Ubiquitination of APOBEC3G by an HIV-1 Vif-Cullin5-Elongin B-Elongin C Complex Is Essential for Vif Function*

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The human immunodeficiency virus type 1 (HIV-1) virion infectivity factor (Vif) overcomes the antiviral activity of APOBEC3G to protect HIV-1 DNA from G-to-A hypermutation. Vif targets APOBEC3G for ubiquitination and proteasomal degradation by forming an SCF-like E3 ubiquitin ligase complex composed of Cullin5, Elongin B, and Elongin C (Vif-BC-Cul5) through a novel SOCS-box motif. In this paper, we have established an in vitro ubiquitination assay with purified Vif-BC-Cul5 complex and reported that the Vif-BC-Cul5 complex could function as an E3 ligase for APOBEC3G in vitro. A Vif-BC-Cul5 complex promotes the in vitro ubiquitination of the wild type, APOBEC3G, but not that of D128K mutant, which does not interact with Vif. We have also investigated several loss-of-function Vif mutants. One mutant, SLQ144/146AAA, lost its activity on APOBEC3G because it could not form a complex due to mutations in SOCS-box motif. Other mutants, C114S and C133S, also lost their activity because of loss of the E3 ligase activity of a Vif-BC-Cul5 complex, although these mutants retained the ability to bind to APOBEC3G as well as Cul5 complex. These findings suggest that the E3 ubiquitin ligase activity of the Vif-BC-Cul5 complex is essential for Vif function against APOBEC3G.

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HIV-1 Vif protein has been shown to play a crucial role during the viral life cycle by regulating the virion infectivity (1) and in vivo pathogenesis (2), which was achieved by overcoming the effect of a host antiretroviral factor, APOBEC3G (3). APOBEC3G is a member of the APOBEC superfamily of cytidine deaminases (4) that is incorporated into HIV-1 virions by an interaction with HIV-1 gag nucleocapsid protein (5) and regulates HIV-1 infectivity by deaminating cytidine to uracil in newly synthesized minus-strand viral DNA, inducing G-to-A hypermutation in the plus-strand viral DNA (6–9). Vif, which is a 24-kDa cytoplasmic protein, binds directly to APOBEC3G. Vif has conserved SLQ residues that share similarity with the SOCS-box motif of SOCS6 (10), and several recent studies have revealed that Vif interacts with cellular proteins, Cullin5 (Cul5), Elongin B (EloB), Elongin C (EloC), and Rbx1 through a novel SOCS-box motif to form a Vif-BC-Cul5 complex, which is a ubiquitin ligase (E3)-like complex (11–13).

The ubiquitin-mediated protein degradation system has been shown to be involved in a wide variety of cellular functions, including cell cycle progression and signal transduction (14). Ubiquitination is a multistep process of a three-enzyme cascade involving the ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) enzymes (15). The E3 binds both the protein target and a cognate E2 to mediate the transfer of ubiquitin from the E2 to the substrate protein and plays a pivotal role in substrate recognition and conferring specificity to the ubiquitination pathway. Since a Vif-BC-Cul5 complex is an E3-like complex, it has suggested that Vif functions as a substrate recognition subunit of the Vif-BC-Cul5 complex and targets APOBEC3G for ubiquitination and subsequent degradation by the proteasome, resulting in inhibition of the incorporation of APOBEC3G into HIV-1 virions and protection of the viral DNA from mutation (10, 16, 17). However, there has been no direct evidence on the ubiquitination of APOBEC3G by a Vif-BC-Cul5 complex. Moreover, there still remain some controversies between the Vif-BC-Cul5 complex formation and/or substrate recognition by Vif and its activity to antagonize APOBEC3G. Here, we have first shown that a Vif-BC-Cul5 complex could indeed work as an E3 ligase complex by an in vitro ubiquitination assay using purified Vif-BC-Cul5 complex. By using the assay, we have shown that a Vif-BC-Cul5 complex induces the ubiquitination of the wild type (WT) APOBEC3G, but not that of D128K APOBEC3G, which does not interact with HIV-1 Vif (18, 19). Moreover, we have also shown the clear relationship between the ability of Vif to overcome APOBEC3G and the ubiquitination of APOBEC3G by the Vif-BC-Cul5 complex using several Vif mutants.

MATERIALS AND METHODS

Expression Vectors and Molecular Clones—The wild type (WT) Vif and mutants (C114S, C133S, and SLQ144/146AAA) were expressed by pgVif, M18, M19, and M29 vectors, respectively (kind gifts from Dr. M. Malim) (20). Expression vector for hemagglutinin (HA)-tagged human APOBEC3G, pcDNA3/HA-huAPOBEC3G, was constructed as described previously (21). APOBEC3G D128K mutant (19, 22) was generated by PCR method and cloned into pcDNA3/hygro/HA vector. pNL43-Luc and pNL43/Δvif-Luc were constructed as described previously (8).

Cell Lines—HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% FCS and penicillin, strep-
His-HA-APOBEC3G protein was also obtained by the same method. Cells were purified by TALON metal affinity resin (Clontech). Recombinant viruses. Sixty-four hours after infection, cells were harvested and the supernatants were collected after 48 h of transfection, and virus titers were measured with an enzyme-linked immunosorbent assay kit for the p24 antigen (RETRO-TEK, Zoetis-Metrix Corp., Buffalo, NY). An adjusted amount of viruses was challenged to target cells, M8166. Twenty-six hours postinfection, the cells were lysed in passive lysis buffer (Promega, Madison, WI), and the luciferase activity was measured with a Luminometer (EG & G Berthold, Bad Wildbad, Germany). Values were presented as percent infectivity relative to the value of wild type virus without expression of APOBEC3G.

Co-immunoprecipitation Assay—To see protein-protein interaction in vivo, we performed an immunoprecipitation assay as described previously (8). Expression vectors for HA-APOBEC3G WT or D128K were cotransfected with expression vectors for Vif WT or its mutants into HEK293T cells by calcium phosphate method. Two days after transfection, cells were lysed in RIPA buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.2% Triton X-100, 0.1% SDS, 0.1% deoxycholate). Complexes were immunoprecipitated with 1 μg of anti-HA monoclonal antibody (mAb) (12CA5) (F. Hoffmann-La Roche Ltd.) for 1 h, followed by addition of 20 μl of protein A-Sepharose™ beads (Amersham Biosciences) for 1 h at 4°C. The beads were washed with RIPA buffer three times and analyzed on immunoblot with anti-HA mAb or rabbit antiserum against Vif (a kind gift from Dr. D. Gabuzda through the AIDS Research and Reference Reagent Program) (23). HA-APOBEC3G and Vif were visualized by ECL detection system (Amersham Biosciences).

Recombinant baculoviruses for Vif WT or its mutants were co-infected Hi Five cells with Cul5, EloB, EloC, and Rbx1 expressing recombinant viruses. Sixty-four hours after infection, cells were harvested and lysed in lysing buffer (0.5% Triton X-100, 25 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 10% glycerol, 2 mM DTT, 1 mM PMSF, 1× protease inhibitor (Nacalai Tesque, Inc., Kyoto, Japan)). Complexes were immunoprecipitated with 1 μg of anti-Vif mAb (319) (a kind gift from Dr. M. Malim through the AIDS Research and Reference Reagent Program) (24) for 1 h, followed by addition of 20 μl of protein G-Sepharose™ beads (Amersham Biosciences) for 1 h at 4°C. The beads were washed with lysing buffer three times and analyzed on immunoblot with anti-c-myc mAb (9E10) (Sigma-Aldrich Corp.) for detection of c-myc-Cul5 or rabbit anti-Vif serum.

Expression of Recombinant Proteins in HEK293T Cells—HEK293T cells were transfected with pcDNA3/HA-WT or D128K APOBEC3G by calcium phosphate method. Two days after transfection, cells were lysed in lysing buffer. Proteins were immunoprecipitated with 1 μg of anti-HA mAb (12CA5) for 1 h, followed by addition of 20 μg of protein G-Sepharose™ beads for 4 h at 4°C. The beads were washed with lysing buffer three times and washing buffer (20 mM Tris-Cl, 10 mM DTT) twice.

Expression of Recombinant Proteins in Insect Cells—N-terminal-(His)6-tagged WT and mutants Vif were cloned into pVL1393 vectors (Invitrogen). Recombinant baculovirus was generated by using BacPAK6 baculovirus expression system (Clontech). N-terminal c-myc-tagged Cul5, C-terminal-HPCL4-tagged EloB, C-terminal herpes simplex virus-tagged EloC, and N-terminal T7-tagged human Rbx1 were described previously (14). Hi Five cells were cultured in Grace’s insect medium (Invitrogen) supplemented with 10% FCS at 27°C and co-infected with Vif WT or mutants, Cul5, EloB, EloC, and Rbx1 recombinant viruses. Sixty-four hours after infection, cells were harvested and lysed in insect cell lysing buffer (2% Triton X-100, 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 10% glycerol, 2 mM DTT, 1 mM PMSF). Vif proteins were purified by TALON metal affinity resin (Clontech). Recombinant His-HA-APOBEC3G protein was also obtained by the same method. In Vivo Ubiquitination Conjugation Assay—N-terminal-(His)6-tagged NEDD8, Ubc12 (E2 for NEDD8) and APP-BP/Ubα3 (E1 for NEDD8) were described previously (25). The plasmid encoding glutathione S-transferase (GST)-ubiquitin was kindly provided by Dr. Peter Howley (Harvard Medical School), and GST-ubiquitin was expressed and purified in our laboratory. Vif WT or mutant complexes were incubated with 4 μl of packed and washed antibody-conjugated beads containing HA-WT or D128K APOBEC3G in reaction buffer (20 mM Tris-Cl, pH 7.5, 5 mM MgCl2, 2 mM DTT, 100 ng of E1, 75 mM E2, 1.2 μg of NEDD8, 160 ng of Ubc12, 50 ng of APP-BP/Ubα3, 2.5 μg of GST-ubiquitin) in the presence of ATP and ATP regeneration system (1 mM ATP; 0.5 mM creatine phosphate, 5 μg of creatine phosphokinase) at 37°C for 1 h. Reactions were stopped by adding 4× SDS sample buffer. After being boiled for 10 min, samples were subjected to Western blot to detect GST-ubiquitin conjugated HA-APOBEC3G. We also performed this assay using recombinant His-HA-APOBEC3G protein purified from insect cells.

RESULTS

APOBEC3G D128K Mutant Is Resistant to HIV-1 Vif—Several groups (18, 19, 26) have reported that the substitution of Asp-128 in human APOBEC3G for Lys (D128K) resulted in the resistance to HIV-1 Vif. Although it is suggested to be attributed to the inability of Vif to bind to APOBEC3G D128K mutant, some groups demonstrate the results against the current notion (26). To confirm these findings, we first tested the antiviral activity of WT and D128K APOBEC3G as well as their functional interaction with HIV-1 Vif. As shown in Fig.
1A, expression of APOBEC3G D128K suppressed the infectivity of wild type as well as ΔVif virions to the same extent, while the antiviral activity of WT APOBEC3G was inhibited by Vif. The amount of WT APOBEC3G protein incorporated into virions as well as in producer cells in the presence of HIV-1 Vif was much lower than that of Δ128K APOBEC3G (Fig. 1B), indicating that HIV-1 Vif prevented virion incorporation of WT APOBEC3G by effectively reducing its intracellular level of the protein in producer cells but not that of Δ128K. These results confirmed the previous reports that APOBEC3G D128K is resistant to HIV-1 Vif. Moreover, HIV-1 Vif was co-immunoprecipitated with APOBEC3G WT, but not Δ128K (Fig. 1C), suggesting that Vif could not down-regulate the expression of Δ128K because Vif cannot bind to the mutant.

Vif C114S, C133S, and SLQ144/146AAA Mutants Could Bind to APOBEC3G but Failed to Exclude APOBEC3G from Virions—Simon et al. (20) reported that introduction of several Vif mutants such as C114S, C133S, and SLQ144/146AAA into ΔVif virions could not restore the infectivity of the virions produced from non-permissive cells. We then tested the function of these mutants in the context of inhibition of the antiviral activity of APOBEC3G. Vif WT could overcome the antiviral activity of APOBEC3G on ΔVif virions, but Vif mutants did not (Fig. 2A). Inability of the Vif mutants to prevent virion incorporation of APOBEC3G was attributed to their defect to down-regulate its expression in producer cells because WT Vif could reduce the amount of APOBEC3G in both virion and producer cells (Fig. 2B). Co-immunoprecipitation assays revealed that APOBEC3G could interact with all these mutants as well as WT Vif (Fig. 2C). These results indicated that interaction between APOBEC3G and Vif could not explain Vif function to antagonize APOBEC3G. We then examined whether these Vif mutants could form a complex with EloB, EloC, and Cul5 in baculovirus-infected insect cells (Fig. 2D). Lysates from Hi Five insect cells infected with Vif WT or mutants encoding baculoviruses together with EloB, EloC, and Cul5 encoding baculoviruses were immunoprecipitated with anti-Vif antibody, and the amount of Cul5 co-immunoprecipitated with Vif was probed. An SLQ144/146AAA mutant, in which highly conserved SLQ residues required for the interaction with EloC were substituted for AAA (27), could not interact with Cul5 as reported previously (11–13), indicating that the mutant cannot inhibit APOBEC3G function because it cannot form a Vif-Bc-Cul5 complex, which is critical for the down-regulation of APOBEC3G. In contrast, C114S and C133S Vif mutants could bind to Cul5 possibly through EloB and EloC, indicating that the reason why both C114S and C133S mutants could not antagonize APOBEC3G was still unclear because these mutants could interact with APOBEC3G (Fig. 2C) as well as form a Vif-Bc-Cul5 complex (Fig. 2D).

A Vif-Bc-Cul5 Complex Had an E3 Ligase Activity for APOBEC3G—Yu et al. (11) have demonstrated that HIV-1 Vif interacted with cellular proteins Cul5, EloB, EloC, and Rbx1 to form a Vif-Bc-Cul5 complex and then induced the ubiquitination and degradation of APOBEC3G. However, the direct evidence whether the Vif-Bc-Cul5 complex is the E3 ligase for APOBEC3G is still missing. To confirm this hypothesis, we tried to establish an in vitro ubiquitin conjugation assay using the purified Vif-Bc-Cul5 complex. The Vif-Bc-Cul5 complex was purified with NTA beads from lysates of Hi Five insect cells co-infected with recombinant baculoviruses expressing His-tagged Vif (His-Vif), Cul5, EloB, EloC, and Rbx1. As shown in Fig. 3A, Vif protein with molecular weight of 24kDa was purified successfully, which was confirmed by immunoblot. We also detected Vif-associated proteins of approximate molecular masses of 85, 18, 15, and 12 kDa. Western blot revealed that these proteins corresponded to Cul5, EloB, EloC, and Rbx1, respectively. These findings clearly showed that Vif could form a complex with Cul5, EloB, EloC, and Rbx1 in insect cells.

With the purified Vif-Bc-Cul5 complex in hand, we examined the E3 ligase activity of Vif-Bc-Cul5 for APOBEC3G by an in vitro ubiquitin conjugation assay. HA-tagged-APOBEC3G immunopurified with anti-HA beads from 293T lysates transfectected with the HA-APOBEC3G expression vector was used as a substrate. Since NEDD8, an ubiquitin-like modifier protein, enhances the E3 activity of cullin-based ligases, we also added purified APP-BP1/Uba3 complex (E1 for NEDD8), Ubc12 (E2 for NEDD8), and NEDD8 to the reaction mixture. As shown in Fig. 3B, APOBEC3G was ubiquitinated efficiently and specifically in the presence of the Vif-Bc-Cul5 complex (lane 6), since APOBEC3G was not ubiquitinated when either GST-ubiquitin, E1, E2, or Vif-Bc-Cul5 was omitted (lanes 2–5). To exclude the possibility that the recognition of APOBEC3G by the complex requires substrate modification and/or a factor copurified, we performed the assay using recombinant APOBEC3G purified from insect cells as a substrate and again obtained the exact same result (Fig. 3C). These indicated that the Vif-Bc-Cul5 complex is the E3 ligase for APOBEC3G and that the complex is sufficient for the ubiquitination of APOBEC3G in vitro. Furthermore, a Vif-Bc-Cul5 complex could not promote the ubiquitination of APOBEC3G D128K (Fig. 3D). Since Vif could not recognize APOBEC3G D128K (Fig. 1C), this result indicated that recognition of APOBEC3G by Vif is crucial for its ubiquitination by the Vif-Bc-Cul5 complex.

Finally, the reason why Vif C114S and C133S mutants cannot antagonize APOBEC3G was still unclear because both mutants could interact with APOBEC3G (Fig. 2C) as well as form a Vif-Bc-Cul5 complex (Fig. 2D). We, therefore, tested the E3 ligase activity of the Vif C114S- or C133S-Bc-Cul5 complex using this assay (Fig. 3E). The WT Vif-Bc-Cul5 complex could ubiquitinate APOBEC3G effectively (lane 3), whereas the Vif C114S- or C133S-Bc-Cul5 complex almost lost the E3 ligase activity toward APOBEC3G (lanes 4 and 5, respectively), although each complex contained similar level of each protein (Fig. 3F). We thus concluded that the ubiquitin ligase activity of Vif mutants through the Vif-Bc-Cul5 complex is strongly correlated with their ability to inhibit APOBEC3G.

DISCUSSION

HIV Vif is known to antagonize the antiviral activity of APOBEC3G by excluding the protein from HIV virion. Although it has been suggested that Vif forms a Vif-Bc-Cul5 complex, and the complex is involved in the ubiquitination and subsequent degradation of APOBEC3G, it has not been proven whether a Vif-Bc-Cul5 ligase directly ubiquitinates APOBEC3G. In the present study, we have established an in vitro ubiquitin conjugation assay for APOBEC3G using the purified Vif-Bc-Cul5 complex and demonstrated that this complex indeed ubiquitinates APOBEC3G and Vif could function as a target recognizing subunit of the E3 ligase. We believe that this is the first direct evidence of ubiquitination of APOBEC3G by a Vif-Bc-Cul5 ligase complex. The result that the Vif-Bc-Cul5 complex did not induce the ubiquitination of APOBEC3G D128K, which could not be recognized by Vif, supports that this complex is the E3 ligase for APOBEC3G. Some viruses utilize ubiquitin-mediated proteolysis in their viral life cycle to enhance viral replication, in which viral proteins function as an adaptor or a substrate recognition subunit of E3 ligase. In the former case, the human papillomavirus E6 protein acts as an adaptor to bring the substrate protein p53 to a cellular ligase, termed E6AP (28), and HIV-1 Vpu protein brings CD4 to an Skp1-Cullin-βTrCP complex by binding to βTrCP (29). In the latter case, adenovirus proteins E4orf6 and E1B55K form an E3
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We have then investigated the ability of several loss-of-function Vif mutants to ubiquitinate APOBEC3G using the in vitro ubiquitin conjugation assay. Vif SLQ144/146AAA mutant lost its activity against APOBEC3G because it could not form a Vif-Bc-Cul5 ligase complex due to mutations in SOCS-box motif, which is critical for binding to EloC. Our findings confirm the notion revealed recently that a novel SOCS-box motif in Vif is responsible for and regulates assembly of a Vif-Bc-Cul5 complex.

More importantly, we have demonstrated that both Vif C114S and C133S mutants can form a complex with Bc-Cul5 (Figs. 2D and 3E) but cannot ubiquitinate APOBEC3G. Two recent reports have shown different results from ours. Mehele et al. (12) has shown that C114/133S double mutant have a weak interaction with Cul5, whereas Yu et al. (13) has shown that C114S and C133S mutants could bind to EloB-EloC complex but not to Cul5. These two reports suggest that these cysteine residues are important for assembly of a Vif-Bc-Cul5 complex. However, our data clearly showed that both Vif C114S and C133S could form the Vif-Bc-Cul5 complex in insect cells, and the complex could be purified. This discrepancy between the previous observations and ours cannot be fully explained at this moment, but we suspect that it is because the amount of components of Vif-Bc-Cul5 is larger in baculovirus-infected insect cells than 293T cells, in which the binding experiments of the previous reports were employed. Alternatively, post-translational modification(s) to strengthen the binding between Vif-Bc and Cul5 might be occurred in insect cells. Moreover, our in vitro ubiquitin conjugation assay clearly showed that purified Vif C114S- and C133S-Bc-Cul5 complexes could not ubiquitinate APOBEC3G. These Vif mutants retained the ability to bind to Bc-Cul5 complex as well as APOBEC3G, namely, these Vif mutants can recognize APOBEC3G as a substrate recognition subunit of a Vif-Bc-Cul5 ligase. If so, why do these complexes containing Vif C114S or C133S fail to ubiquitinate APOBEC3G? The crystal structure of the SCF$^{skp2}$ ligase, a family of culin-based ligases, has shown that the ligase has a rigid structure and the positioning between E2 and the substrate is crucial for the ligase activity (32, 33). When N-terminal domain, critical for substrate binding, and C-terminal half (E2-binding site) of culin-1 (Cul1) were linked with a flexible linker, the ligase could not ubiquitinate the substrate, although the mutated Cul1 could bind both E2 and the percent infectivity relative to the ΔVif virus without expression of APOBEC3G. A, Vif WT could overcome the antiviral activity of APOBEC3G on ΔVif virions, but Vif mutants did not. B, protein expression of APOBEC3G in producer cells (top panel) and its incorporation into virions (bottom panel) were detected by immunoblotting with anti-HA mAb. Vif prevented virion incorporation of APOBEC3G by reducing its intracellular level in producer cells (lane 2), but Vif mutants did not (lanes 4–6). C, APOBEC3G could interact with all these mutants as well as WT Vif. HEK293T cells were cotransfected with expression vectors for APOBEC3G and Vif WT or mutants. The lysates were immunoprecipitated with anti-HA mAb and analyzed by immunoblotting with anti-HA mAb (top panel) or anti-Vif antibody (middle panel). Cell lysates were also analyzed by immunoblotting with anti-Vif serum (bottom panel). D, Hi Five cells were co-infected with recombinant baculoviruses expressing His-Vif (WT or mutants), myc-Cul5, EloB, EloC, and Rbx1. The lysates were immunoprecipitated with anti-HA mAb and analyzed by immunoblotting with anti-c-myc mAb (top panel) or anti-HA mAb (middle panel). Cell lysates were also analyzed by immunoblotting with anti-c-myc mAb (bottom panel). Vif WT as well as C114S and C133S mutants could bind to Cul5 through EloB and EloC (lanes 1–3, respectively), but the SLQ144/146AAA mutant did not (lane 4).

Fig. 2. Loss-of-function Vif mutants could bind to APOBEC3G but failed to exclude APOBEC3G from HIV-1 virions. HEK293T cells were cotransfected with pNL43/ΔVif-Luc (ΔVif) plus pcDNA3/HA-based vectors (a mock or APOBEC3G) and Vif expression vectors (WT, ΔVif, C114S, C133S, or SLQ144/146AAA). Viruses from these cells were challenged to target M8166 cells, and productive infection was measured by luciferase activity. Values are presented as the percent infectivity relative to the ΔVif virus without expression of APOBEC3G. A, Vif WT could overcome the antiviral activity of APOBEC3G on ΔVif virions, but Vif mutants did not. B, protein expression of APOBEC3G in producer cells (top panel) and its incorporation into virions (bottom panel) were detected by immunoblotting with anti-HA mAb. Vif prevented virion incorporation of APOBEC3G by reducing its intracellular level in producer cells (lane 2), but Vif mutants did not (lanes 4–6). C, APOBEC3G could interact with all these mutants as well as WT Vif. HEK293T cells were cotransfected with expression vectors for APOBEC3G and Vif WT or mutants. The lysates were immunoprecipitated with anti-HA mAb and analyzed by immunoblotting with anti-c-myc mAb (top panel) or anti-HA mAb (middle panel). Cell lysates were also analyzed by immunoblotting with anti-Vif serum (bottom panel). D, Hi Five cells were co-infected with recombinant baculoviruses expressing His-Vif (WT or mutants), myc-Cul5, EloB, EloC, and Rbx1. The lysates were immunoprecipitated with anti-HA mAb and analyzed by immunoblotting with anti-c-myc mAb (top panel) or anti-HA mAb (middle panel). Cell lysates were also analyzed by immunoblotting with anti-c-myc mAb (bottom panel). Vif WT as well as C114S and C133S mutants could bind to Cul5 through EloB and EloC (lanes 1–3, respectively), but the SLQ144/146AAA mutant did not (lane 4).
Fig. 3. In vitro ubiquitination of APOBEC3G. A, Hi Five cells were co-infected with recombinant baculoviruses expressing His-Vif, Cul5, EloB, EloC, and Rbx1 and His-Vif protein was purified with NTA beads. Purified His-Vif and associated proteins were visualized by staining with Coomassie Brilliant Blue (left panel) and immunoblotting with appropriate antibodies (right panel). We detected Cul5, Vif, EloB, EloC, and Rbx1 (indicated by arrowheads). B, an in vitro ubiquitin (Ub) conjugation assay was performed using immunopurified HA-APOBEC3G from transfected 293T cells as a substrate with E1, E2, a Vif-BC-Cul5 complex (E3), GST-ubiquitin (as indicated), Nedd8, Ubc12, APP-BP1/Uba3, and ATP regeneration system for 1 h at 37 °C. GST-ubiquitin-conjugated APOBEC3G was specifically detected as a ladder (arrows) in the presence of a Vif-BC-Cul5 complex (lane 6) by immunoblotting with anti-HA mAb. C, an in vitro ubiquitin (Ub) conjugation assay was performed using recombinant His-HA-APOBEC3G purified from insect cells as a substrate by the same method as described above. GST-ubiquitin-conjugated APOBEC3G was specifically detected as a ladder (arrows) in the presence of a Vif-BC-Cul5 complex (lane 5) by immunoblotting with anti-HA mAb. D, WT or D128K APOBEC3G was purified from HEK293T cells and used as a source of a substrate for an in vitro ubiquitin (Ub) conjugation assay as described above. A Vif-BC-Cul5 complex promoted the in vitro ubiquitination of APOBEC3G (lane 2) but not that of D128K (lane 3). E, C114S and C133S Vif mutants resulted in a disruption of ligase activity. An in vitro ubiquitin (Ub) conjugation assay was performed with a Vif-BC-Cul5 complex (WT, C114S, or C133S) (as indicated), as described in the legend to Fig. 3B. GST-ubiquitin-conjugated APOBEC3G was specifically detected as a ladder (arrows) only in a WT Vif-BC-Cul5 complex (lane 3) but not in C114S and C133S Vif-BC-Cul5 complex (lanes 4 and 5, respectively). F, Hi Five cells were co-infected with recombinant baculoviruses expressing His-Vif (WT, C114S, or C133S), Cul5, EloB, EloC, and Rbx1, and His-Vif protein was purified with NTA beads. Purified His-Vif and associated proteins were visualized by immunoblotting with the appropriate antibodies.
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substrate (32). This suggested that Cul1 ensures the fine positioning between E2 and the substrate because it is a rigid protein. Thus, in the case of Vif-BC-Cul5, the mutation of C114 or C133 may affect the conformation of Vif and perturb the position of APOBEC3G in the ligase complex, resulting in the loss of the E3 activity of the Vif-BC-Cul5 complex. However, it might be possible that stability of these complexes containing C114S or C133S is too weak to ubiquitinate APOBEC3G. The mechanism for loss of the E3 activity of C114S- and C133S-containing complexes should be fully elucidated in the future.

Finally, our results clearly suggested that loss of ubiquitin ligase activity of the Vif-BC-Cul5 complex is tightly linked to the loss of Vif function against APOBEC3G. In other words, ubiquitination of APOBEC3G by the Vif-BC-Cul5 complex is essential for Vif function. Our in vitro ubiquitin conjugation assay is a powerful tool to analyze the molecular mechanisms of ubiquitination of APOBEC3G by the Vif-BC-Cul5 complex, which will provide us with new insights into Vif function and identification of new targets for therapeutic strategy.

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