chased as described above, and the degradation kinetics of two forms of Vif were monitored. As shown in Fig. 2, whereas Vif of a soluble form was degraded similarly rapidly, Vif of an insoluble form was quite stable and accumulated as observed in 293T cells.

Based on the effects of MG-132, Bafilomycin A1, EST, and calpeptin, we next assessed whether the ubiquitin–proteasome pathway was required for Vif degradation. Proteins that are selected for proteasomal degradation are marked by polyubiquitination (for review, see Refs. [32,33]). We co-

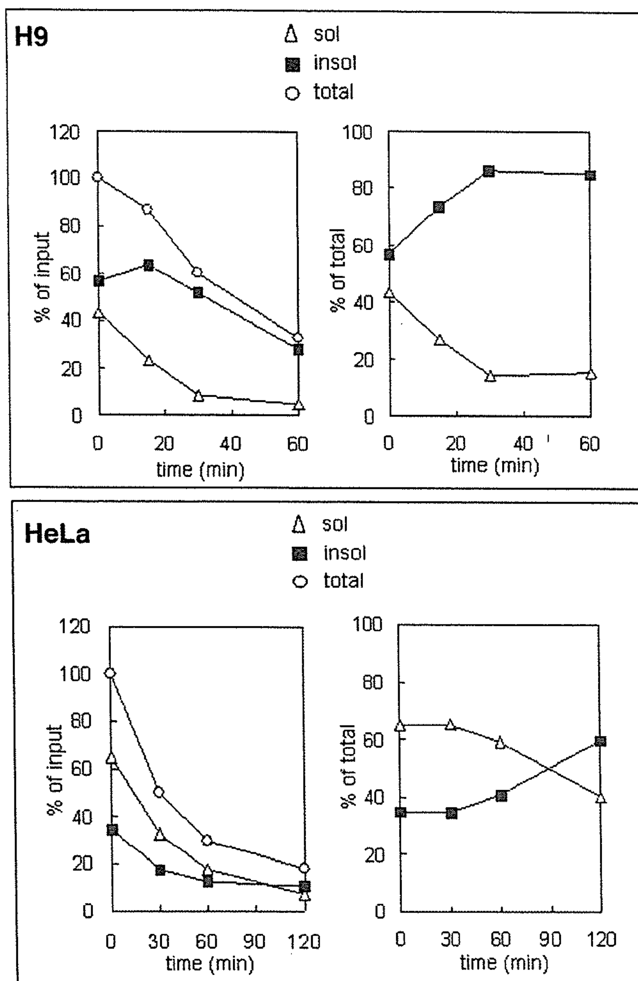

Fig. 2. Stability analysis of HIV-1 Vif in H9 and HeLa cells. Cells were transfected with 10 μg of pNL-A1 plus 10 μg of pNL432 (H9) or 5 μg of pNL-A1 (HeLa). Cells were collected 24 h after transfection, labeled for 5 min with [^{35}S]-methionine and [^{35}S]-cysteine, and chased for up to 60 min, as indicated. Cell lysates were prepared as described in Section 2 and precipitated with a Vif-specific polyclonal antibody Vif93 [29]. Vif proteins were identified by SDS-PAGE followed by fluorography. Vif-specific bands were quantified by scanning, and relative values were plotted as a function of time; sol: soluble fraction; insol: insoluble fraction; total: sol plus insol.

transfected 293T cells with pNL-A1 and a vector that expresses HA-tagged ubiquitin, and the cells were analyzed by immunoprecipitation/western blot, as described in Section 2. As shown in Fig. 3, co-transfected cells contained large polyubiquitinated Vif proteins in amounts that were increased by prolonged incubation time with the proteasome inhibitor MG-132.

3.2. *Formation of β-strand structures is important for regulated expression and function of Vif*

We have recently shown that amino acid residues 88 and 89 in NL432 Vif are critical for steady-state expression of Vif and for viral infectivity [22]. The two residues are located within a β-strand structure (residues 86–89) as predicted by the PredictProtein (http://www.embl-heidelberg.de/predictprotein/predictprotein.html). We wanted to evaluate

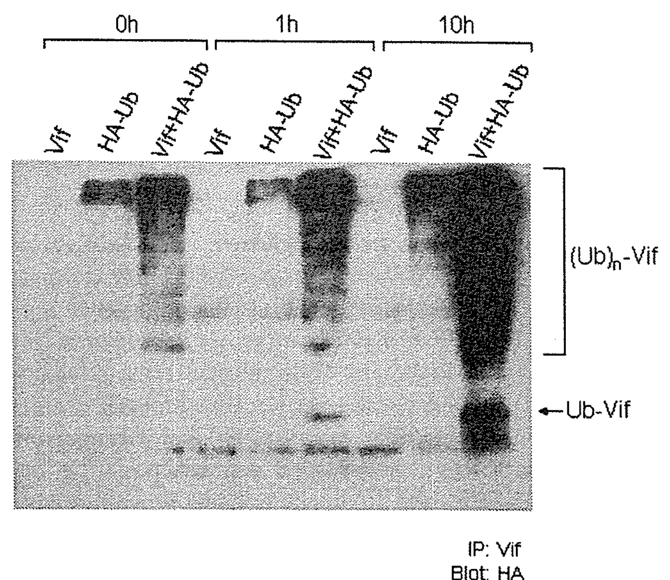

Fig. 3. Ubiquitination of HIV-1 Vif. 293T cells were singly transfected with 3 μg of pNL-A1 (Vif) or pcDNA3.1-HA-Ub (HA-Ub), or were double-transfected with pNL-A1 (1.5 μg) plus pcDNA3.1-HA-Ub (1.5 μg) (Vif + HA-Ub), and cultured for 48 h. Cells were cultured in the presence of MG-132 (50 μM) during the last incubation period, as indicated above the lanes. Cell lysates were prepared, immunoprecipitated with a polyclonal anti-Vif antibody (NIH catalog no. 2221), and the precipitated proteins were analyzed by western blotting using a monoclonal anti-HA antibody HA.11 (BAbCO, Berkeley, CA, USA), as described in Section 2. $(Ub)_n$-Vif, polyubiquitinated Vif; Ub-Vif, monoubiquitinated Vif; IP:Vif, immunoprecipitation by anti-Vif antibody; Blot:HA, western blot analysis using anti-HA antibody.

the importance of the amino acids in this β-strand and in an adjacent one (residues 63–70) for Vif expression and for viral infectivity by deletion analysis (Fig. 4). Proviral deletion mutants designated pNL-fL64*del*, -fV65*del*, -fI66*del*, -fT67*del*, -fS86*del* and -fI87*del* were newly constructed from pNL432, as described in Section 2. Various proviral clones were transfected into 293T and H9 cells and monitored for expression of Vif and for viral infectivity, respectively. As shown in Fig. 4A, amino acids within the β-strand structures, but not those on the outside, were indispensable for normal expression of Vif in 293T cells. Growth potentials of the deletion mutants in H9 cells were then monitored. As shown in Fig. 4B, the mutants, which express Vif at a very low level in 293T cells, were not infectious for H9 cells. We then determined whether the observed low expression level of mutant Vif is due to rapid degradation rate. A pNL-A1 version of mutant Vif (pNLA1-E88*del*) was constructed, and pulse/chase experiments using 293T cells were carried out, as described above. As can be clearly seen in Fig. 5A and B, E88*del* mutant Vif was more rapidly degraded than wt Vif. To ascertain the effect of proteasome inhibitors MG-132 and *clasto*-lactacystin β-lactone [30,31], steady-state levels of wt and mutant Vif proteins expressed by full-length proviral clones were determined in transfected 293T cells in the presence or absence of the inhibitors. As shown in Fig. 5C, E88*del* mutant Vif was expressed at a level similar to that of wt Vif only when the inhibitors were present.

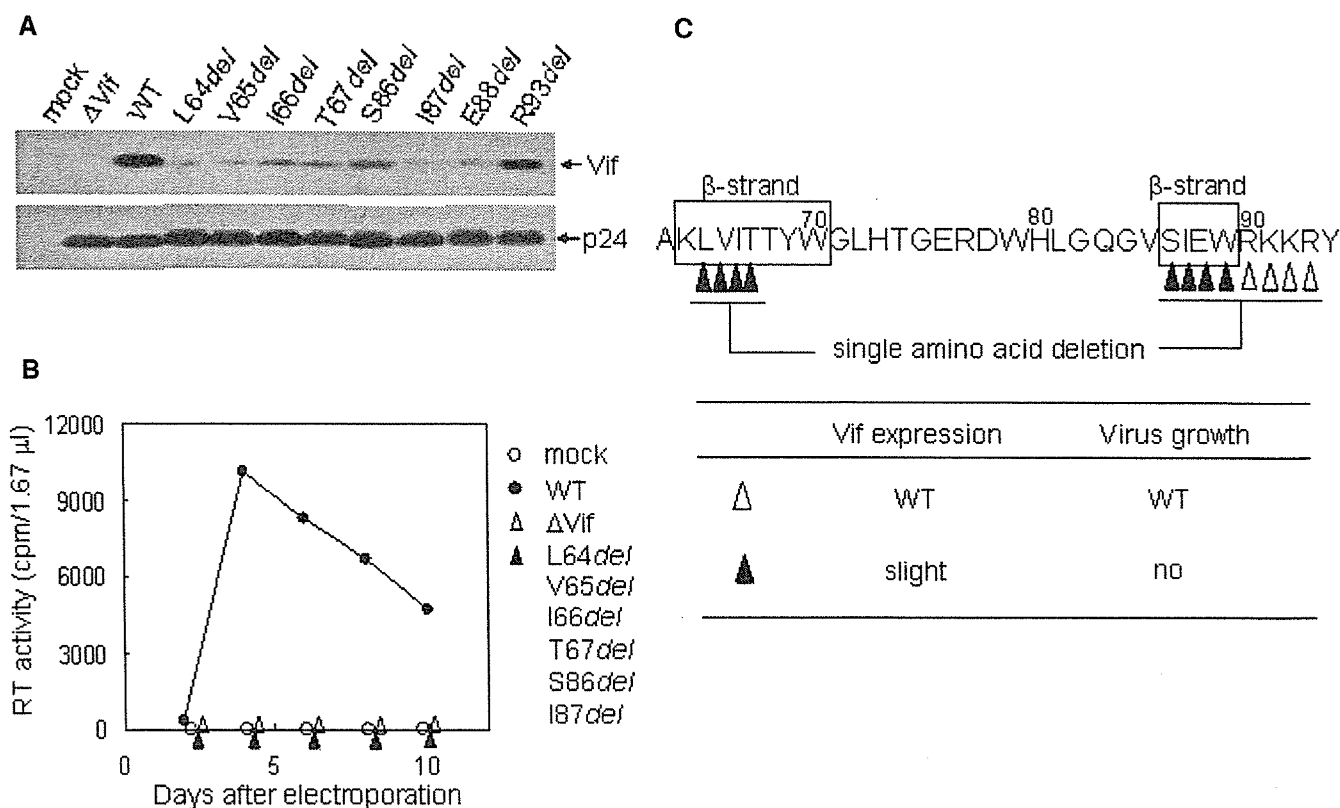

Fig. 4. Deletion analysis of HIV-1 Vif. (A) Steady-state expression of Vif in 293T cells transfected with various proviral mutant clones. 293T cells were transfected with 20 µg of various pNL clones indicated, and cell lysates were prepared at 48 h post-transfection for western blot analysis using appropriate anti-Vif and anti-Gag (p24) antibodies, as previously described [22,23]. ΔVif, pNL–Nd [34]; WT, pNL432 [21,34]. (B) Growth kinetics in H9 cells of various proviral mutant clones. H9 cells were electroporated with 10 µg of various pNL clones indicated, and RT production in the culture supernatants was determined at intervals. WT, pNL432 [21,34]; ΔVif, pNL–Nd [34]. (C) Phenotype of various mutant clones. The results in (A) and (B) are summarized. Characteristics of E88*del*, W89*del*, R90*del*, K91*del*, K92*del*, and R93*del* mutants have been previously described [22] and are included in this summary.

3.3. Rapid and extensive degradation is unique to Vif among accessory proteins

We finally asked whether Vif is unique among the four HIV-1 accessory proteins with respect to its high sensitivity to proteasome degradation. To address this question, we performed pulse/chase analyses using wt full-length proviral clone pNL432. Transfected 293T cells were pulse-labeled and chased as described above, and the degradation kinetics of Vif, Vpr, Vpu and Nef were monitored. Gag-p24 was used as a control. As shown in Fig. 6A, Vif was unstable and decayed much more rapidly and extensively than Vpr, Vpu and Nef. The blocking effect of the proteasome inhibitor MG-132 on the degradation of accessory proteins was then examined (Fig. 6B). Transfected 293T cells were pulse-labeled for 5 min and chased for 30 min in the presence or absence of MG-132, and the ratio of expression level of accessory proteins (soluble fraction) was determined. As clearly seen in Fig. 6B, the effect was the greatest for Vif.

4. Discussion

We have recently shown that rapid turn-over of Vif in cells may be virologically important [20]. Excess amounts of Vif exert harmful effects on virus infectivity, and therefore HIV-1 has apparently evolved to control the intracellular expression level of Vif. In this study, we report for the first time on the mechanism, the cell-type independency, and the region in Vif critical for the rapid intracellular degradation. We also show that this unstable nature of Vif is unique among HIV-1 accessory proteins.

Our results here strongly suggest that the mechanism of Vif degradation involves the cellular proteasome pathway. The proteasome inhibitors MG-132 and *clasto*-lactacystin β-lactone specifically blocked the degradation (Figs. 1, 5, and 6). We have detected large amounts of polyubiquitinated derivatives of Vif that are greatly increased by the proteasome inhibitor MG-132 (Fig. 3). These results are quite consistent with those on the presence of APOBEC3G recently reported by Mehle et al. [14]. In the absence of APOBEC3G, however, they found that only a minor fraction of Vif is monoubiquitinated, and that Vif is a relatively stable protein with a half-life of approximately 90 min [14]. These observations were very different from our results presented here. In contrast to the soluble cytosolic Vif, which is rapidly degraded, the insoluble cytoskeleton-associated Vif was quite stable and accumulated in cells (Figs. 1 and 2). This phenomenon was observed in permissive 293T and HeLa cells, and in non-permissive H9 cells (Figs. 1 and 2). Whether

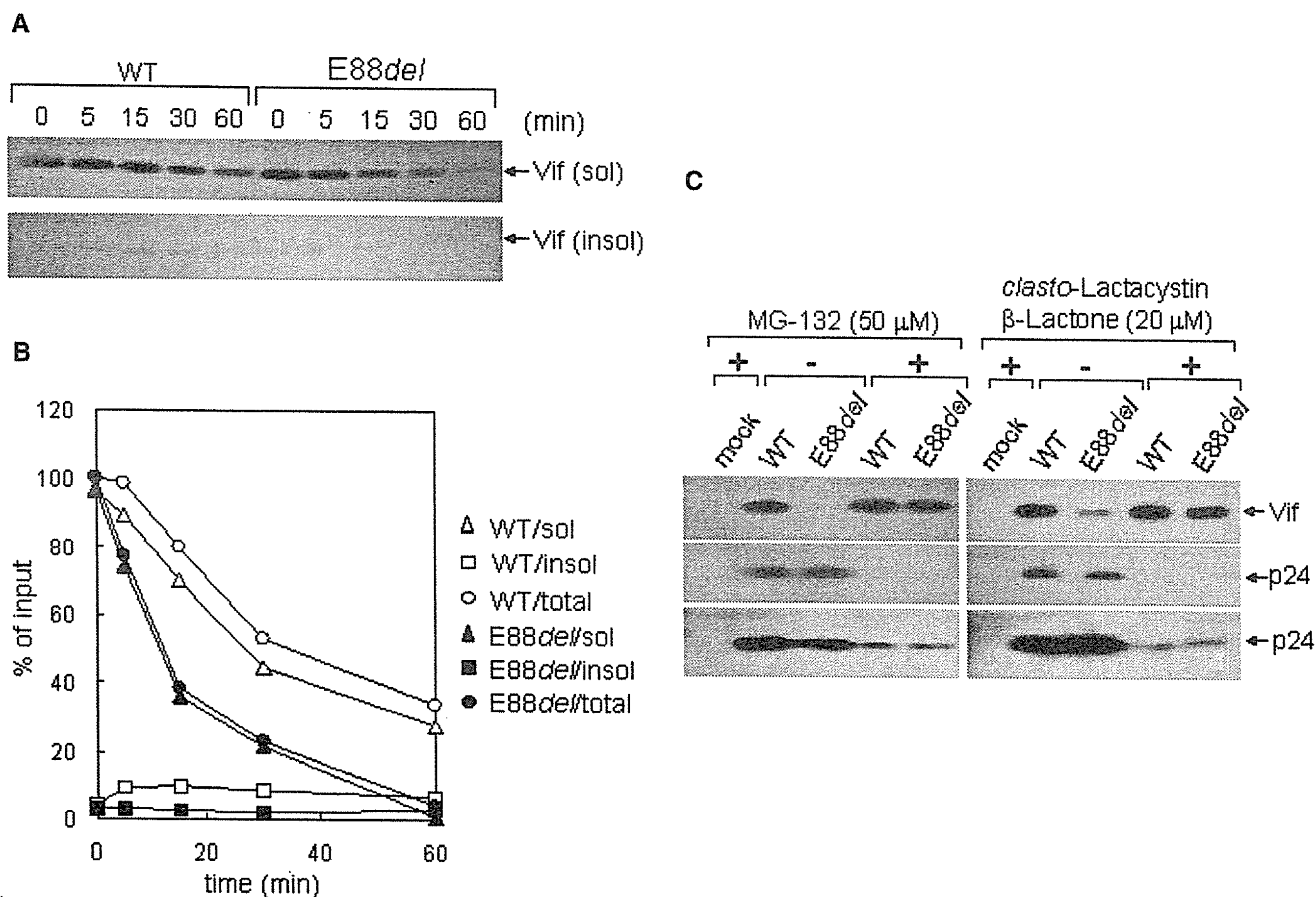

Fig. 5. Comparative analysis of wt and E88*del* mutant Vif proteins. (A) Stability of wt and mutant Vif proteins. 293T cells were transfected with 5 μg of pNL-A1 (WT) or pNLA1-E88*del*. Cells were collected 24 h after transfection, labeled for 5 min with [^{35}S]-methionine and [^{35}S]-cysteine, and chased for up to 60 min, as indicated above the lanes. Cell lysates were prepared as described in Section 2 and precipitated with a Vif-specific polyclonal antibody Vif93 [29]. Vif proteins were identified by SDS-PAGE followed by fluorography; sol, soluble fraction; insol, insoluble fraction. (B) Degradation kinetics of wt and mutant Vif proteins. Vif-specific bands in (A) were quantified by scanning, and relative values were plotted as a function of time. Total, sol plus insol. (C) Steady-state expression of wt and mutant Vif proteins in the presence or absence of proteasome inhibitors. The 293T cells were transfected with 3 μg of wt pNL432 or pNL-fE88*del* and cultured in the presence (+) or absence (–) of proteasome inhibitors MG-132 and *clasto*-lactacystin β-lactone. Cells were harvested at 36 h post-transfection for western blot analysis using appropriate anti-Vif and anti-Gag (p24) antibodies, as previously described [22,23]. Appropriate adjustments in sample volumes were made to give bands of similar intensity (wt Vif). Also, results of two different blots for Gag-p24 are presented to show that each lane for comparison contained approximately similar amounts. Expression level of viral proteins in cells in the presence of the inhibitors was low relative to that in the absence of the inhibitors.

the cytoskeletal Vif represents a population refractory to ubiquitination and has a pivotal role for Vif function remains to be determined. It would be vitally important to generate degradation-resistant Vif mutants and characterize them biochemically and biologically.

Deletion analysis of Vif in this study has demonstrated that there are some amino acids that confer stability on Vif. We have recently reported that mutations affecting residues 88 and 89 of NL432 Vif, located within a β-strand structure, as predicted by the PredictProtein, resulted in a reduced level of Vif expression [22]. Based on this observation, we performed mutational analysis of the area. Deletion of amino acids in the predicted β-strand structures (residues 63–70 and 86–89 in NL432 Vif), but not those located outside of the regions, diminished Vif expression to a negligible level and abolished viral infectivity (Fig. 4). Apparently, formation of the β-strand structures is important for stable expression of Vif. In addition, we showed that the E88*del* mutant Vif is degraded more rapidly than wt Vif, and that this degradation is proteasome-dependent (Fig. 5). Together with the results in our recent work [20], we can conclude that there is an optimal level of Vif expression in cells for viral replication.

Recent work proposed that Vif induces proteasome-dependent degradation of APOBEC3G [13–16], but the precise molecular mechanism for this activity of Vif is still unclear. We have already shown by pulse/chase analyses that there is no significant difference in the stability of APOBEC3G in the presence or absence of Vif [17] and that there is no significant difference in the stability of Vif in the presence or absence of APOBEC3G [20]. Furthermore, we found that the half-life of Vif in non-permissive H9 cells (Fig. 2) is very different from that of APOBEC3G in the presence of Vif, which was recently reported [13,15,16, 18,19]. Based on these findings, it seems unlikely that Vif and APOBEC3G form a complex that leads to subsequent simultaneous degradation of both proteins by the proteasome path-

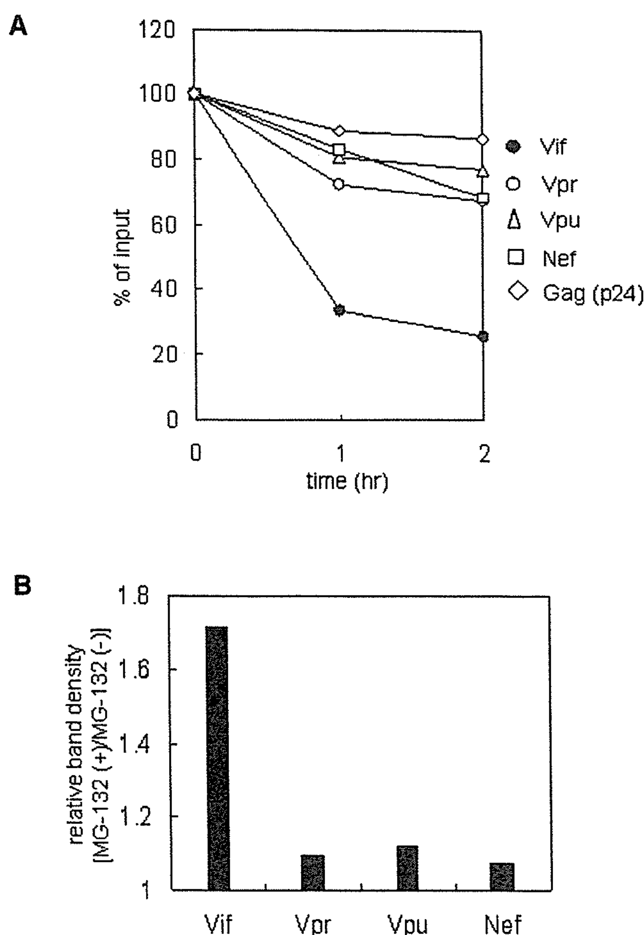

Fig. 6. Comparative analysis of HIV-1 accessory proteins. (A) Degradation kinetics of the HIV-1 accessory proteins. The 293T cells were transfected with 5 µg of pNL432 or a control Gag-p24 expression vector pSG-Gag (p24) cFLAG. Cells were collected 24 h after transfection, labeled for 5 min with [^{35}S]-methionine and [^{35}S]-cysteine, and chased for up to 2 h as indicated. Cell lysates (soluble fraction) were prepared and precipitated with an antibody against each viral protein (anti-Vif, Vif93; anti-Vpr, raised against full-length synthetic Vpr peptide (Strebel et al., unpublished); anti-Vpu, NIH catalog no. 969; anti-Nef, NIH catalog no. 2949; anti-Gag-p24, NIH catalog no. 6521). Viral proteins were identified by SDS-PAGE followed by fluorography. Specific bands of viral proteins were quantified by scanning, and relative values were plotted as a function of time. (B) Blocking effect of proteasome inhibitor MG-132 on the degradation of accessory proteins. 293T cells were transfected with various clones, as described in (A). Cells were collected 24 h after transfection, labeled for 5 min with [^{35}S]-methionine and [^{35}S]-cysteine, and chased for 30 min. Part of cells were incubated in the presence of MG132 (50 µM) during the labeling and chasing time, as indicated. Cell lysates were prepared and precipitated with an antibody against each accessory protein as above. Accessory proteins were identified by SDS-PAGE followed by fluorography. Specific bands of accessory proteins were quantified by scanning, and the ratio of values for each accessory protein is shown.

way. We and others have reported that Vif affects APOBEC3G translation [17–19]. However, the reduction of intracellular APOBEC3G in the presence of Vif does not fully account for the Vif-induced reduction of virus-associated APOBEC3G [17]. Further investigations are required to elucidate the molecular mechanism by which Vif counteracts the activity of APOBEC3G.

Acknowledgments

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Rapid Communication

Ubiquitination of APOBEC3 proteins by the Vif–Cullin5–ElonginB–ElonginC complex

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Abstract

APOBEC3 proteins are antiviral host factors for a wide variety of retroviruses. HIV-1 Vif overcomes the antiviral activity of APOBEC3G by ubiquitinating the protein. In this study, we examined the ability of Vif to antagonize other family members of APOBEC3 proteins, together with its mechanism. Using HIV infectivity, virion incorporation, immunoprecipitation, and in vitro ubiquitin conjugation assays, we show that the ability of Vif to inhibit antiviral activity of APOBEC3 proteins positively correlates with its ability to bind and ubiquitinate these proteins by a Vif–Cullin5–ElonginB–ElonginC (Vif–BC–Cul5) complex. These results suggest that Vif exhibits its anti-APOBEC3 activity by the ubiquitin ligase activity of the Vif–BC–Cul5 complex.
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Keywords: HIV-1; Cytidine deaminase; Virion incorporation; Ubiquitin; Cullin5–ElonginB–ElonginC complex

Introduction

APOBEC3G (A3G) is a potent antiretroviral host factor (Sheehy et al., 2002). It deaminates cytidine to uridine in nascent minus-strand viral DNA, inducing G-to-A hypermutation in the plus-strand viral DNA (Harris et al., 2003; Mangeat et al., 2003; Shindo et al., 2003; Zhang et al., 2003). HIV-1 Vif protein overcomes the antiviral activity of A3G by targeting it for ubiquitin-dependent degradation (Marin et al., 2003; Sheehy et al., 2003; Stopak et al., 2003). Vif interacts with cellular proteins, Cullin5 (Cul5), Elongin B (EloB), Elongin C (EloC), and Rbx1 through its novel SOCS-box motif to form a ubiquitin ligase (E3) complex (Vif–BC–Cul5) and functions as a substrate recognition subunit of the complex (Kobayashi et al., 2005; Mehle et al., 2004; Yu et al., 2003, 2004b). A3G belongs to the APOBEC superfamily of cytidine deaminases (Jarmuz et al., 2002), and several studies have reported that other members of this family such as APOBEC3B (A3B) and APOBEC3F (A3F) also have an antiviral activity on HIV-1, while the involvement of Vif in antagonizing these enzymes remains controversial (Bishop et al., 2004; Liddament et al., 2004; Wiegand et al., 2004; Zheng et al., 2004). We have recently reported that the Vif–BC–Cul5 complex could indeed work as an E3 ligase by an in vitro ubiquitin conjugation assay using the purified Vif–BC–Cul5 complex (Kobayashi et al., 2005). Using the assay, here, we show that the Vif–BC–Cul5 complex ubiquitinates A3F as well as A3G, but not A3B. We also demonstrate that the ability of Vif to inhibit antiviral activity of APOBEC3 proteins positively correlates with its ability to bind and ubiquitinate these proteins by the Vif–BC–Cul5 complex, indicating that Vif exhibits its anti-APOBEC3 activity by the ubiquitin ligase activity of the Vif–BC–Cul5 complex.

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Results

We first tested the antiviral activity of A3G, A3B, and A3F on HIV-1 as well as their incorporation into HIV-1 virions. As shown in Fig. 1A, expression of APOBEC3 proteins suppressed the infectivity of ΔVif virions to various extents. HIV-1 Vif overcame the antiviral activity of A3G and A3F, but not that of A3B, suggesting that APOBEC3B was resistant to HIV-1 Vif. The infectivity of the wild type (WT) virion was 8 to 47 times higher than that of ΔVif virion with A3G, and 2 to 3 times higher with A3F, suggesting that HIV-1 Vif antagonized A3G more effectively as compared to A3F. An immunoblotting of APOBEC3 proteins in virions and producer cells (Fig. 1B) revealed that all APOBEC proteins were incorporated into ΔVif virions (Fig. 1B, top panel, lanes 3, 5, and 7). An immunoblot with anti-β-actin mAb revealed no incorporation of this abundant cellular protein into virions, suggesting the specific incorporation of APOBEC3 proteins (data not shown). Vif could inhibit virion incorporation of A3G and A3F effectively (top panel, lanes 4 and 8, respectively), but not that of A3B (top panel, lane 6). In parallel, Vif reduced intracellular levels of A3G and A3F, but not that of A3B (Fig. 1B, middle panel), and reduction of the intracellular level of A3G by Vif was stronger than that of A3F. We next examined the physical interaction of Vif with APOBEC3 proteins by an immunoprecipitation assay. HIV-1 Vif was co-immunoprecipitated with A3G and, to a lesser extent, with A3F (Fig. 1C, top panel, lanes 2 and 4, respectively), but not with A3B (lane 3). These results suggested that Vif inhibited the virion incorporation of A3F as well as A3G, but not that of A3B because Vif could not bind to A3B. This also suggested that the ability of Vif to bind these proteins corresponded to the extent of reduction of their intracellular levels. Finally, we tested the E3 activity of Vif–BC–Cul5 ligase complex on APOBEC3 proteins by an in vitro ubiquitin conjugation assay using the purified Vif–BC–Cul5 as previously reported (Kobayashi et al., 2005). As shown in Fig. 2, the Vif–BC–Cul5 (E3) complex specifically ubiquitinated A3G (lane 7) and, to a lesser extent, A3F (lane 9) since it did not ubiquitinate these when E2 was omitted. The magnitude of ubiquitination of these proteins corresponded to the extent to which Vif overcame the antiviral

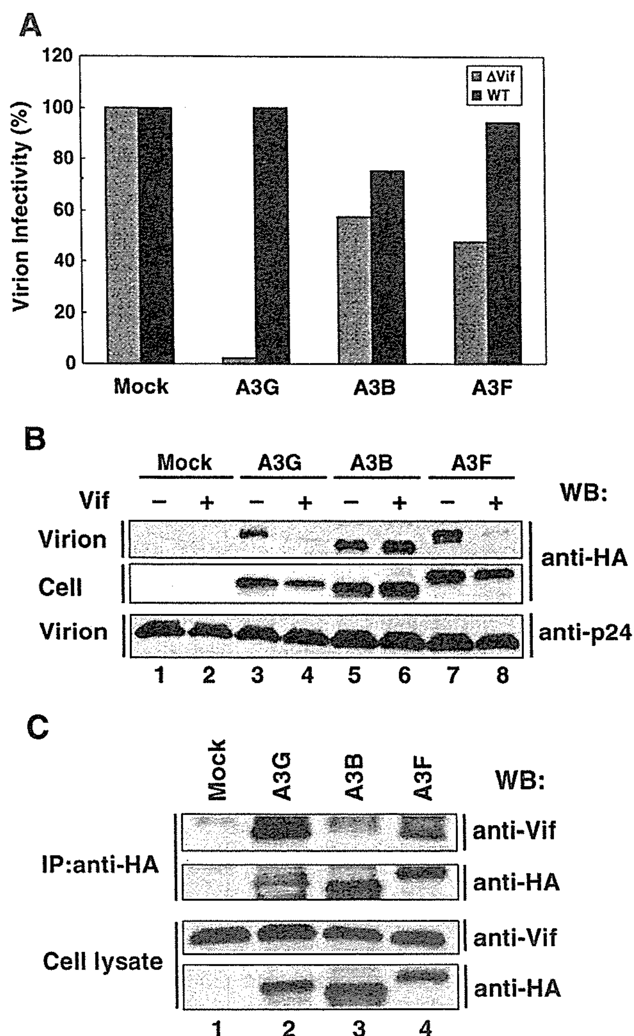

Fig. 1. Antiviral activity of APOBEC3 proteins on HIV-1. (A) A3B is resistant to HIV-1 Vif. We transfected HEK293T cells with pNL43/ΔEnv-Luc (WT) or pNL43/ΔEnvΔvif-Luc (ΔVif) plus pVSV-G in the presence of pcDNA3/HA-based vectors (a mock, A3G, A3B, and A3F). Viruses from these cells were challenged to M8166 cells, and productive infection was measured by luciferase activity. Values are presented as the percent infectivity relative to the values of each virus without expression of APOBEC3 proteins. Expression of APOBEC proteins suppressed the infectivity of ΔVif virions, and HIV-1 Vif overcame the antiviral activity of A3F as well as A3G, but not that of A3B. (B) Vif inhibited virion incorporation of A3G and A3F, but not that of A3B. HIV-1 virions prepared as described above were precipitated by ultracentrifugation and subjected to immunoblot with anti-HA (top panel) and anti-p24 (bottom panel) mAbs. Cell lysates of producer cells were also subjected to immunoblot with anti-HA mAb (middle panel). (C) Vif could bind to A3G and A3F, but not to A3B. HEK293T cells were co-transfected with expression vectors for APOBEC3 proteins and Vif. The lysates were immunoprecipitated with anti-HA mAb and analyzed by immunoblotting with anti-Vif mAb (top panel) or anti-HA mAb (2nd top panel). Vif was coprecipitated with A3G and, to a lesser extent, with A3F, but not with A3B. Cell lysates were also subjected to immunoblot with anti-Vif mAb (3rd top panel) or anti-HA mAb (bottom panel).

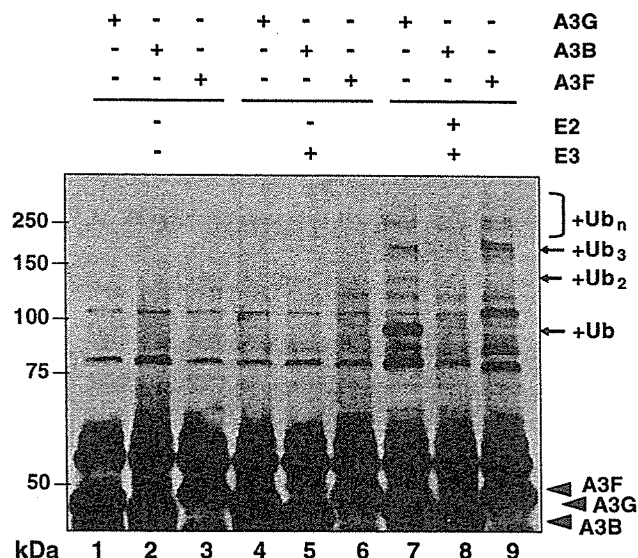

Fig. 2. In vitro ubiquitination of APOBEC3 proteins. An in vitro ubiquitin conjugation assay was performed as described in Materials and methods. GST-ubiquitin-conjugated A3G and A3F proteins were specifically detected as ladder (arrows) by immunoblotting with anti-HA mAb.

activity of these proteins. In contrast, the assay showed no ubiquitination of A3B (lane 8).

Discussion

HIV Vif is known to antagonize the antiviral activity of A3G by excluding the protein from HIV virion, which is attributed to the ubiquitination of A3G by the Vif–BC–Cul5 complex as previously reported (Kobayashi et al., 2005). In this study, we show the clear correlation between the function of Vif to antagonize APOBEC3 proteins and the ubiquitination of these by Vif–BC–Cul5 ubiquitin ligase complex using the in vitro ubiquitin conjugation assay. Vif overcomes the antiviral activity of A3F by ubiquitinating it through the Vif–BC–Cul5 complex as reported with A3G although to a lesser extent. However, Vif cannot overcome the antiviral activity of A3B because it cannot bind to A3B. The magnitude of inhibitory activity of Vif against the proteins corresponds to the extent of ubiquitination of APOBEC3 proteins by the Vif–BC–Cul5 complex as well as the binding ability of Vif to APOBEC3 proteins. This suggests two possibilities. One is that the binding of Vif to APOBEC3 proteins might induce the changes in its conformation or subcellular localization leading to unpackaging into virions as reported by the Strebel laboratory (Kao et al., 2004) because the binding ability of Vif to APOBEC3 proteins correlates to the inhibitory activity on APOBEC3. The other is, as we reported previously, that the ubiquitination of APOBEC3 proteins by the Vif–BC–Cul5 complex is essential for Vif function against the proteins. Although we cannot fully exclude the former possibility at this time, we believe that the latter is more likely because our in vitro ubiquitination assay showed the clear correlation between the in vitro ubiquitination of APOBEC3 proteins and the inhibitory effect of Vif on the proteins. This could not be fully explained by the former mechanism alone. Further study is necessary to fully elucidate this mechanism.

The antiviral activity of A3B on HIV-1 has been controversial. Some groups reported a weak inhibitory effect of A3B on HIV-1 (Bishop et al., 2004; Yu et al., 2004a), while others recently reported a strong inhibition (Doehle et al., 2005). In this study, we found only a weak antiviral activity of A3B on HIV-1. By sequencing, we found some SNPs in the coding region of A3B according to National Center for Biotechnology Information database. Although we could not fully explain the discrepancies of the anti-HIV-1 activities of A3B among studies, one explanation might be that SNPs in the coding region of A3B might affect its antiviral activity. Further study on this matter is also be warranted.

We previously demonstrated that ubiquitination of A3G by the Vif–BC–Cul5 complex is essential for Vif function against A3G. In this study, we further extend this notion by showing that the ability of Vif to inhibit antiviral activity of APOBEC3 positively correlates with its ability to bind and ubiquitinate APOBEC3 by Vif–BC–Cul5. This will provide us with new insights into the mechanism of Vif function to antagonize APOBEC proteins and to identify new targets for therapeutic strategy.

Materials and methods

Plasmids and cell lines

Expression vector for hemagglutinin (HA)-tagged human A3G, pcDNA3/HA-A3G, was constructed as previously described (Kobayashi et al., 2004). pcDNA3/HA-A3F was constructed in the same way. pNLA1-43Vif was constructed by inserting a Vif fragment from NL4-3 into the subgenomic expression vector pNL-A1 (a kind gift from Dr. K. Strebel), which expresses all HIV-1 proteins except for *gag* and *pol* products (Strebel et al., 1987). pcDNA3/HA-A3B was a kind gift from Dr. K Imada (Kyoto University) (Hishizawa et al., 2005). pNL43/ΔEnv-Luc and pNL43/ΔEnvΔvif-Luc were constructed as previously described (Shindo et al., 2003). HEK293T and M8166 cells were maintained as previously described (Shindo et al., 2003).

Infectivity assay with luciferase reporter viruses

Luciferase reporter viruses with or without Vif were prepared by cotransfection of pNL43/ΔEnv-Luc (WT) or pNL43/ΔEnvΔvif-Luc (ΔVif) plus pVSV-G together with a mock vector or expression vectors for A3G, A3B, and A3F by calcium phosphate method as previously described (Shindo et al., 2003). Productive infection was measured by luciferase activity. Values were presented as percent infectivity relative to the value of each virus without expression of APOBEC3 proteins.

Co-immunoprecipitation assay

To see protein–protein interaction in vivo, we performed an immunoprecipitation assay as described previously (Shindo et al., 2003). pcDNA3/HA-A3G, A3B, or A3F was co-transfected with pNLA1-43Vif into HEK293T cells by calcium phosphate method. Two days after transfection, cells were lysed in lysis buffer (25 mM HEPES pH 7.4/150 mM NaCl/1 mM $MgCl_2$/0.5% TritonX-100/10% Glycerol), and complexes were immunoprecipitated with anti-HA monoclonal antibody (mAb) (12CA5) (F. Hoffmann-La Roche Ltd.) and protein A–Sepharose beads (Amersham Biosciences Corp., Piscataway, NJ) at 4 °C. The beads were washed with lysis buffer and analyzed on immunoblot with anti-HA mAb or anti-Vif mAb (#319) (A kind gift from Dr. M. Malim through the AIDS Research and Reference Reagent Program) (Simon et al., 1995).

In vitro ubiquitin conjugation assay

In vitro ubiquitin conjugation assay was performed as previously described (Kobayashi et al., 2005). In brief, a Vif–BC–Cul5 complex was purified from insect cells and incubated with immunopurified HA-A3G, A3B, or A3F from 293T cells in reaction buffer containing E1, E2, GST-ubiquitin, NEDD8, Ubc12 (E2 for NEDD8), and APP-BP/Uba3 (E1 for NEDD8) at 37 °C for 1 h. Samples were subjected to

immunoblot to detect GST-ubiquitin-conjugated HA-APOBEC3 proteins.

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Original article

Development of a rapid and convenient method for the quantification of HIV-1 budding

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Abstract

In cells, the expression of Gag protein, one of the major structural proteins of retroviruses, is sufficient for budding virus-like particles (VLPs) from the cell surface. We have previously reported that spheroplasts of *Saccharomyces cerevisiae* expressing HIV-1 Gag proteins from an episomal plasmid constitutively released a large amount of VLPs into culture media; however, commercially available ELISA kits which detect mature capsid of HIV-1 could not detect uncleaved 55-kDa Gag proteins released from budding yeast. We therefore developed a method to quantitate VLP levels released from budding yeast by using fusion protein from HIV-1 Gag and Firefly Luciferase. This system is useful for screening cellular factor(s) involved in retrovirus budding from *S. cerevisiae*.
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Keywords: HIV-1; *Saccharomyces cerevisiae*; Luciferase

1. Introduction

All replication-competent retroviruses have three major structural proteins (i.e. Gag, Pol and Env), and some have regulatory proteins. In human immunodeficiency virus type 1 (HIV-1), Gag protein is synthesized as a 55-kDa precursor protein in the cytosol and cleaved to MA (matrix), CA (capsid), p2, NC (nucleocapsid), p1 and p6 by a viral encoded protease during budding from the cell surface. There is a myristoylation signal at the amino terminus of MA, so that precursor Gag proteins can target plasma membranes, where virus protein assembly and particle budding occur. The viral envelope is lined with MA, and CA forms a cone-shaped core encapsidating the RNA genome associated with NC. Pol proteins are initially synthesized as a 160-kDa precursor of Gag–Pol protein via a −1 translational frameshift, and the ratio of Gag and GAG–Pol proteins is about 20:1 [1]. Gag protein alone could be assembled into virus-like particles (VLPs) when expressed inside cells by recombinant vaccinia virus [2,3], baculovirus [4], and budding yeast [5]. These VLPs contain only the p55 Gag precursor because of the absence of protease encoded in the pol gene. When authentic *gag* and *pol* open reading frames were inserted in those expression vectors, VLPs with mature Gag and Pol proteins were produced by recombinant vaccinia virus vector, but not by baculovirus [4] or budding yeast (Sakuragi and Morikawa, unpublished data), probably due to higher expression levels of protease by baculovirus or budding yeast than by vaccinia virus. Significant protease expression would cleave Gag–Pol protein inside the cells [6], so that essential domains for virus assembly were lost before precursor proteins targeted the

Abbreviations: VLP, virus-like particle; MA, matrix; CA, capsid; NC, nucleocapsid; PEG, polyethylene glycol.

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plasma membrane. Similarly, an artificial Gag–Pol fusion protein generated by inserting four nucleotides at the natural frameshift region failed to produce VLPs even if expressed by a vaccinia virus vector [2]. When Gag–Pol fusion protein, whose protease was in an inactive form [7], or Gag protein fused with β-galactosidase at the C-terminus [8] was expressed in cells alone, VLP production was significantly reduced. These modified Gag fusion proteins could be incorporated into VLPs when co-expressed with authentic Gag proteins. [8,9]. In contrast, relatively short C-terminal extensions, such as the V3 loop of HIV-1 envelope protein (~100 bp long) [10] or green fluorescent protein (GFP; ~720 bp long) [11], on Gag proteins were tolerated for VLP formation.

It has been previously reported that several cellular proteins play important roles in transporting Gag protein to the plasma membrane and in budding from cells. For example, the MA region of Gag was shown to interact with AP-3 complex, which is involved in protein trafficking to certain cell compartments [12]. It has also been reported that Rab9, which mediates late endosome-to-*trans*-Golgi-network trafficking, plays an important role in viral assembly [13]. Moreover, Tsg101, which is localized in the multi-vesicular body (MVB), is involved in virus budding in a coordinated manner with the L domain, corresponding to the PTAP motif in the p6 region of HIV-1 Gag protein [14]. Proteasome inhibitors such as lactacystin or MG132 restricted virus budding from the cells [15], but they are not candidates for practical therapeutic use because the proteasome function is indispensable for cell survival. In addition, HP68, a cellular ATP binding protein, was essential for the assembly of immature HIV-1 capsids in vitro [16]. Furthermore, a peptide which bound to the C-terminal region of HIV-1 CA could inhibit the assembly of virion [17]. We speculate that other cellular factors may also be involved in the transport, assembly, and/or budding of HIV-1. We previously developed a yeast system which releases VLPs from cells when their cell walls were removed [5], and envisage that this yeast system could be applied to search for novel host factor(s) of retrovirus particle formation; however, commercially available ELISA kits to quantitate levels of mature CA protein in HIV-1 virion cannot detect precursor 55-kDa Gag protein, probably because the mature CA epitope used in this ELISA kit is absent or masked in the precursor 55-kDa Gag protein. We therefore developed a VLP system consisting of Gag–Firefly Luciferase fusion protein released from yeast for the rapid and convenient quantification of VLPs in culture media. The C-terminal extension to Gag protein in our system was approximately 1.65 kbp. In the expression system of vaccinia virus or baculovirus, it is not easy to completely remove infectious vector viruses from culture supernatant. In contrast, culture media of the budding yeast system do not contain any infectious vector viruses, because Gag proteins are expressed from episomal plasmids. Thus, this yeast system is safe to handle. We propose that this system would be useful to search for novel host factors involved in retrovirus budding by using the powerful genetics of budding yeast.

2. Materials and methods

2.1. Plasmid constructs

The entire *gag* coding sequence was amplified from an infectious proviral clone pNL-4-3 [18] by PCR with a primer pair of G5 (5′-GGCTAGAAGGAGAGCCATGGGTGCGAGAGC-3′) and G3 (5′-GCCGCTCACCAT*GGTACC*TTGTGACGAGGG-3′). The firefly luciferase gene was amplified from pGL3-basic (Promega Co., Madison, WI) by PCR using a primer pair of L5 (5′-CGG*GGTACC*ATGGAAGACGCCAAAAACATA-3′) and L3 (5′-CGGGGTACCTACCACATTTGTAGAGGTTTT-3′). Italicizing in G3 and L5 shows *Kpn*I restriction endonuclease recognition sites used for the ligation of *gag* and *luciferase* fragments. *gag* and a *firefly luciferase* fusion open reading frame were inserted into the poly-linker region of pGEM3Zf(+), resulting in pGEM3Zf(+)-NLgag-FL. In this construct, Gag and the Firefly Luciferase fusion protein were driven by the T7 promoter. A myristoylation signal mutant of the *gag* gene was generated using primer G5M (5′-TGCGGGATCCATGGCTG CGAGAGCGTCGG-3′) instead of G5, and the resultant plasmid was designated pGEM3Zf(+)-NLgagmyr(−)-FL. The wild type of the *gag–firefly luciferase* gene and its myristoylation signal mutant versions were also ligated to pRS425 [19] to be expressed in budding yeast.

2.2. Transfection and infection of mammalian cells

Human embryonic kidney 293T cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Cells were inoculated with vaccinia virus expressing T7 polymerase (VTF7-3) at 10 PFUs/cell [20] and then transfected with pGEM3Zf(+), pGEM3Zf(+)-NLgag-FL or pGEM3Zf(+)-NLgagmyr(−)-FL by using DMRIE-C (Invitrogen, Carlsbad, CA, USA). Transfected cells were cultivated in the presence of 40 ng/ml of cytosine arabinonucleoside (AraC; Sigma, St. Louis, MO, USA). At 48–72 h after transfection, the cells were harvested and assayed for the levels of intracellular Gag–Firefly Luciferase protein by Western blot using anti-HIV-1 CA monoclonal antibody (Advanced Biotechnologies Inc., Columbia, MD, USA) and horseradish peroxidase-conjugated anti-mouse IgG (H + L) (Vector Laboratories, Inc., Burlingame, CA, USA). Levels of bound antibody were measured using a chemiluminescence imager, LAS-1000plus (Fuji, Tokyo, Japan). The supernatant of transfected cells was clarified by centrifugation at $1600 \times g$ for 20 min at 4 °C. VLPs were pelleted by ultracentrifugation at 35,000 rpm for 1 h at 4 °C on a Beckmann SW41Ti rotor through a 20% (wt/vol) sucrose cushion. The pellet lysates were analyzed by Western blot probing with anti-CA monoclonal antibody as described above.

2.3. Culture and transformation of yeast

S. cerevisiae BY4743 (*MATa/αhis3D1/his3D1 leu2DO/leu2DO MET15/met15DO LYS2/lys2DO ura3DO/ura3DO*) cells were transformed using the one-step transformation method [21] with some modifications. Briefly, BY4743 cells

were patched on a YPD plate (1% yeast extract, 2% polypeptone, 2% glucose and 2% agar) and incubated for 16–24 h at 30 °C. A loopful of cells was suspended in 100 µl of one-step buffer [40% PEG (polyethylene glycol) 3350, 0.2 M LiAc, 0.1 M β-mercapto-ethanol, 0.5 mg/ml RNAs from Trula Yeast (Nacalai Tesque, Kyoto, Japan), and ~1 µg of plasmid DNA], and incubated at 45 °C for 1 h. Some of the transformants were spread on a plate containing 2% agar and leucine drop-out synthetic media (0.67% yeast nitrogen base without amino acids, 2% glucose, and amino acid mixtures without leucine) and incubated at 30 °C to select transformants with leucine. Lysates of yeast transformants were prepared using acid-washed glass beads (Sigma, St. Louis, MO, USA) and subjected to Western blot with the serum of an HIV-1 patient and horseradish peroxidase anti-human IgG (H + L) (Vector Laboratories, Inc., Burlingame, CA, USA). A spheroplast of the yeast transformant was prepared as described previously [5].

2.4. Purification of Gag–Firefly Luciferase VLP budding from yeast

Culture media of spheroplasts were clarified by centrifugation at 1650 × g for 20 min at 4 °C, and the supernatants were layered onto a 30% (wt/vol) sucrose cushion and ultracentrifuged in SW41 rotor (Beckmann Coulter) at 35,000 rpm for 1 h at 4 °C. The pellets were resuspended with 200 µl of PBS(−) and applied onto a 20–70% (W/V) linear sucrose density gradient centrifugation in SW55 rotor (Beckmann Coulter) at 45,000 rpm for 16 h at 4 °C. Ten fractions were collected from the bottom and subjected to Western blot analysis using serum from an HIV-1 patient. The luciferase activity of each fraction was measured by luminometer (DIAYATORON, Tokyo, Japan) using the Bright Glo Luciferase assay system (Promega Co., Madison, WI, USA). The refractive index of each fraction was measured using a hand-held refractometer R-5000 (Atago Co., Tokyo, Japan) and converted to relative density.

2.5. Precipitation of VLPs by PEG

Culture media of spheroplasts were centrifuged at 1650 × g for 20 min at 4 °C, and supernatants were mixed with PEG 10,000 to a final concentration of 2.5, 5 or 10% (W/W) and put on ice for 16 h. After centrifugation at 1294 × g for 45 min at 4 °C, supernatants were removed, and the precipitated pellets were suspended with 100 µl of 1× Glo Lysis Buffer. Each suspension was reacted with an equal volume of Bright Glo System for 2 min at 25 °C, and the luciferase activity of each sample was measured using a luminometer (FLUOstar Optima, BMG LABTECH, Offenburg, Germany).

3. Results

3.1. VLP budding of HIV-1 Gag–Firefly Luciferase from higher eukaryotes

We first investigated whether VLPs consisting of Gag–Firefly Luciferase could be produced from higher eukaryotes. For this purpose, the entire *gag* gene derived from HIV-1 molecular clone NL4-3 and the *firefly luciferase* gene were fused and ligated to the poly-linker region of pGEM3Zf (+), and consequently, the *gag–firefly luciferase* fusion gene was driven by T7 promoter (Fig. 1A). A substitution mutant at the myristoylation signal of the *gag* gene was also fused to *firefly luciferase* [myr(−)-FL] and used as a negative control for VLP budding, because this mutation caused budding defects from the cellular membrane [22]. After inoculation with Vaccinia virus vTF7-3 expressing T7 RNA polymerase [20] for 1 h, 293T cells were transfected with pGEM3Zf(+)-NLgag-FL, pGEM3Zf(+)-NLgagmyr(−)-FL or pGEM3Zf(+) using cationic lipid reagent DMRIE-C. Transfected cells were incubated for 2 days to allow the release of VLPs into the culture media in the presence of cytosine arabinonucleoside to inhibit the DNA synthesis of vaccinia virus. Cells were lysed for the detection of Firefly Luciferase activity using the Bright Glo System. Both cells transfected with either pGEM3Zf(+)-NLgag-FL or pGEM3Zf(+)-NLgagmyr(−)-FL expressed high levels of Luciferase activity

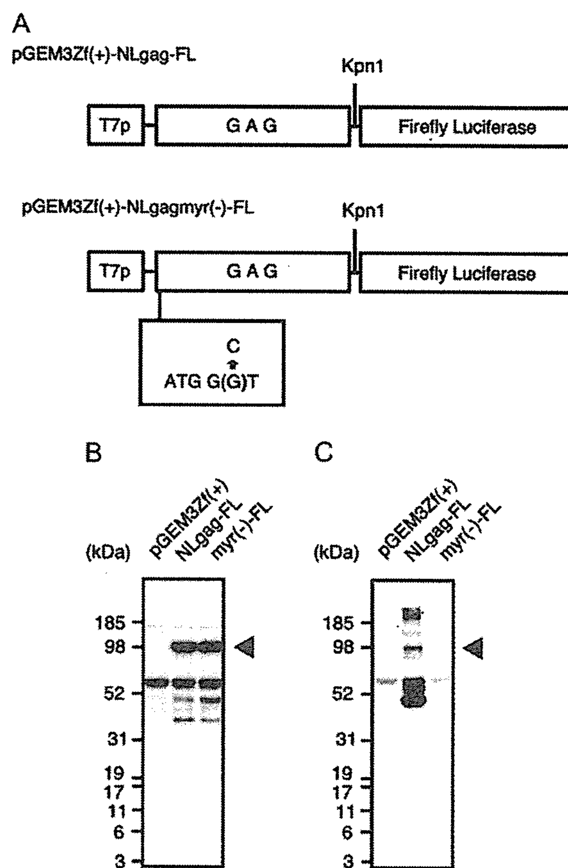

Fig. 1. Expression of Gag–Firefly Luciferase fusion protein in higher eukaryote cells. (A) Schematic representation of expression constructs for 293T cells. In the case of myristoylation signal mutants of Gag protein, the second amino acid glycine was substituted with alanine. (B) Western blotting of cell lysates of transfected cells. Twenty micrograms of each lysate was loaded on SDS-PAGE gel and probed with anti-CA mAb. (C) Western blotting of VLPs. Culture media pellets of transfected cells through a 20% sucrose cushion were probed with anti-CA mAb. An arrowhead indicates the positions of Gag–Firefly Luciferase fusion protein. Vector, WT, and myr(−) denote pGEM3Zf(+), pGEM3Zf(+)-NLgag-FL, and pGEM3Zf(+)-NLgagmyr(−)-FL, respectively.

[3×10^3–1.2×10^4-fold to pGEM3Zf(+)] (data not shown). Western blot analysis of the cell lysate using anti-CA monoclonal antibody (Fig. 1B) revealed the presence of approximately 100-kDa protein in cells transfected with pGEM3Zf(+)-NLgag-FL and pGEM3Zf(+)-NLgagmyr(−)-FL but not in cells transfected with empty vector pGEM3Zf(+). The size of this protein was in a good agreement with that of Gag–Firefly Luciferase.

We then examined whether VLPs consisting of Gag–Firefly Luciferase were produced from transfected cells. The culture media of transfected cells were collected and clarified by low-speed centrifugation 2 days after transfection. Ultracentrifugation was performed to remove soluble forms of Gag–Firefly Luciferase from the culture supernatants, and a sucrose cushion pellet was assayed for Gag–Firefly Luciferase fusion proteins by Western blotting probed with anti-CA monoclonal antibody. As shown in Fig. 1C, we could detect Gag–Firefly Luciferase fusion protein only from the culture supernatants of cells transfected with NLgag-FL, but not from those of cells transfected with NLgagmyr(−)-FL or empty vector. These results indicated that VLPs were released only from cells transfected with NLgag-FL, and no VLP was released from cells transfected with NLgagmyr(−)-FL. This result was consistent with the previous report on VLPs consisting of only Gag protein [22]. We also detected at least two discrete signals around 52 kDa with anti-CA monoclonal antibody only from the culture supernatants of NLgag-FL-transfected cells. Those are most likely the degraded products of Gag–Firefly Luciferase. Alternatively, cross-reactive cellular proteins (see Fig. 1B) may be incorporated into or co-purified with VLPs.

3.2. Expression of Gag–Firefly Luciferase protein in S. cerevisiae

We then constructed two expression vectors of *gag–firefly luciferase* in *S. cerevisiae*. The wild type and myristoylation signal mutant of *gag–firefly luciferase* fusion genes were driven under the control of TDH3 (glyceraldehyde-3-phosphate dehydrogenase) promoter derived from pKT10 [23] (Fig. 2A). These two constructs or parental plasmid, pRS425, were introduced into *S. cerevisiae* diploid strain BY4743 by the one-step transformation method. Cell lysates of the transformants were prepared with acid-washed glass beads with lysis buffer and then assayed for levels of luciferase activity. The luciferase activity of cells transformed with pRS425-NLgag-FL and pRS425–NLgagmyr(−)-FL was $5–6 \times 10^5$ times higher than that of transformants of empty vector pRS425 (data not shown). This result indicated that each transformant expressed an active form of luciferase. The cell lysates were then subjected to immunoblotting and probed with serum from an HIV-1 patient to identify whether intact Gag–Firefly Luciferase was expressed inside the cells. As shown in Fig. 2B, we could detect Gag–Firefly Luciferase fusion protein around the 98-kDa marker in cells with NLgag-FL and NLgagmyr(−)-FL. In order to compare the expression level of Gag–Firefly Luciferase fusion protein to Gag protein alone inside the cells, we also transformed budding yeast with

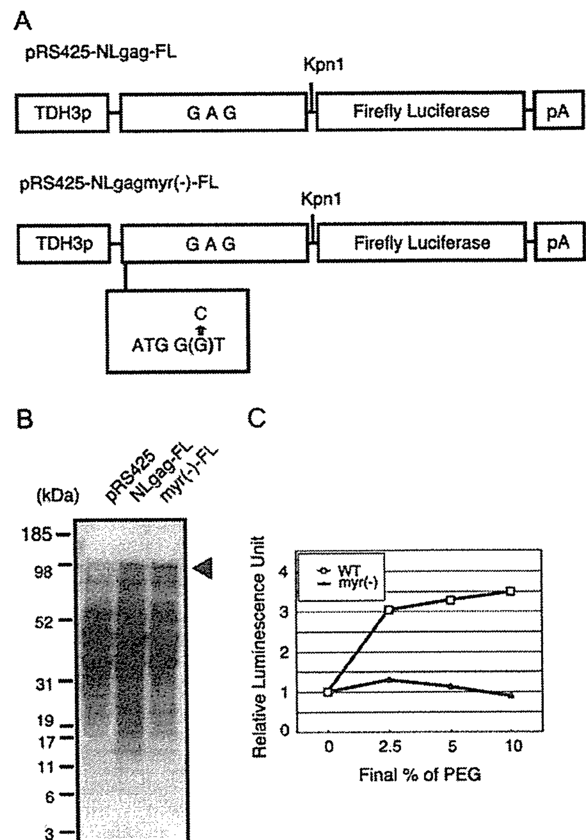

Fig. 2. Expression of Gag–Firefly Luciferase fusion protein in *S. cerevisiae*. (A) Schematic presentation of constructs for expression in *S. cerevisiae*. In the case of myristoylation signal mutants of Gag protein, the second amino acid glycine was substituted with alanine. (B) Western blotting of cell lysates of transformants. Forty micrograms of each lysate was loaded onto SDS-PAGE gel and probed with serum from an HIV-1 patient. The digitizing of band intensity corresponding to Gag–Firefly Luciferase fusion protein is shown below the blot. (C) Luciferase assay of PEG precipitants of VLPs in culture media. One millilitre of each culture medium of spheroplasts was mixed with PEG 10,000, and each precipitant was reacted with the Bright Glo System and measured with a luminometer. The value of each sample is divided by that of 0% PEG and shown as relative luminescence unit. Representative data of three independent experiments are shown. Vector, WT, and myr(−) denote pRS425, pRS425-NLgag-FL and pRS425-NLgagmyr(−)-FL, respectively.

pRS425-NLgag, an expression vector containing only *gag* open reading frame. Western blot analysis of cell lysate showed that expression of Gag protein alone was about 15-fold higher than Gag–Firefly Luciferase fusion protein (data not shown).

3.3. VLPs released from budding yeast

We then tried to purify VLPs carrying Firefly Luciferase from *S. cerevisiae*. Transformants of pRS425, pRS425-NLgag-FL, and pRS425-NLgagmyr(−)-FL were treated with Zymolyase-100T to remove their cell walls, and the resultant spheroplasts were cultured in YPD supplemented with 1 M sorbitol, used to optimize osmotic conditions for 16 h to allow the release of VLPs. The culture media were clarified by low-speed centrifugation, and VLPs in the supernatants were

precipitated with PEG 10,000. After low-speed centrifugation, pellets were assayed for luciferase activity levels using a luminometer. Raw values of luciferase activities of the pellets were divided by that of the pellets without PEG. As shown in Fig. 2C, we detected much higher luciferase activity of wild-type fusion protein in pellets with 2.5, 5, and 10% of PEG 10,000 than in those with 0% PEG 10,000. On the other hand, the level of luciferase activity of the myristoylation signal mutant in PEG 10,000 pellets was almost identical to that in 0% PEG 10,000. These results suggested that only the wild-type version of fusion protein could form VLPs in the culture media of spheroplasts, and that the majority of luciferase activity in the culture medium of the myristoylation signal mutant was a soluble form of fusion protein.

3.4. Properties of VLPs of Gag–Firefly Luciferase

We then measured the density of pelleted luciferase activity to identify whether pellets with luciferase activity really formed VLPs. The culture media of yeast spheroplasts were clarified by low-speed centrifugation and then ultracentrifuged through a 30% sucrose cushion to remove the soluble forms of Gag–Firefly Luciferase. The resultant pellets were resuspended with PBS and laid on the top of a 20–70% sucrose density gradient. After ultracentrifugation, 10 fractions were collected from the bottom of the tube and each fraction was assayed for VLP levels by Western blotting probed with serum from an HIV-1 patient. As shown in Fig. 3A (Western blotting) and Fig. 3B (quantification of Gag–Luciferase fusion protein), wild-type Gag–Firefly Luciferase fusion protein was distributed between densities of 1.2241 and 1.1764 (W/W), almost identical to that of VLPs composed of authentic Gag protein in budding yeast [5]. When we performed the same experiments on the myristoylation signal mutant or empty vector (Fig. 3B), no signal was detected on the immunoblotting membrane with serum from an HIV-1-positive patient (data not shown). We then measured the luciferase activity of each fraction of these three samples. The result showed that luciferase activity was detected only in fractions with Gag–Luciferase fusion proteins (Fig. 3C). These results clearly indicated that VLPs composed of Gag–Luciferase fusion proteins were successfully produced by spheroplasts of budding yeast.

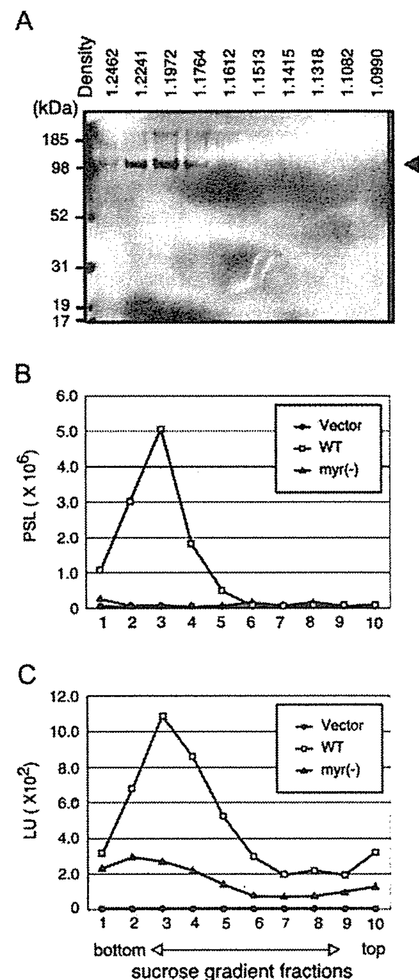

Fig. 3. Detection of VLPs released from spheroplasts of *S. cerevisiae.* (A) Western blotting of fractions of sucrose density gradient centrifugation with serum from an HIV-1 patient. (B) Digitizing of band intensity corresponding to Gag–Firefly Luciferase fusion protein on Western blotting. (C) Luciferase assay of each fraction of sucrose density gradients. Total luminescence counts detected in 10 s are shown as LU. Vector, WT, and myr(−) denote pRS425, pRS425-NLgag-FL and pRS425-NLgagmyr(−)-FL, respectively.

4. Discussion

Budding yeast was used in a model system of mammalian cells because it possesses approximately 6000 genes and the basic mechanisms of vital activity inside the cells are highly conserved from budding yeast to humans. The strong power of yeast genetics has unveiled the precise molecular mechanisms underlying several biological phenomena such as oncogenesis and signal transduction cascade [24]. Furthermore, budding yeast is easy and safe to handle.

We previously developed a budding yeast system releasing VLPs of HIV-1 Gag. We next wanted to apply this system to the exploration of cellular factor(s) involved in VLP budding. We initially used a commercially available ELISA kit which detects mature CA of HIV-1; however, this kit failed to detect precursor 55-kDa Gag proteins. It is possible, but would be expensive, to construct a new ELISA system by using the anti-Gag antibody which reacts to the precursor Gag protein. We then tried to produce VLPs composed of Gag–EGFP fusion protein; however, this was unsuccessful because of the autofluorescence of the culture medium. In this study, we developed a rapid and convenient method to quantitate HIV-1 VLPs by expressing Gag–Firefly Luciferase fusion protein in spheroplasts of *S. cerevisiae.*

In the culture supernatants of 293T cells and spheroplasts of budding yeast expressing wild-type Gag–Firefly Luciferase fusion protein, we could successfully detect Gag–Firefly Luciferase proteins which could be pelleted through a sucrose cushion by ultracentrifugation. When we used a myristoylation mutant, no Gag–Firefly Luciferase protein could be detected in the ultracentrifugation pellets. Furthermore, both Gag–Firefly Luciferase protein and luciferase activity were detected

in the density range of 1.2241–1.1764 mg/ml. These results clearly indicated that Gag protein could assemble into VLPs, although a 1.6 kbp-long *firefly luciferase* gene was fused at the 3′ end of the *gag* gene.

The expression levels of Gag–Firefly Luciferase fusion protein inside budding yeast was 15-fold lower than that of Gag protein expressed by a vector carrying *gag* open reading frame alone. This result suggested that Luciferase-tag might affect the expression in budding yeast. Nevertheless, the luciferase system has an apparent advantage in sensitivity. Furthermore, as shown in Fig. 2C, we successfully concentrated VLPs by PEG. The PEG sedimentation procedure could be performed by low-speed centrifugation and enabled us to use a high-throughput method using a 96-well deep-dish plate.

In Fig. 3B and C, the values for fraction 2 and 4 were inconsistent. This was most likely caused by a slight inhibitory effect of sucrose on luciferase activity, since the presence of 70% sucrose reduced luciferase activity to nearly 70% (data not shown).

There were weak luciferase activities in fractions 1–4 of myristoylation signal mutant. However, the peak of luciferase activity of myristoylation signal mutant was detected in the higher-density fraction than the peak of the wild type (Fig. 3C). So, it is possible that this myristoylation signal mutant of Gag–Firefly Luciferase fusion proteins with luciferase activity was an aggregate without lipid bilayer.

In summary, we showed that both higher eukaryotic cells and budding yeasts could release HIV-1 VLPs consisting of Gag–Firefly Luciferase fusion proteins. The property of VLPs consisting of Gag–Firefly Luciferase fusion protein examined here was consistent with the nature of the wild-type virion of HIV-1. Thus, we suggest that the VLP budding system described here can be applied in the search for novel host factors essential for the transport, assembly and/or budding of HIV-1 Gag protein. There are several precedents for such studies, in the viral RNA replication of Brome mosaic virus [25] or in the viral RNA recombination of Tomato Bushy Stunt Virus [26]. We also suggest that HIV VLPs produced by our system may be useful for screening inhibitors of HIV-1 virion budding.

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Topoisomerase I dissociates human immunodeficiency virus type 1 reverse transcriptase from genomic RNAs

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Abstract

Both HIV-1 reverse transcriptase (RT) and topoisomerase I bind to structural RNAs and they cooperate to synthesize cDNA during the replication of HIV-1. In this study, we find that human topoisomerase I exclusively dissociated HIV-1 reverse transcriptase, which strongly binds to structural RNAs. Meanwhile, topoisomerase I did not dissociate either HIV-1 nucleocapsid proteins or murine leukemia virus RT which was bound to RNA. We propose that human topoisomerase I may regulate the binding of RT to RNAs and play a pivotal role in HIV-1 replication.
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Keywords: HIV-1; Reverse transcriptase; Topoisomerase I; Dissociation; RNA

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is a multifunctional enzyme that has an RNA- and DNA-dependent polymerase activity for synthesis of the minus or plus strand DNA and ribonuclease H (RNase H) activity, which degrades RNA complementary to the newly synthesized DNA for plus strand DNA synthesis and second-strand primer formation [1]. The RT-associated RNase H activity causes cleavage of both double-stranded (ds) RNAs [2,3] and RNA templates of DNA–RNA hybrids, suggesting that RT has a high affinity for structured RNAs containing double-stranded regions with RNAs or DNAs.

The cDNA synthesis of HIV-1 at the post-entry steps is regulated either by the cellular factor(s) incorporated into HIV-1 virions from producer cells or by the factor present in target cells. Topoisomerase I is incorporated into retroviral particles from the producer cells during replication [4] and thus is a candidate for the cellular regulatory factor. HIV-1 [5,6] and Rous sarcoma virus RNAs [7] have been reported to be tightly bound with topoisomerase I. We reported that topoisomerase I bound to stem-loop RNAs and appeared to be incorporated into HIV-1 virions with structured gnomic RNAs [5,6], and also reported that topoisomerase I enhances HIV-1 cDNA synthesis in vitro and in vivo [8,9]. In addition, expression of topoisomerase I mutant lacking ligation activity in the producer cells remarkably reduced the infectivity of virions originating from the producer cells to target cells, and purified topoisoemerase I mutant inhibited viral cDNA synthesis [5]. Similarly as with RT, topoisomerase I can recognize structured genomic RNA and play important roles in cDNA synthesis.

Based on these findings we hypothesized that RT and topoisomerase I compete for the binding to structured RNAs. Here, we show that topoisomerase I dissociates RT from structured RNAs in a specific manner.

Materials and methods

Cloning and expression of His$_6$-tagged RT proteins and topoisomerase I. For expression of RT proteins, the primers 66F

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