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The Vif protein of human immunodeficiency virus type 1 is posttranslationally modified by ubiquitin☆,☆☆

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Abstract

The viral infectivity factor (Vif), one of the six HIV-1 auxiliary genes, is absolutely necessary for productive infection in primary CD4-positive T lymphocytes and macrophages. Vif overcomes the antiviral function of the host factor APOBEC3G. To better understand this mechanism, it is of interest to characterize cellular proteins that interact with Vif and may regulate its function. Here, we show that Vif binds to hNedd4 and AIP4, two HECT E3 ubiquitin ligases. WW domains present in those HECT enzymes contribute to the binding of Vif. Moreover, the region of Vif, which includes amino acids 20–128 and interacts with the hNedd4 WW domains, does not contain proline-rich stretches. Lastly, we show that Vif undergoes post-translational modifications by addition of ubiquitin both in cells overexpressing Vif and in cells expressing HIV-1 provirus. Vif is mainly mono-ubiquitinated, a modification known to address the Gag precursor to the virus budding site.

Keywords: HIV; Ubiquitin; Vif; HECT; Viral budding; Assembly; APOBEC3G/CEM15; Nedd4; AIP4

HIV-1 replicates preferentially in $CD4^+$ T lymphocytes and in macrophages. The virally encoded Vif protein is required to establish productive infection in primary cells [1]. The lack of a functional Vif protein results in the production of virus particles that are not infectious, since a defect in the reverse transcription step occurs in the next round of replication [2]. This phenotype results from the encapsidation of APOBEC3G, an antiviral host factor, within virus particles [3]. In the absence of Vif, APOBEC3G induces the deamination of cytosines incorporated in the minus single-strand of the viral DNA during the reverse transcription [4–7]. The HIV-1 Vif protein counteracts APOBEC3G by decreasing its packaging into virions [8,9]. Moreover, Vif decreases the intracellular level of APOPEC3G in the infected cells probably by several independent mechanisms [10]. One of them implicates the poly-ubiquitination of APOBEC3G induced by Vif and its degradation by the proteasome [8,9].

In order to determine how Vif limits APOBEC3G encapsidation into HIV-1 virion, it is necessary to determine which are the cellular and viral partners of Vif during the late stages of virus cycle. Vif was reported to interact with the Gag and Gag–Pol precursors [11,12] and with the viral genomic RNA [13]. In addition, besides APOBEC3G, other cellular factors interacting with Vif have been identified. These include the vimentin [14], Sp140 [15], and the tyrosine kinase Hck [16].

Since we have shown that Hck interacts with Vif through its SH3 domain [16] and because SH3 domains share convergent structural features with WW modules [17], we addressed the question of the possible recruitment of proteins containing WW domains by Vif.

In this work, we tested WW domains belonging to different classes [18] for their ability to bind to Vif. Our findings show that Vif of HIV-1 is recognized by the WW

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☆☆ *Abbreviations:* GST, glutathione *S*-transferase; AIP4, atrophin-1 interacting protein 4; HECT, homologous to E6-AP carboxyl terminus; Nedd4, neuronal precursor cell-expressed developmentally down-regulated 4.

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domains of Nedd4. Moreover, we confirmed the interaction with HECT E3 ubiquitin ligases, by co-immunoprecipitation of hNedd4-1 and AIP4. Since HECT ubiquitin ligases transfer ubiquitin to their ligands, we investigated ubiquitination of Vif. We demonstrated that Vif is ubiquitinated when expressed in vaccinia virus expression system and in cells expressing HIV-1 provirus.

Materials and methods

Cell culture transfection and infection. Human kidney 293T and epithelial HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), antibiotics (penicillin–streptomycin 100 μg/ml), and 2 mM glutamine and transfected with appropriate plasmids as previously reported [16]. Infection with T7 recombinant vaccinia virus followed by transfection was performed as described [16].

Plasmid constructions. The *vif* gene, derived from the pNL4.3 strain, was expressed as a GST fusion protein or under the control of the T7 promoter as previously described [11,16]. hNedd4-1 cDNA was a gift from T. Nagase and was subcloned into the pCDNA3.1 vector. HA-tagged AIP4 cDNA was a gift from J.R. Courbard [19] and GST-WW derivatives were a gift from M. Sudol. hNedd4-1 WW domains were mutated by PCR and cloned into the pGEX 2TK. The HA-tagged ubiquitin cDNA was a gift from D. Bohmann and was subcloned into the pOS7 expression vector.

GST pull down. GST fusion proteins were expressed in BL21 RIL (Stratagen) and purified on GSH beads as described [16]. The TNT coupled wheat germ extract system (Promega) was used to produce ^{35}S-labeled proteins. In vitro protein–protein interactions were performed as described in [16] except that lysis and binding were carried out in a 20 mM Hepes, pH 7.5, 150 mM NaCl, containing respectively 1% and 0.2% NP40 buffer in the presence of protease inhibitor cocktail (Roche).

Co-immunoprecipitation. Plasmids carrying the hNedd4-1, HA-tagged AIP4, and *vif* genes under the T7 promoter were transfected into HeLa cells using the vaccinia virus expression system. Twenty hours after transfection, cells were lysed in ice-cold lysis buffer (20 mM Hepes, pH 7.6, 120 mM NaCl, 0.5 % NP40, 1 mM EDTA, and 0.5 mM DTT) containing protease inhibitor cocktail (Roche) and 50 μM *N*-Ethylmaleimide (Sigma). Whole cell lysates were clarified and precleared on protein G–Sepharose before incubation with anti-Nedd4 antibodies or anti-HA Affinity Matrix (Roche). After several washes in lysis buffer containing 0.2% NP40, immunoprecipitates were boiled in sample buffer before separation of proteins on 12% SDS–PAGE.

Western blotting. Following SDS–PAGE, proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes (NEN). Blots were incubated with the following primary antibodies, mouse monoclonal antibody anti-GST Z-S sc-459 (Santa Cruz Biotechnology), rabbit polyclonal anti-Nedd4 (BD), mouse monoclonal anti-Vif (NIH, AIDS Research and Reference Reagent Program) [20], and rat polyclonal anti-HA (Boehringer–Mannheim). Bound primary antibodies were revealed by ECL+ technique (Amersham) following immunoreaction with appropriate secondary antibodies.

Results

Interaction of HIV-1 Vif with the WW domains of the Nedd4-1 ubiquitin ligase

WW domains displaying distinct ligand specificities were bacterially expressed as GST fusion proteins and immobilized on glutathione–agarose beads. For GST pull-down assays, each of the GST-derivatives was incubated with ^{35}S-labeled Vif and bound proteins were resolved by SDS–PAGE and revealed by autoradiography. As shown in Fig. 1A, Vif bound mainly to the WW2 and WW3 domains of hNedd4-1, but not to the WW4 domain or to its mouse orthologue WW3. The binding of Vif to the WW1 domain of hNedd4-1 was

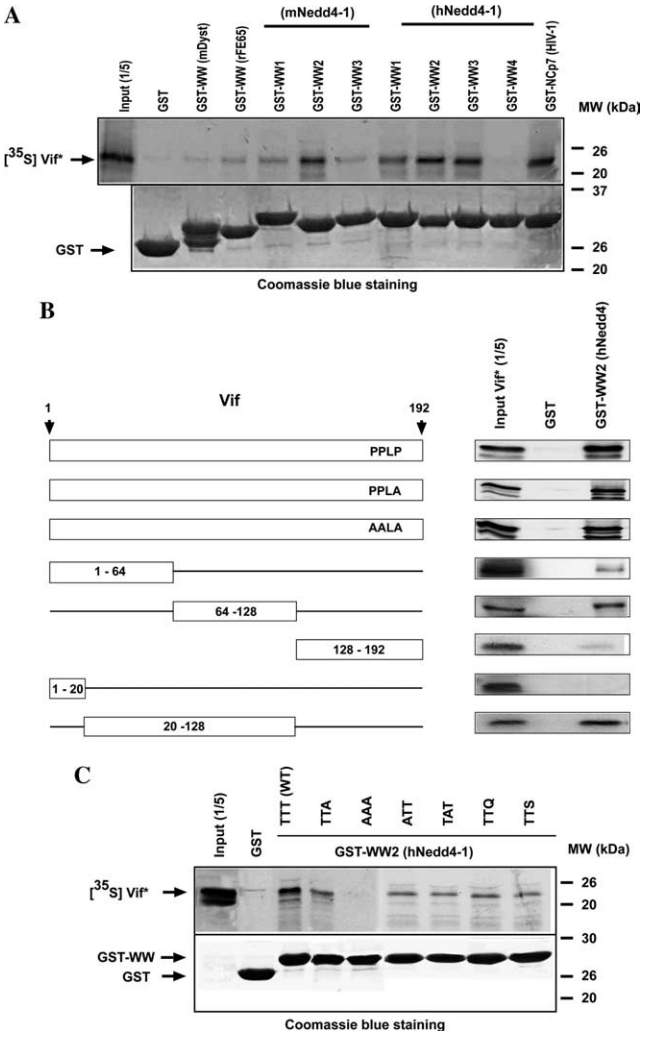

Fig. 1. HIV-1 Vif protein interacts with WW domains of hNedd4-1. (A) GST pull-down analysis was performed upon incubation of 5 μg bacterially expressed GST-WW fusion protein with ^{35}S-labeled Vif protein obtained by in vitro translation. After several washes in Hepes buffer, the Vif protein bound to the GST fusion protein was separated by SDS–PAGE and revealed by autoradiography. Coomassie blue staining of GST derivatives was showed in the bottom panel. (B) Interaction between in vitro translated ^{35}S-labeled Vif containing point mutations in the PPLP motif or containing C- and N-terminus deletions upon incubation with GST-WW2 of hNedd4-1 or GST. Bound proteins were revealed by autoradiography. (C) The TTT motif upstream the second tryptophan of WW2 domain of hNedd4-1 protein was mutated by PCR before GST pull-down assay was performed with ^{35}S-labeled Vif. Bound proteins were revealed by autoradiography. Coomassie blue staining of GST-derivatives is shown in the bottom.

also weakly detectable. No significant interaction of Vif with the WW domains of FE65 and dystrophin was detected. As controls, we showed that Vif bound to the nucleocapsid protein of HIV-1 NCp7 in fusion with GST, but not to GST. Similar amounts of GST-derivatives were used as judged by Coomassie blue staining of the gel. These data indicate that hNedd4 may interact with Vif through its WW domains.

Regions of Vif involved in the binding to hNedd4-1 WW domains

To determine whether the highly conserved PPLP motif of Vif, located in the C-terminal portion, contributes to the binding to the WW domains of hNedd4-1, we performed GST pull-down assays with ^{35}S-labeled Vif that contained mutations in the PPLP motif, and the different GST-WW of hNedd4-1 protein. Results, as illustrated for the GST-WW2 domain in Fig. 1B, indicate that mutations in the PPLP motif of Vif did not alter its interaction with the hNedd4-1 WW2 domain. Similar results were obtained for WW3 of hNedd4-1 (not shown), indicating that the PPLP motif of Vif is not necessary for this interaction. This observation was confirmed by performing the reverse binding assay in which the GST–Vif mutated in the PPLP motif was incubated with the cell lysate of HeLa cells transfected with Nedd4-1 construct (supplementary data S1). All these data allowed us to conclude that Vif interacts with hNedd4-1 in a PPLP-independent manner.

Since no other proline-rich motif was identified within the primary sequence of Vif, we sought for the Vif region implicated in this interaction by analyzing N- and C-terminus deletion mutants of Vif for their binding abilities with WW domains of hNedd4-1. Each of the truncated Vif proteins was tested for its ability to bind GST derivatives. GST and GST-WW4 (not shown) failed to pull down the different Vif fragments, indicating the specificity of the interactions. Fig. 1C shows that the central third (64–128) of Vif interacted much stronger than its N-terminal third (1–64) with GST-WW2 domain of hNedd4-1. In contrast, the C-terminal third (128–192) failed to bind to the WW2 domain. Vif deleted from 20 residues at the N-terminus did neither interact with the GST-WW2. Altogether, these data indicate that the core domain of Vif (20–128), but not the C-terminal domain containing the PPLP motif, is important for the binding to hNedd4-1.

Mapping of the epitope of WW domain of hNedd4-1 involved in the Vif binding

The WW2 and WW3 domains of hNedd4-1 that preferentially interact with Vif displayed in their primary sequences a conserved TTT motif upstream the C-terminus tryptophan of the WW domain suggesting a possible involvement of this motif in the binding of Vif. We thus tested, in GST pull-down assays, the interaction of the GST-WW2 hNedd4-1 proteins containing mutations within this motif with the ^{35}S-labeled Vif. Results from Fig. 1C indicate that the substitution of the three threonines into alanines in the TTT motif of WW2 abolished the interaction with Vif. In contrast, single mutations decreased, but did not abrogate, the Vif binding. These data suggest that the threonines present upstream from C-terminus tryptophan of the WW may facilitate the interaction with Vif. hNedd4-1 belongs to a large family of ubiquitin ligases that have in common the presence of an HECT catalytic site necessary for the ubiquitination of proteins targeted by their WW domains. Other HECT ubiquitin ligases as AIP4/ITCH and BUL1/NEDL2 display WW domains containing TTT motif. This observation suggests that HECT proteins, other than hNedd4-1, could also interact with Vif.

Binding of Vif to full-length hNedd4 and AIP4 HECT E3 ubiquitin ligases

We focused our study on two HECT ubiquitin ligases expressed in T cells: Nedd4-1 and AIP4 (data not shown). To determine their interaction with Vif, we performed in vitro interaction assays. First, we produced ^{35}S-labeled hNedd4-1 and AIP4 proteins by in vitro translation. Full-length proteins were incubated with bacterially expressed GST or GST–Vif bound on GSH–agarose beads. GST pull-down analysis revealed by autoradiography indicated that GST–Vif interacts directly with hNedd4-1 and AIP4 (Fig. 2A). No interaction occurs between GST and hNedd4 or AIP4, which demonstrates the specificity of these interactions. We next confirmed these interactions by performing GST pull-down assays with hNedd4-1 or HA-tagged AIP4 proteins expressed by transfection in 293T cells (Fig. 2B).

To examine whether untagged Vif protein physically interacts with hNedd4-1 and AIP4 in eukaryotic cells, we performed co-immunoprecipitations. For this purpose, Vif was co-expressed with hNedd4-1 or HA-tagged AIP4 in HeLa cells. The whole cell lysates were, respectively, immunoprecipitated with anti-Nedd4-1 polyclonal antibody or affinity bound immunoglobulins raised against HA epitope, followed by immunoblotting with the appropriate polyclonal antibodies. Western blots performed on 1/25 of the cell extract indicated that equal amounts of each HECT ligase were expressed in the presence or in the absence of Vif. Fig. 2C shows that overexpressed hNedd4-1 is efficiently immunoprecipitated. We also precipitated a low amount of endogenous hNedd4-1. Anti-Vif immunoblot revealed a band corresponding to Vif protein when hNedd4-1 and Vif were co-expressed. Moreover, overexposed blots revealed that Vif co-precipitates also with endogenous hNedd4-1

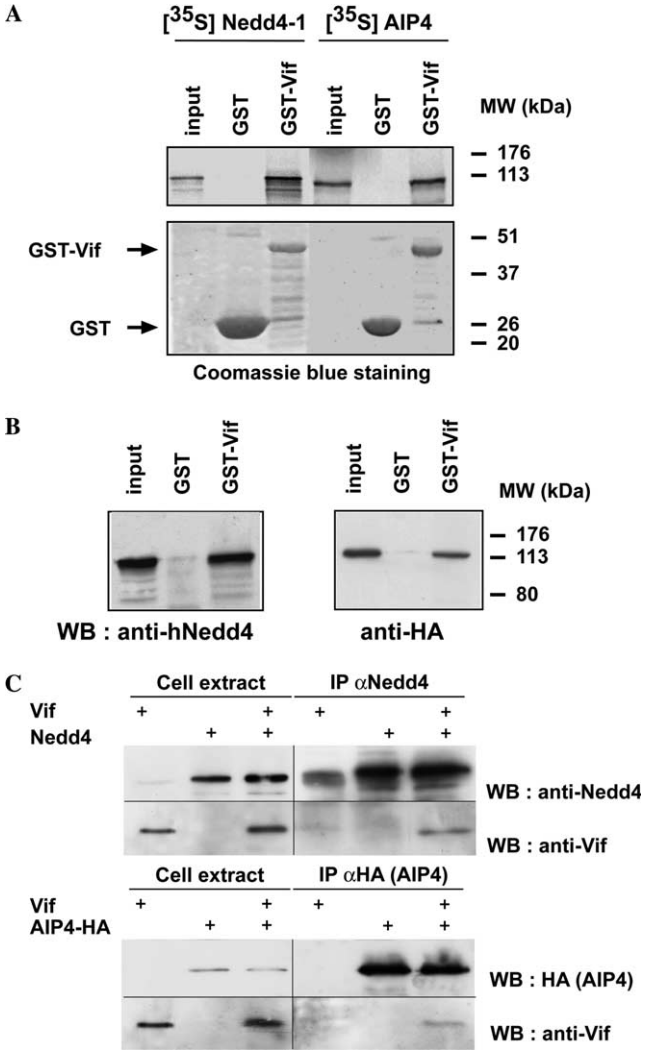

Fig. 2. Vif binds to full length hNedd4-1 and AIP4. (A) Both hNedd4-1 and AIP4-HA were in vitro translated in the presence of [^{35}S]methionine. The radiolabeled proteins interacting with GST or GST–Vif were pulled down, separated by SDS–PAGE, and revealed by autoradiography (upper). GST and GST–Vif fusion proteins were controlled by Coomassie blue staining (bottom). (B) 293T cells were transfected by pcDNA3.1 carrying hNedd4-1 and AIP4-HA cDNAs. Forty-eight hours after transfection, cells were lysed before pull-down assay with 10 μg GST or GST–Vif. hNedd4-1 and AIP4-HA interacting with GST–Vif were separated by SDS–PAGE and revealed by Western blot analysis using appropriate antibodies. (C) Human HeLa cells infected by vaccinia virus expressing the T7 polymerase were transfected with Vif, hNedd4-1, and AIP4-HA expression plasmid. Twenty-four hours post-transfection, cells were lysed in Hepes buffer containing 0.5% NP40. Clarified cell lysates were immunoprecipitated by anti-Nedd4 antibodies or anti-HA affinity matrix. The immunoprecipitated proteins were separated on SDS–PAGE and analyzed by Western blot using appropriate antibodies.

(data not shown). Similarly, we also observed that Vif co-precipitates with AIP4 in a lower extent compared to the co-immunoprecipitation obtained with hNedd4-1. These data confirm that, in human cells, Vif interacts with HECT E3 ubiquitin ligases as hNedd4-1 and AIP4.

Ubiquitination of HIV-1 Vif

The interaction of Vif with members of the HECT ubiquitin ligase family suggests that Vif may be an ubiquitinated protein. To test this hypothesis, human cells were cotransfected with plasmids expressing HA-tagged ubiquitin and GST or GST–Vif under the control of the T7 polymerase promoter, following infection by T7 recombinant vaccinia virus. Cell lysates were incubated with GSH–agarose beads to affinity purified GST or GST–Vif. Western blotting with anti-GST controlled the expression of GST and GST–Vif fusion protein (Fig. 3A). Ubiquitination of Vif was revealed by Western blotting with anti-Vif and anti-HA antibodies. Anti-Vif antibody Western blot revealed that co-expression of HA-tagged ubiquitin and GST–Vif led to the appearance of two additional species of GST–Vif proteins. According to their apparent molecular weight, these two forms correspond to ubiquitin-modified Vif proteins containing one or two ubiquitin molecules. We noted that, the additional GST–Vif species were also revealed with anti-HA antibody. No ubiquitination of the GST protein was observed, confirming the specificity of the assay. These data indicate that Vif expressed as a fusion with GST is ubiquitinated in cells. To exclude that the fusion of Vif with GST can promote abnormal levels of ubiquitination of Vif, we performed similar experiments but with cellular extracts overexpressing the Vif protein under a non-fused form and HA-tagged ubiquitin. As shown in Fig. 3B, different polypeptides immunoreacting with an anti-Vif antibody were observed by Western blot. These different proteins were not detected in the absence of overexpressed HA-tagged ubiquitin, indicating that they indeed correspond to ubiquitin-modified forms of Vif. According to the apparent molecular weight of the different polypeptides, it can be concluded that Vif is expressed as mono- or poly-ubiquitinated forms, confirming the data obtained with GST–Vif.

To ensure that Vif, expressed in the context of HIV-1 infected cells, was indeed ubiquitinated, human cells were co-transfected with wild-type or Vif-deficient HIV-1 molecular clones together with HA-tagged ubiquitin expressing plasmid. Cell lysates from transfected cells were analyzed by Western blotting with anti-Vif antibody (Fig. 3C). An unmodified form of Vif with an apparent molecular weight of 23 kDa was detected in wild type HIV-1 infected cells. An additional form of Vif with an apparent molecular weight of 31 kDa was detected only when HA-tagged ubiquitin was co-expressed. These molecular species were not detected in cell lysates transfected with Vif-deficient HIV-1 virus. These data indicate that the Vif protein, through interactions with the cellular ubiquitin machinery, undergoes mainly mono-ubiquitination.

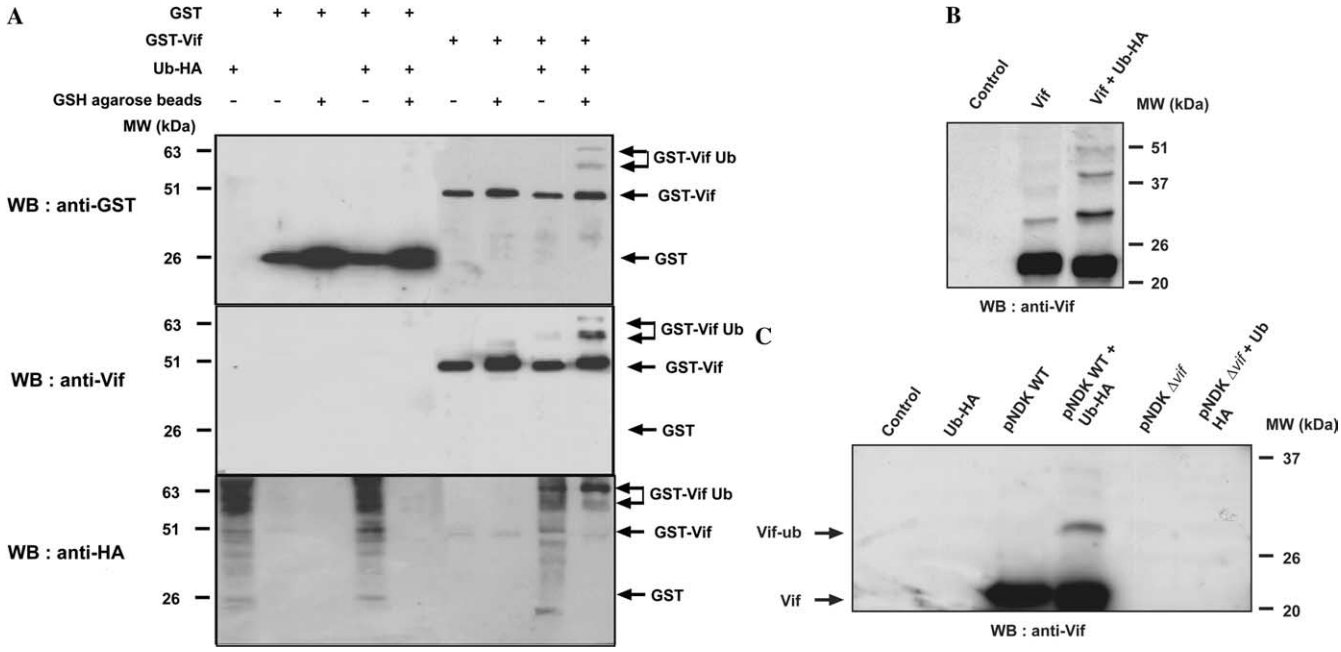

Fig. 3. GST–Vif is ubiquitinated both in cellular overexpression system and in HIV-1 transfected cells. (A) Human HeLa cells infected by vaccinia virus expressing the T7 polymerase were transfected with pOS7–ubiquitin–HA and pOS7-GST–Vif or pOS7-GST. Twenty-four hours post-infection, cell extracts expressing GST or GST–Vif in the presence or not of HA-tagged ubiquitin were analyzed by Western blot using antibodies against Vif and HA epitope. The cell lysates were analyzed directly (5%) or upon GST pull down (95%). (B) Human HeLa cells infected by vaccinia virus expressing the T7 polymerase were transfected with pOS7, pOS7–ubiquitin–HA, and pOS7-Vif. Twenty-four hours post-infection, cell extracts were analyzed by Western blot with anti-Vif antibody. (C) Human 293T cells were transfected with WT or Vif-deficient HIV molecular clones in the presence or not of HA-tagged ubiquitin expressing vector. Cell lysates were analyzed by Western blot with anti-Vif monoclonal antibody.

Discussion

In this study, we reported the interaction of HIV-1 Vif with proteins belonging to the HECT E3 ubiquitin ligase family, namely with hNedd4-1 and AIP4. In addition, we found that Vif could be recovered under a ubiquitin-modified form in the cells that express HIV-1.

The Vif protein was shown to interact with different WW domains of hNedd4-1 including the WW2 and WW3 and in a lower extent with the WW1 domain. Similarly WW domains of AIP4 also interact with Vif (M. Courcoul, personal communication). Mutagenesis of the threonines upstream from C-terminus tryptophan of the WW2 domain of hNedd4-1 limits the Vif binding. We observed that this motif is present in other WW domains shared by several HECT E3 ubiquitin ligases, suggesting that Vif could recognize different members of this multigenic family. Accordingly, we showed that full-length AIP4 interacted with GST–Vif. Co-immunoprecipitations confirmed the intracellular interaction between Vif and hNedd4-1 and in a lower extent with AIP4.

The interaction between Vif and hNedd4-1 did not involve the proline-rich domain of Vif, but the core region of Vif (20–128). This feature is consistent with a previous study showing that the interaction of hNedd4-1 with some ligands can occur independently of a proline-rich domain [21], and, in some cases, may depend on the presence of phosphorylated serine and/or threonine residues [22]. Since Vif has been reported to be phosphorylated on serine and threonine [23,24], it will be important to determine if the phosphorylation status of Vif may influence the hNedd4 recruitment.

In addition to the interaction of Vif with the HECT ubiquitin ligases presently described, Vif was also reported to interact with a ring finger E3 ubiquitin complex containing Elongin B and C, Cullin 5, and Rbx1 [25]. Since all these E3 ubiquitin ligases are expressed in T lymphocytes, we addressed the question of Vif ubiquitination.

We demonstrated that GST–Vif and Vif expressed by the vaccinia virus expression system undergo post-translational modifications by addition of ubiquitin moieties with a majority of mono-ubiquitinated forms. Similar modifications were observed in HIV-1 transfected cells. Concomitant with this work, Vif expressed by transfection was also reported to be mono-ubiquitinated [26]. This modification is insensitive to proteasome inhibitors and does not target Vif to the degradation pathway, suggesting that it may affect its subcellular localization, its structure, or its activity. In both studies, the mono-ubiquitination of Vif concerned only a low proportion of the expressed Vif molecules, like it was previously observed for the HIV-1 Gag

precursor polypeptide [27]. Mono-ubiquitination is a necessary transient modification for sorting cargo proteins into the multivesicular bodies (MVB), where ubiquitin is removed by de-ubiquitinating enzymes [28]. Interestingly, retroviruses bud from infected cells by appropriating this ubiquitin dependent MVB machinery [29].

The role of the ubiquitination of Vif in the HIV-1 life cycle is still an open question. Vif was reported to be colocalized with intracellular complexes and a few molecules of Vif are incorporated into the viral particles [30]. Accordingly, it is possible that the mono-ubiquitination of the Vif protein facilitates its addressing to the virus budding site, enriched in Gag polypeptides. At this site, Vif may efficiently protect HIV-1 virions from the encapsidation of the antiviral factor APOBEC3G. Indeed, recent reports indicate that Vif interacts with APOBEC3G in HIV-1 infected cells and reduces its expression level [31] by a proteasome pathway. The poly-ubiquitination of APOBEC3G [9] requires the interaction of Vif with the Cul5 ring finger E3 ligase complex [25]. Since a ring finger E3 ligase (Cblc) and an HECT E3 ligase (AIP4) cooperate for the EGFR down-regulation mediated by ubiquitination [19], it is also possible that hNedd4 or AIP4 might cooperate with the Cul5 ring finger E3 ligase complex in the presence of Vif. Further work will be needed to address this possibility, which might provide promising information to develop drugs restoring APOBEC3G antiviral activity.

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