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

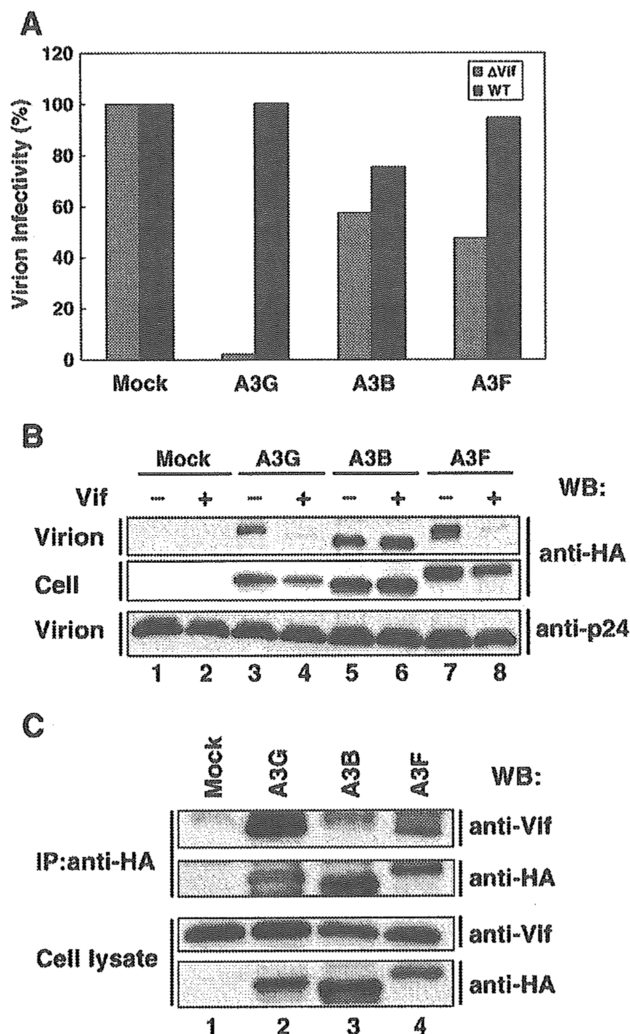

Fig. 1. Antiviral activity of APOBEC3 proteins on HIV-1. (A) A3B is resistant to HIV-1 Vif. We transfected HEK293T cells with pNL43/ΔEnv-Luc (WT) or pNL43/ΔEnvΔvif-Luc (ΔVif) plus pVSV-G in the presence of pcDNA3/HA-based vectors (a mock, A3G, A3B, and A3F). Viruses from these cells were challenged to 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).

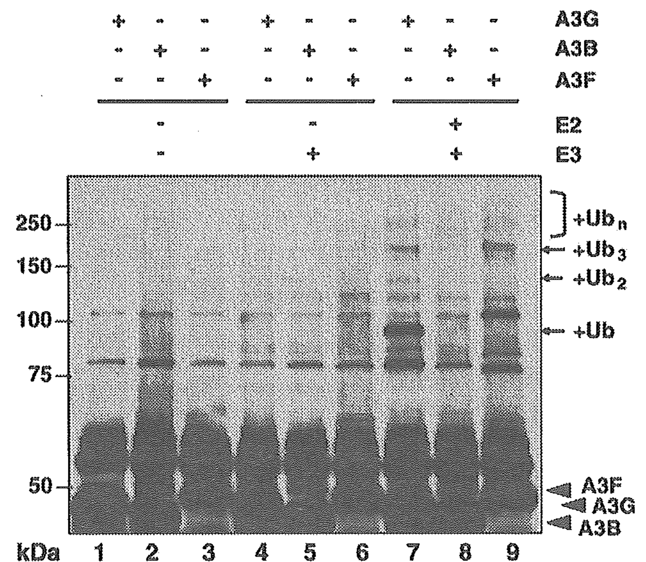

Fig. 2. In vitro ubiquitination of APOBEC3 proteins. An in vitro ubiquitin conjugation assay was performed as described in Materials and methods. GST-ubiquitin-conjugated A3G and A3F proteins were specifically detected as ladder (arrows) by immunoblotting with anti-HA mAb.

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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