Results

Auto poly-ubiquitination of TRIM5α impaired by mutations in RING domain

The RING finger domain of TRIM5α comprises eight potential metal ligands and binds two atoms of zinc, with each zinc atom ligated tetrahedrally by either four cysteins or three cysteines and a single histidine. Based on the three-dimensional structure of the RING domains of TRIM5 (Abe et al., 2007) and the promyelocytic leukemia (PML) protein (Borden et al., 1995; Borden and Freemont, 1996), the first pair of metal ligands of the AGM TRIM5α RING domain (C15 and C18) would share a zinc atom with the third pair (C35 and C38), and the second (C30 and H32) and fourth pairs (C56 and C59) would share another zinc atom (Fig. 1A). To determine whether anti-HIV-1 and anti-SIVmac activities of AGM TRIM5α are similarly affected by RING domain mutations, several AGM TRIM5α constructs with mutations in the RING domain were generated (Fig. 1B). In the mutant TRIM5α constructs with C15AC18A, C30AH32A, or C15AC18AC30AH32A, two key amino acid residues in the first or second, or in both the first and

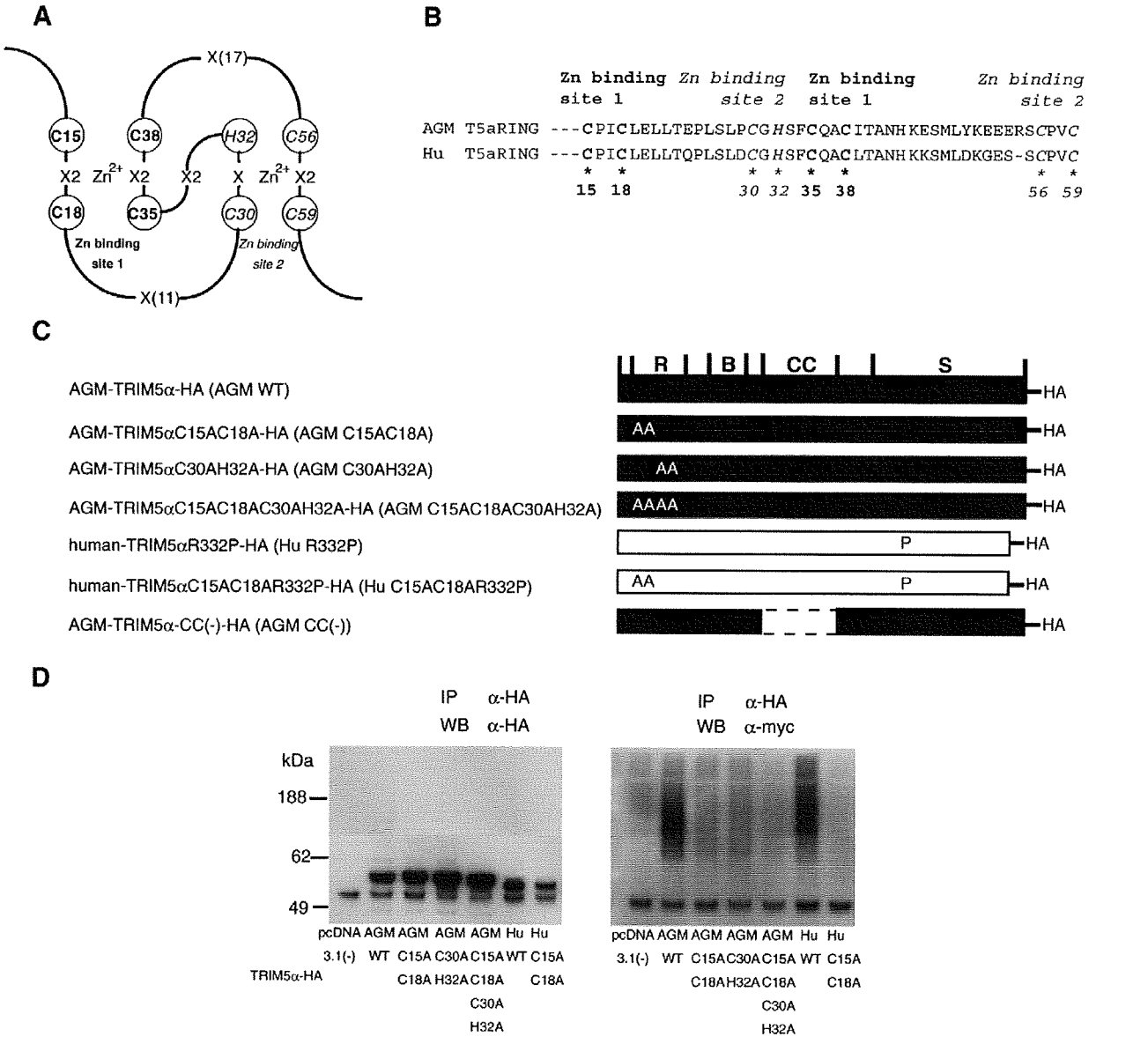


Fig. 1. Auto poly-ubiquitination of TRIM5α was impaired by RING domain mutations. (A) The RING finger zinc binding motif. The numbered AGM TRIM5α zinc-binding ligands are shown in circles. Each zinc atom is coordinated tetrahedrally by four ligands. Zinc-binding site 1 (**bold**) and site 2 (*italic*) are indicated. The numbers of amino acid residues between the zinc-binding cystein and histidine ligands in AGM TRIM5α are also indicated. (B) Primary amino acid sequences of the RING domains of AGM TRIM5α (AGM T5aRING) and human TRIM5α (Hu T5aRING) are aligned. Zinc-binding site 1 (**bold**), site 2 (*italic*), and cystein and histidine ligands (large numbers) are indicated. (C) Schematic representation of TRIM5α constructs. Black and white bars denote AGM and Hu sequences, respectively. Abbreviations for domains: R, RING; B, B-box 2; CC, Coiled-coil; S, SPRY. A dotted box denotes deletion of corresponding amino acid. The positions of individual amino acid changes are also indicated. (D) 293 T cells were transfected with plasmids encoding HA-tagged AGM TRIM5α (TRIM5α-HA) or its RING mutants together with a plasmid expressing myc-tagged ubiquitin (myc-Ub). Forty-eight hours after transfection, the cells were lysed and TRIM5α proteins in the lysates were precipitated with an anti-HA antibody. The immunoprecipitates were Western blotted and probed with anti-HA antibody for TRIM5α detection or with anti-myc antibody for ubiquitin detection. Abbreviations: WB, Western blot; IP, immunoprecipitation. The representative results of two independent experiments with similar results are shown.

second zinc-binding sites within the RING domain of AGM TRIM5α were replaced with alanine residues, respectively. All mutant TRIM5α constructs contained the HA-tag at their C-terminus (Fig. 1C).

To determine the effects of TRIM5α RING mutations on its ubiquitin ligase activity, 293T cells were transfected with plasmids encoding HA-tagged TRIM5αs together with plasmid expressing myc tagged ubiquitin. Forty-eight hours later, the cells were lysed and TRIM5α proteins were precipitated with the anti-HA antibody followed by Western blot analysis using anti-HA and anti-myc antibodies. Poly-ubiquitinated forms of the wild type AGM TRIM5α were observed (Fig. 1D). AGM TRIM5α with C15AC18A or C30AH32A was less poly-ubiquitinated than the wild type AGM TRIM5α, and AGM TRIM5α with C15AC18AC30AH32A was the least poly-ubiquitinated among the mutant constructs tested. These results confirmed the previously published report (Diaz-Griffero et al., 2006) that the TRIM5α RING zinc-binding site mutations impaired auto poly-ubiquitination of TRIM5α.

Contribution of RING domain to retrovirus restriction by AGM TRIM5α

We next examined anti-viral activities of zinc-binding site mutants of TRIM5α. The HA-tagged wild type and mutant AGM TRIM5α proteins were expressed by Sendai virus (SeV) in MT4 cells (Fig. 2A). CV1 cells were then used for a confocal microscopic examination of cytoplasmic bodies, since the cytoplasm of MT4 cells is not large enough for observation of cytoplasmic bodies. Each of the TRIM5αs with RING mutations formed uniformly larger cytoplasmic bodies than did the wild type (Fig. 2B), although the size of cytoplasmic

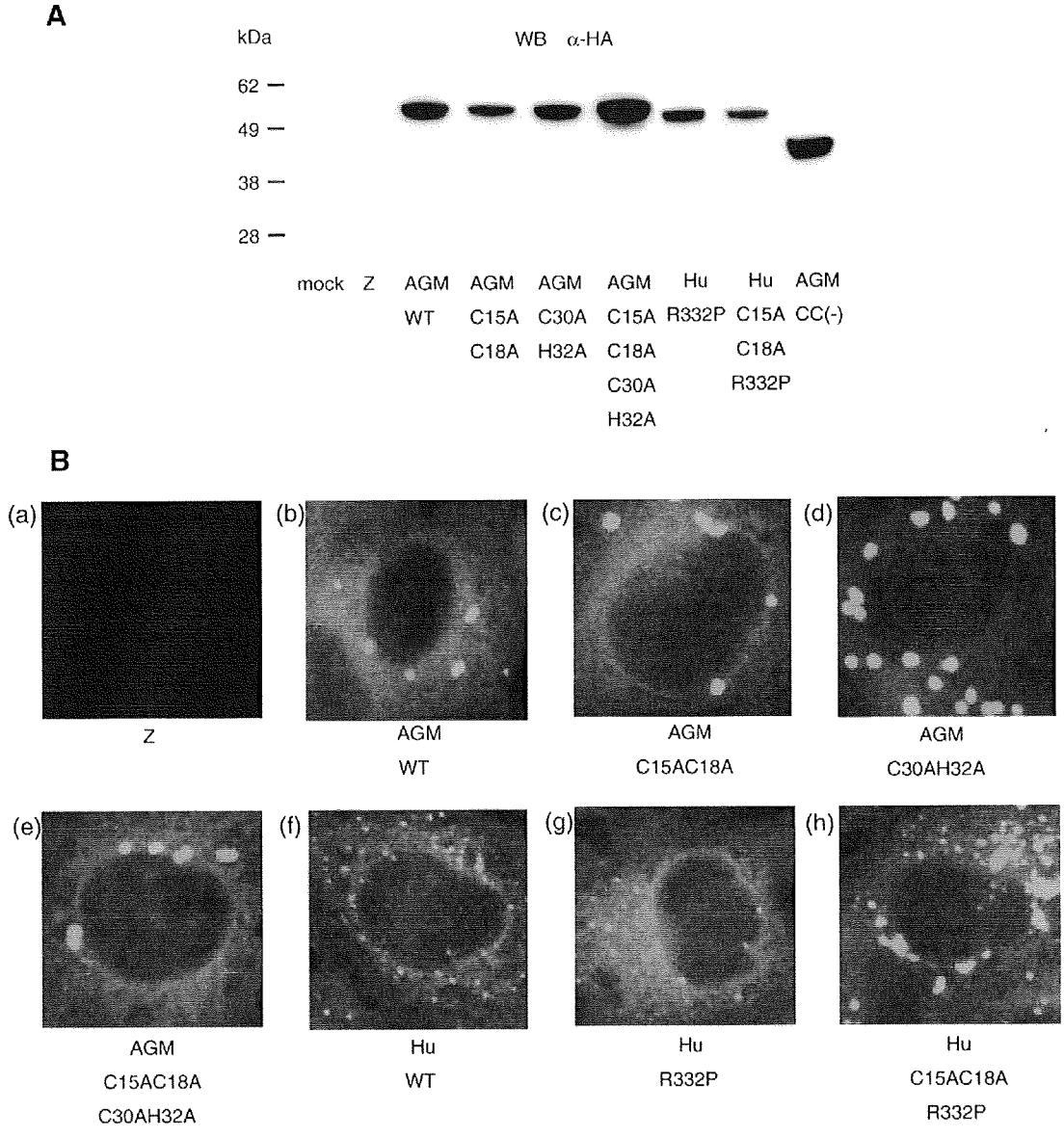


Fig. 2. Expression of RING mutant TRIM5α proteins. (A) Expression of various TRIM5αs. TRIM5α proteins in MT4 cells mock infected (mock) or infected with parental Z strain of SeV (Z), SeVs expressing AGM TRIM5α (AGM WT), AGM TRIM5α C15AC18A (AGM C15AC18A), AGM TRIM5α C30AH32A (AGM C30AH32A), AGM TRIM5α C15AC18AC30AH32A (AGM C15AC18AC30AH32A), human TRIM5α R332P (Hu R332P), human TRIM5α C15AC18AR332P (Hu C15AC18AR332P), or AGM-TRIM5α-Coiled-coil(−) (AGM CC(−)), were visualized by Western blotting with antibody to HA. (B) Subcellular localization of TRIM5αs. CV1 cells infected with SeV expressing HA-tagged TRIM5 proteins were analyzed as described in "Materials and methods". Representative confocal microscopic images are shown of parental Z strain of SeV (a), or with SeV expressing AGM WT (b), AGM C15AC18A (c), AGM C30AH32A (d), AGM C15AC18AC30AH32A (e), Hu WT (f), Hu R332P (g), or Hu C15AC18AR332P (h).

bodies slightly varied among different RING mutants of TRIM5α. These results confirmed the previous observations on Rh TRIM5α (Javanbakht et al., 2005). Specifically, AGM TRIM5α with C30AH32A showed the highest numbers of cytoplasmic bodies and the least levels of diffuse staining of cytoplasm among the three RING mutants (Fig. 2B).

For the viral replication assay, MT4 cells infected with SeVs expressing the wild type and mutant TRIM5αs were also superinfected with the NL43 strain of HIV-1, GH123 strain of HIV-2 or SIVmac239. Three days after infection, culture supernatants were collected and assayed for their levels of p24, p25 or p27 viral CA protein, respectively. AGM-TRIM5α-CC(−) was used as a negative control. AGM TRIM5α with C15AC18A, C30AH32A, or C15AC18A-C30AH32A moderately inhibited HIV-1 growth, while these variants completely lost their inhibitory effect on SIVmac growth (Fig. 3A). These results indicated that effects of cysteine substitutions in RING domain on anti-HIV-1 activity of AGM TRIM5α differ from those on anti-SIVmac activity, suggesting that SIVmac restriction by AGM TRIM5α was totally dependent on the intact RING domain of TRIM5α, while HIV-1 restriction was at least in part independent from this domain as reported previously (Javanbakht et al., 2005; Perez-Caballero et al., 2005b; Stremlau et al., 2004). It has been proposed that both proteasome-dependent and -independent pathways are involved in HIV-1 restriction by Rh TRIM5α, since disrupting the proteasome function by adding a proteasome inhibitor enabled the generation of normal levels of HIV-1 late reverse transcribed products, although HIV-1 infection and the generation of nuclear imports of 1-LTR and 2-LTR viral cDNA forms remained impaired by Rh TRIM5α (Anderson et al., 2006; Wu et al., 2006). We therefore concluded that AGM TRIM5α restricts SIVmac mainly via the RING-proteasome-dependent pathway.

We then tested the third virus, human immunodeficiency virus type 2 (HIV-2), which is more closely related to SIVmac than to HIV-1 (Gao et al., 1999). AGM TRIM5α clearly inhibited HIV-2 GH123 replication and all the RING domain mutants showed reduced anti-HIV-2 activity. AGM TRIM5α with C30AH32A completely lost its anti-HIV-2 activity (Fig. 3A). Unlike SIVmac, however, AGM TRIM5α with C15AC18A or C15AC18AC30AH32A still moderately inhibited HIV-2 GH123 growth (Fig. 3A). These results indicate that the RING domain contribution to HIV-2 restriction by TRIM5α was also distinct from its contributions to HIV-1 and SIVmac restrictions.

In a single round infection assay, MT4 cells infected with SeVs expressing the wild type or mutant TRIM5αs variants were superinfected with HIV-1-GFP or SIVmac-GFP. The wild type AGM TRIM5α potently restricted both HIV-1-GFP and SIVmac-GFP infection (Fig. 3B) as reported previously (Nakayama et al., 2005). On the other hand, AGM TRIM5α with C15AC18A, C30AH32A, or C15AC18A-C30AH32A only moderately inhibited HIV-1-GFP infection, while these variants completely lost their inhibitory effect on SIVmac-GFP infection (Fig. 3B). AGM C30AH32A exhibited the weakest anti-HIV-1 activity among the generated mutant constructs, probably due to its limited localization within the cytoplasm. However, the number of HIV-1-infected cells was still lower in AGM C30AH32A expressing cells than in those expressing negative control AGM-TRIM5α-CC(-) or cells infected with the parental SeV Z strain (Fig. 3B). The same results as above were obtained when we use canine Cf2Th cell line lacking endogenous TRIM5α expression (Sawyer et al., 2007) (Fig. 3C). These results confirmed our results in viral replication assay described in Fig. 3A.

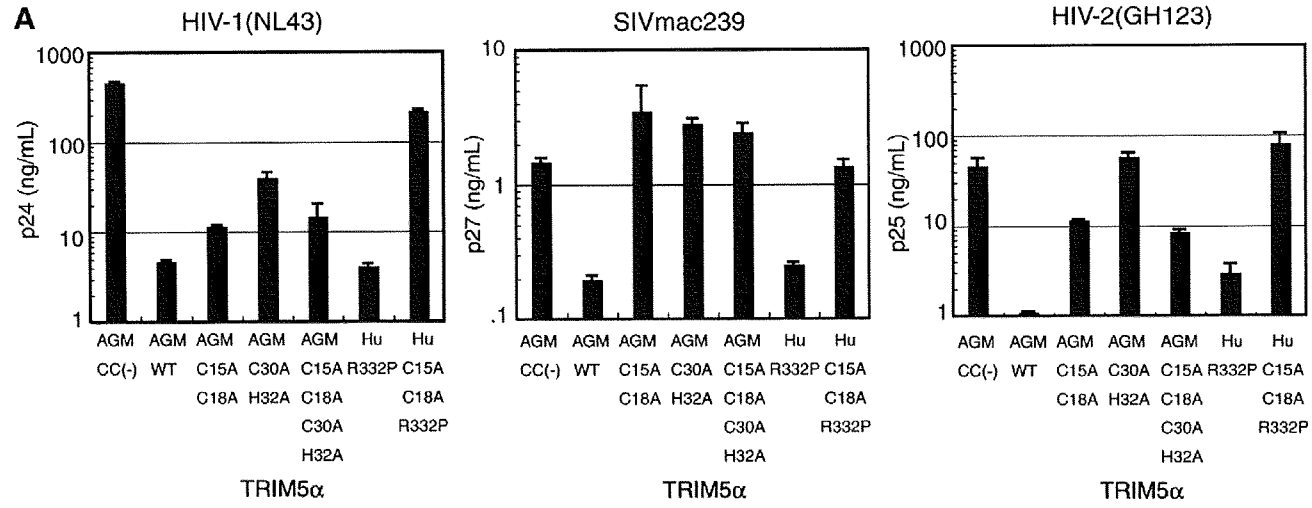
Contribution of RING domain to retrovirus restriction by human TRIM5α with arginine-to-proline substitution at the 332nd position

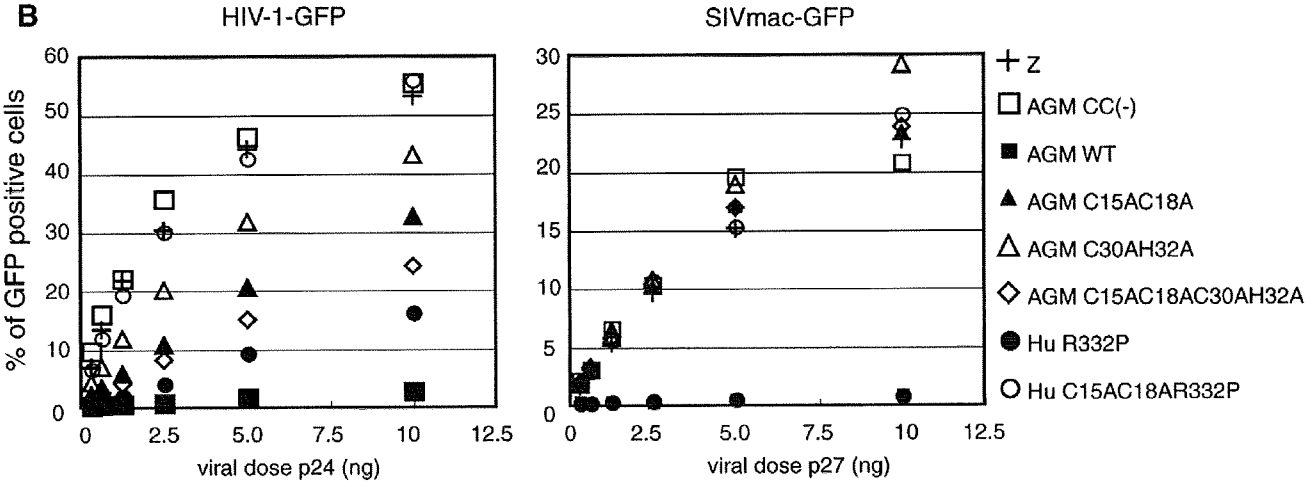
An arginine-to-proline substitution at the 332nd position (R332P) in the SPRY domain reportedly conferred strong anti-HIV-1 and anti-SIVmac activities to human TRIM5α (Stremlau et al., 2005; Yap et al., 2005). To determine whether cystein residue substitutions in the RING domain of human TRIM5α with R332P (Hu-R332P) have similar effects on its anti-HIV-1 and anti-SIVmac activities to those of AGM TRIM5α described above, C15AC18A substitutions were introduced in Hu-R332P. The protein expression levels of Hu-R332P with C15AC18A were comparable to those of Hu-R332P without C15AC18A (Fig. 2B). In addition, Hu-R332P inhibited both HIV-1 and SIVmac infection (Fig. 3A, B and C), which confirmed previous findings (Stremlau et al., 2005; Yap et al., 2005). As expected, Hu-R332P with C15AC18A completely lost its auto poly-ubiquitination (Fig. 1D) and anti-SIVmac activity (Fig. 3A, B and C) indicating that SIVmac restriction by Hu-R332P also strongly depends on the intact RING domain of TRIM5α. In contrast to AGM TRIM5α, however, Hu-R332P with C15AC18A completely lost its anti-HIV-1 activity (Fig. 3A, B and C). These findings suggest that, unlike AGM TRIM5α, Hu-R332P TRIM5α restricted both HIV-1 and SIVmac mainly via a RING-proteasome-dependent pathway. Hu-R332P TRIM5α with C15AC18A also failed to restrict HIV-2 GH123 (Fig. 3A). Taken together with results on AGM TRIM5α described above, our results indicated that the extent of RING domain contribution to retrovirus restriction by TRIM5α could be determined by a combination of virus and host species. We speculate that the intact RING domain is required for the proteasome-dependent but not for the proteasome-independent pathway of TRIM5α restriction of retroviruses.

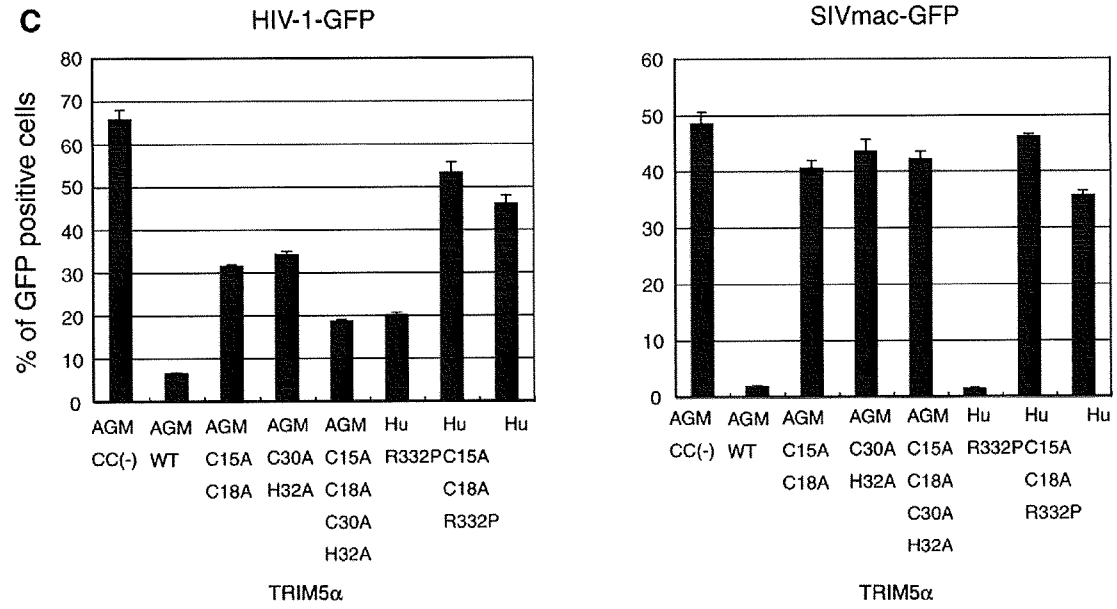
Effect of proteasome inhibition on antiviral activity of TRIM5α

For a direct investigation of whether AGM TRIM5α restricts SIVmac and Hu-R332P TRIM5α restricts both HIV-1 and SIVmac mainly via proteasome-dependent pathway, we used a proteasome inhibitor MG132. MT4 cells infected with SeVs expressing various TRIM5α were superinfected with HIV-1-GFP or SIVmac-GFP in the presence or absence of MG132. After infection, the cells were thoroughly washed and incubated in MG132-free medium. As shown in Fig. 4, MG132 had no effect at all on the anti-HIV-1 activity of AGM, Rh or cynomolgus monkey and of human/AGM chimeric TRIM5α carrying the SPRY domain of AGM TRIM5α and the RING, B-box 2, and coiled-coil domains of human TRIM5α. In contrast, and as expected, MG132 at least partially disrupted the anti-HIV-1 activity of Hu-R332P TRIM5α. Rh and cynomolgus monkey TRIM5α could not restrict SIVmac infection and that addition of MG132 did not affect the numbers of GFP-positive cells, indicating that the condition for MG132 treatment used in our study did not affect cell viability (Fig. 4). AGM, Hu-R332P and human/AGM chimeric TRIM5α restricted SIVmac infection while MG132 partially disrupted the anti-SIVmac activity of those TRIM5α. When we used Cf2Th cells, MG132 also disrupted the anti-HIV-1 activity of Hu-R332P TRIM5α at least partially (data not shown). These results support our conclusions that AGM TRIM5α restricted SIVmac mainly via the proteasome-dependent pathway, and that Hu-R332P TRIM5α restricted both HIV-1 and SIVmac mainly via the proteasome-dependent pathway (see Table 1 for summary of these results).

As described above, the previous studies have shown that disrupting the proteasome function by adding a proteasome inhibitor enabled the generation of HIV-1 late reverse transcribed products, even though HIV-1 infection and the generation of nuclear imports of 1-LTR and 2-LTR viral cDNA forms remained impaired by Rh TRIM5α (Anderson et al., 2006; Wu et al., 2006). We therefore examined levels of late reverse transcribed products and 2-LTR forms of HIV-1 cDNA in TRIM5α-expressing cells by real time PCR method. Mean C_T values (SD) of late reverse transcribed products were 29.80 (0.27), 29.30 (0.15), and 28.11 (0.10) in cells expressing Rh, AGM, and Hu-R332P TRIM5αs, respectively, while that in control cells was 24.73 (0.08). These results clearly indicated that synthesis of late reverse transcribed products were suppressed in cells expressing functional TRIM5α. When we added MG132, mean C_T values (SD) of late reverse


A
HIV-1(NL43)
SIVmac239
HIV-2(GH123)
p24 (ng/mL)
p27 (ng/mL)
p25 (ng/mL)
AGM CC(-)
AGM WT
AGM C15A C18A
AGM C30A H32A
AGM C15A C18A C30A H32A
Hu R332P
Hu C15A C18A R332P
TRIM5α


B
HIV-1-GFP
SIVmac-GFP
% of GFP positive cells
viral dose p24 (ng)
viral dose p27 (ng)
+ Z
AGM CC(-)
AGM WT
AGM C15AC18A
AGM C30AH32A
AGM C15AC18AC30AH32A
Hu R332P
Hu C15AC18AR332P


C
HIV-1-GFP
SIVmac-GFP
% of GFP positive cells
AGM CC(-)
AGM WT
AGM C15A C18A
AGM C30A H32A
AGM C15A C18A C30A H32A
Hu R332P
Hu C15A C18A R332P
TRIM5α

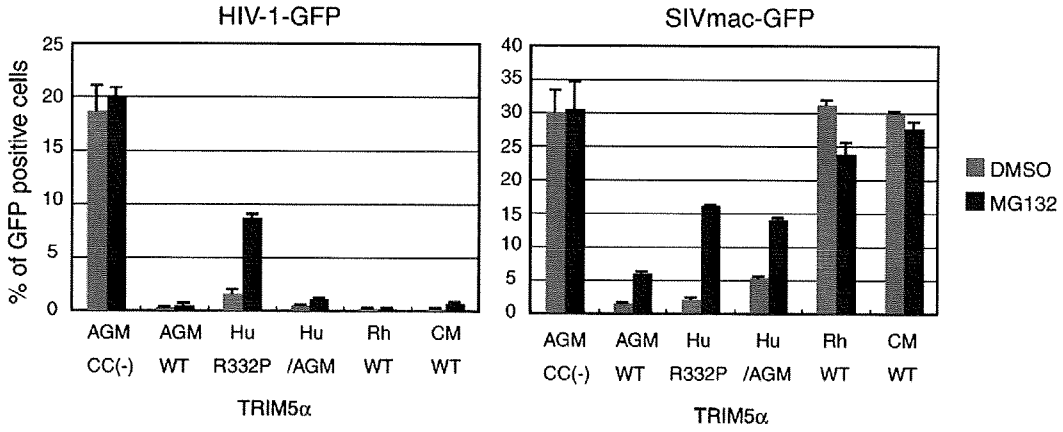


Fig. 4. The effect of proteasome inhibition on antiviral activity of TRIM5α depends on combinations of TRIM5α and viruses. MT4 cells were infected with SeVs expressing AGM CC (−), AGM WT, Hu R332P, Hu/AGM, Rh WT or CM WT. Cells were then superinfected with HIV-1-GFP or SIVmac-GFP in the presence of 10 μM MG132 in 0.1% DMSO (black bar) or 0.1% DMSO (gray bar). The representative results of three independent experiments with similar results are shown. Error bars denote actual fluctuations of duplicate samples.

transcribed products were 29.16 (0.13), 28.72 (0.10), and 26.96 (0.15) in cells expressing Rh, AGM, and Hu-R332P TRIM5αs, respectively, and that in control cells was 24.63 (0.11). Differences between C_T values in the presence of MG132 and those in the absence of MG132 were statistically significant ($P<0.05$) in cells expressing Rh, AGM, and Hu-R332P TRIM5αs but not in control cells. We therefore concluded that slight but significant levels of late reverse transcribed products were recovered by MG132 treatment in cells expressing Rh, AGM, and Hu-R332P TRIM5αs. It should be noted that we failed to obtain complete recovery of late reverse transcribed products by MG132 treatment in our experimental system. This was most likely caused by incomplete suppression of proteasome function in our system since SeV-infected MT4 cells could be treated with MG132 for only 2 h to maintain cell viability, while Hela cells were treated with MG132 for 15 h in the previous studies (Anderson et al., 2006; Wu et al., 2006).

With respect to 2-LTR forms of HIV-1 cDNA, mean C_T values (SD) in the absence of MG132 were 39.99 (1.74), 38.32 (2.36), and 37.81 (1.80) in cells expressing Rh, AGM, and Hu-R332P TRIM5αs, respectively, and that in control cells was 33.68 (0.64). In the presence of MG132, mean C_T values (SD) were 40.02 (1.71), 38.71 (1.39), and 36.46 (2.03) in cells expressing Rh, AGM, and Hu-R332P TRIM5αs, respectively, and that in control cells was 33.80 (0.28). Significant recovery of 2-LTR forms of HIV-1 cDNA was thus observed only in cells expressing Hu-R332P TRIM5α ($P<0.05$) but not in cells expressing either AGM or Rh TRIM5αs. These results confirmed that HIV-1 restriction by Rh and AGM TRIM5αs was both proteasome dependent and independent while that by Hu-R332P TRIM5α was mainly proteasome dependent.

Discussion

Deletion of the RING domain or amino acid changes within the RING domain of Rh TRIM5α has been shown to attenuate anti-HIV-1 activity, but such a mutated TRIM5α still exhibits moderate HIV-1 restriction (Javanbakht et al., 2005; Perez-Caballero et al., 2005b; Stremlau et al., 2004). Both proteasome-dependent and -independent pathways have been proposed in HIV-1 restriction by Rh TRIM5α, since proteasome inhibitor MG132 allows HIV-1 to generate late reverse transcribed products, even though HIV-1 infection and the generation of nuclear 1-LTR and 2-LTR viral cDNA forms remain impaired by Rh TRIM5α (Anderson et al., 2006; Wu et al., 2006). In the study presented here, we demonstrated that the contribution of the RING domain of TRIM5α to retrovirus restriction differed among viral species. SIVmac completely escaped attacks by RING mutants of TRIM5α that could still moderately restrict HIV-1 and HIV-2 infection. Addition of proteasome inhibitor MG132 had no effect at all on the anti-HIV-1 activity of AGM TRIM5α, whereas it disrupted at least partly the anti-SIVmac activity of AGM TRIM5α. These results indicate that SIVmac is restricted by AGM TRIM5α mainly in a proteasome-dependent manner, whereas HIV-1 restriction by AGM, Rh, and cynomolgus monkey TRIM5α is both proteasome dependent and independent. In case of Hu-R332P TRIM5α, however, both HIV-1 and SIVmac restrictions were completely eliminated by mutations in the RING domain. Furthermore, both anti-HIV-1 and anti-SIVmac activities of Hu-R332P TRIM5α could also be disrupted by the proteasome inhibitor. These findings indicate that Hu-R332P TRIM5α restricts both HIV-1 and SIVmac mainly via the proteasome-dependent pathway.

It was found that TRIM5α could be poly-ubiquitinated and degraded by the proteasome (Diaz-Griffero et al., 2006). Furthermore, accelerated turnover of TRIM5α was observed during HIV-1 restriction (Rold and Aiken, 2008). Although there is no direct evidence for ubiquitination of the virus core by TRIM5α, it is highly likely that reverse transcription complexes containing viral CA proteins recognized by poly-ubiquitinated TRIM5α would be degraded by the proteasome in combination with TRIM5α. On the other hand, the exact molecular mechanism of the proteasome-independent pathway is still unclear at present. It was previously shown that the incubation of *in vitro* assembled CA proteins composed of recombinant HIV-1

Fig. 3. Contribution by RING domain to retrovirus restriction by TRIM5α depends on combination of host and viral species. (A) MT4 cells were infected with SeV expressing AGM CC (−), AGM WT, AGM C15AC18A, AGM C30AH32A, AGM C15AC18AC30AH32A, Hu R332P, or Hu C15AC18AR332P. The cells were then superinfected with HIV-1 NL43, HIV-2 GH123 or SIVmac239. The culture supernatants were collected three days after infection for measurement of the p24, p25 or p27 levels. The representative results of two independent experiments with similar results are shown. Error bars denote actual fluctuations of duplicate samples. (B) MT4 cells were infected with parental Z strain of SeV (crosses), or with SeVs expressing AGM WT (black squares), AGM CC(−) (white squares), AGM C15AC18A (black triangles), AGM C30AH32A (white triangles), AGM C15AC18AC30AH32A (white diamonds), Hu R332P (black circles), or Hu C15AC18AR332P (white circles). The cells were then superinfected with serially diluted HIV-1-GFP or SIVmac-GFP. The representative results of two independent experiments with similar results are shown. (C) Canine Cf2Th cells were infected with SeVs expressing indicated TRIM5α protein. The cells were then superinfected with HIV-1-GFP or SIVmac-GFP. The representative results of four independent experiments with similar results are shown. Error bars denote standard deviation in triplicate samples.

Table 1
Summary of TRIM5α-mediated restriction.

TRIM5α	Anti-HIV-1 activity		Anti-SIVmac activity	
	proteasome-dependent	proteasome-independent	proteasome-dependent	proteasome-independent
AGM	yes	yes	yes	no
AGM C15AC18A	no	yes	no	no
AGM with MG132	no	yes	no	no
Hu-R332P	yes	no	yes	no
Hu-R332P C15AC18A	no	no	no	no
Hu-R332P with MG132	no	no	no	no

Yes, presence of the pathway; no, absence of the pathway.

CA–NC fusion proteins with the TRIM5-21R protein containing the Rh TRIM5α B-box, coiled-coil, and SPRY domains and the TRIM21 RING domain caused apparent breaks in the CA structure without any other cellular components (Langelier et al., 2008). It is thus likely that direct binding of Rh TRIM5α proteins to incoming HIV-1 CA proteins causes CA disassembly, which is observed as proteasome-independent restriction. AGM TRIM5α would bind both HIV-1 and SIVmac CA, while it may cause disassembly of the HIV-1 CA but not that of the SIVmac CA. Similarly, Hu-R332P TRIM5α would bind both HIV-1 and SIVmac CA but may fail to cause disassembly of both HIV-1 and SIVmac CAs. We therefore speculate that the proteasome-independent pathway requires specific SPRY–CA interaction that can lead to CA disassembly.

Although the proteasome inhibitor clearly disrupted anti-HIV-1 activity of Hu-R332P and anti-SIVmac activity of AGM, Hu-R332P, and human/AGM TRIM5αs, the number of infected cells never reached the levels of the negative control AGM-TRIM5α-CC(−). Longer exposure of cells expressing the TRIM5αs with the proteasome inhibitor did not increase the number of infected cells (data not shown). In contrast, anti-HIV-1 activity of Hu-R332P and anti-SIVmac activity of AGM and Hu-R332P TRIM5αs were completely eliminated by mutations in the RING domain. The reason for this discrepancy is not clear at present, but it is possible that TRIM5α also exerts a proteasome-independent but RING-dependent restrictive effect.

The RING-proteasome-independent restriction pathway was observed only in anti-HIV-1 but not in anti-SIVmac activity of AGM TRIM5α. It is known that cyclophilin A (CypA) binds to HIV-1 CA via the loop between the 4th and 5th α-helices (L4/5) but not to SIVmac CA (Luban et al., 1993). Since CypA was reported to restrict HIV-1 in monkey cells (Berthoux et al., 2005; Keckesova et al., 2006; Nakayama et al., 2008; Sokolskaja et al., 2006; Stremlau et al., 2006b), it is possible that CypA binding to HIV-1 CA regulates the RING-proteasome-independent restriction mechanism of TRIM5α (Berthoux et al., 2004). This hypothesis prompted us to examine the effect of the RING mutation of TRIM5α on its restrictive effect on NL-ScaVR, an HIV-1 derivative containing SIVmac L4/5 of CA and vif (Kamada et al., 2006). However, NL-ScaVR was similarly restricted by AGM TRIM5α with C15AC18A to HIV-1 (data not shown), indicating that neither the CypA-binding site nor vif is the determining factor in RING-proteasome-independent restriction of HIV-1. Further studies using various chimeric viruses between HIV-1 and SIVmac will also be needed to elucidate the exact molecular mechanisms of the RING-proteasome-independent pathway of TRIM5α mediated HIV-1 restriction.

Conclusion

AGM TRIM5α restricted SIVmac mainly via the proteasome-dependent pathway, whereas HIV-1 and HIV-2 restriction by AGM TRIM5α was both proteasome dependent and independent. In contrast, Hu-R332P restricts both HIV-1 and SIVmac mainly via the proteasome-dependent pathway. We concluded that the mechanisms of retrovirus restriction by TRIM5α vary depending on the combination of host and virus.

Materials and methods

Plasmid construction and protein expression

Previous reports have described recombinant Sendai viruses (SeVs) expressing C-terminally HA-tagged AGM TRIM5α (GenBank accession number AB210050), Rh TRIM5α (GenBank accession number AY625001), cynomolgus monkey (CM) TRIM5α (GenBank accession number AB210052), human TRIM5α (This human TRIM5α cDNA was obtained from T cell line MT4 and there was a single glycine-to-aspartic acid substitution at an amino acid position 249 compared with GenBank accession number NM033034.1), human TRIM5α with R332P, human and AGM chimeric TRIM5α and AGM TRIM5α lacking the coiled-coil domain (AGM-TRIM5α-CC(−)) (Kono et al., 2008; Maegawa et al., 2008; Nakayama et al., 2005, 2007). In the present study, a PCR-based mutagenesis was used to generate cDNA of the following C-terminally HA-tagged AGM TRIM5α RING domain mutants: AGM TRIM5α with C15AC18A, AGM TRIM5α with C30AH32A, AGM TRIM5α with C15AC18A-C30AH32A, and human TRIM5α with R332P and C15AC18A mutations. The entire coding sequences of those TRIM5αs were then transferred to the NotI site of pSeV18+b(+). Recombinant SeVs expressing various TRIM5αs were obtained with a previously described method (Shioda et al., 2001).

The plasmid expressing myc-tagged ubiquitin (myc-Ub) was generated according to the previous publication (Ellison and Hochstrasser, 1991). Briefly, human ubiquitin cDNA (GenBank accession number NM_018955) was amplified by reverse transcription-PCR from the human epithelial carcinoma cell line HeLa by using 5′-GCGAATGCCATGACTGAAG-3′ and 5′-GACGTGGTTGGTGATTGGC-3′ followed by nested PCR using 5′-ATGCAGATCTTCGTGAAAACC-3′ and 5′-CTAACCACCTCTCAGACGCAGGACC-3′. The amplified products were then cloned into pCR-2.1 TOPO (Invitrogen, Carlsbad, CA). The entire coding sequences of the myc-Ub were then transferred to the NheI and NotI site of pcDNA3.1(-) (Invitrogen, Carlsbad, CA).

Immunoprecipitation and Western blot analysis

For protein expression analysis, human T-cell line MT4 was infected with SeV at a multiplicity of infection (MOI) of 10 plaque forming units (PFU) per cell, and incubated at 37 °C for 16 h. The cells were then lysed in RIPA buffer (10 mM Tris–HCl (pH 7.4) containing 100 mM NaCl, 1% Sodium deoxycholate, and 0.1% sodium dodecyl sulfate), and the cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel were transferred to a membrane (Immobilon; Millipore, Billerica, MA), and blots were blocked and probed with an anti-HA High Affinity rat monoclonal antibody (Roche, Indianapolis, IN) overnight at 4 °C. Blots were then incubated with peroxidase-

conjugated anti-rat IgG (American Qualex, San Clemente, CA). Bound antibodies were visualized with ChemiLumi-One L chemiluminescent kit (Nacalai Tesque, Kyoto, Japan).

For ubiquitination analysis, the NotI and EcoRI sites were used to construct the plasmid expressing HA-tagged TRIM5α RING mutants in pcDNA3.1(-). DMRIE-C reagent (Invitrogen, Carlsbad, CA) was used to transfect 293T cells with 1 μg of plasmid encoding HA-tagged wild-type or mutant TRIM5αs together with 1 μg of plasmid expressing myc-Ub in six-well plates. Forty-eight hours later, the cells were lysed and TRIM5α proteins in the lysates were precipitated with a Protein G-immunoprecipitation kit (Roche, Indianapolis, IN) using the anti-HA rat monoclonal antibody. After overnight incubation at 4 °C, beads were washed three times in RIPA buffer. Precipitated proteins were detected with the same procedure as above except that an anti-myc mouse monoclonal antibody and peroxidase-conjugated anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were used for visualizing the myc-tagged Ub protein.

Virus preparation

HIV-1 NL43, HIV-2 GH123 or SIVmac239 was prepared by transfection of 293T cells with pNL432 (Adachi et al., 1986), pGH123 (Shibata et al., 1990), or pBRmac239 (Kestler et al., 1991), respectively. The vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped HIV-1 vector expressing green fluorescence protein (GFP) (HIV-1-GFP) was prepared as described previously (Miyoshi et al., 1997, 1998) as was VSV-G pseudotyped SIVmac vector expressing GFP (SIVmac-GFP) (Hofmann et al., 1999). The viral titer was determined by measuring viral CA protein, p24, p25 or p27, with a RetroTek antigen ELISA kit (ZeptoMetrix, Buffalo, NY).

Viral infection

MT4 or canine Cf2Th cells were infected with SeV expressing various TRIM5αs. Nine hours after SeV infection, 1.0×10^5 cells per dose were superinfected with serially diluted HIV-1-GFPs or SIVmac-GFPs in 48-well plates and incubated at 37 °C. Forty hours after infection, the infected cells were fixed with 1% formaldehyde and counted with a flow cytometer (FACScaliber; Becton Dickinson, Franklin Lakes, NJ). For the HIV-1, HIV-2 or SIVmac replication assay, 2.0×10^5 MT4 cells were infected with SeV expressing various TRIM5αs and 9 h after SeV infection, the cells were superinfected with 20 ng of p24 of HIV-1 NL43, p25 of HIV-2 GH123 or p27 of SIVmac. The culture supernatants were collected periodically for measurement of the p24, p25 or p27 levels.

Proteasome inhibition and infection with HIV-1-GFP or SIVmac-GFP

MT4 cells were infected with SeV expressing various TRIM5αs. Nine hours after SeV infection, 1.0×10^5 cells were superinfected with 10 ng of p24 of HIV-1-GFP or 100 ng of p27 of SIVmac-GFP in the presence of 10 μM MG132 (CALBIOCHEM) in 0.1% DMSO or 0.1% DMSO only. Two hours after the HIV-1-GFP or SIVmac-GFP infection, the cells were washed in fresh medium and incubated at 37 °C for 40 h. The infected cells were fixed with 1% formaldehyde and then counted with a flow cytometer.

Immunofluorescence confocal microscopy

AGM CV1 cells infected with SeV expressing several TRIM5αs at an MOI of 10 PFU per cell were fixed 24 h after infection in 3% paraformaldehyde in PBS, permeabilized with 0.05% saponin and 0.2% bovine serum albumin in PBS, and incubated with the anti-HA rat monoclonal antibody. Bound antibodies were then detected with a FITC-conjugated goat antibody directed against rat IgG (American Qualex Antibodies, San Clemente, CA). Indirect immunofluorescence was visualized with a Radiance 2000 laser confocal microscope system (Bio-Rad Laboratories, Hercules, CA).

Real-time PCR analysis

To prepare high titer virus stock of HIV-1 NL43, MT4 cells were infected with NL43 virus and the culture supernatant was harvested at its peak titer (1250 ng/ml of p24) at 12 days after infection. Five x 10^6 MT4 cells were infected with SeV expressing TRIM5α. Twenty hours after SeV infection, cells were superinfected with 500 μl (625 ng of p24) of NL43 stock virus with 10 μM MG132 (CALBIOCHEM) in 0.1% DMSO or with 0.1% DMSO only for 2 h. After washing out of inoculated virus containing MG132, cells were suspended in 10 ml of flesh media and incubated at 37 °C for 12 h. Total DNA was extracted by using QIAamp DNA Blood kit. Real-time PCR was performed with an Applied Biosystems 7500 Real-Time PCR System to analyze viral cDNA synthesis. Primers and Taqman probes for late reverse transcribed products and 2-LTR forms were designed according to Julias et al. (2001) and Van Maele et al. (2003), and 0.6 μg DNA were subjected to 40 cycles of PCR in 10 μl reaction mixture. Threshold cycle (C_T) values were calculated by 7500 Fast System SDS software (Applied Biosystems). Mean C_T values and their standard deviation (SD) were calculated in triplicate (late reverse transcribed product) or septuplicate (2-LTR) samples. In a few cases we failed to detect amplification of 2-LTR forms, the C_T values were assigned as 41 cycles. Statistical significance of observed difference in mean C_T values was evaluated by Mann-Whitney *U* test.

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Review

Role of HIV-1 Nef protein for virus replication *in vitro*

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Abstract

The Nef protein of primate lentiviruses (simian and human immunodeficiency viruses; SIV/HIVs) appears to be multi-functional and plays a pivotal role in viral persistence and pathogenesis *in vivo*. Of its numerous functions reported to date, the ability to enhance virion infectivity in indicator cell lines and to augment viral replication in peripheral blood mononuclear cells (PBMCs) and lymphocytes (PBLs) is very well conserved among various SIV/HIVs. This review summarizes and organizes current knowledge of HIV-1 Nef with respect to this particularly virological activity for understanding the basis of its *in vivo* function.

Keywords: Nef; HIV-1; SIV; Replication *in vitro*

1. Introduction

The *nef* gene is present in primate lentiviruses but not in the other retroviruses. Early studies have demonstrated the importance of Nef for efficient viral replication and pathogenesis *in vivo*; *nef*-deleted SIVmac239 displays attenuated viral replication and pathogenicity in rhesus macaques [1]. Mutations and deletions of HIV-1 Nef also have been found in virus isolates from several long-term non-progressors of HIV-1 infection [2,3]. Even though it has been reported that *nef*-deleted HIV-1 [4] and SIVmac239 [5] finally induce AIDS symptoms, Nef is obviously required for maintenance of high viral loads in individuals, and accelerates the disease progression.

Nef appears to be a multi-functional protein that involves in: (1) down-regulation of cell surface molecules such as CD4, major histocompatibility complex class I and class II, CD28, and CD3, (2) enhancement of virion infectivity and stimulation of viral replication, and (3) modulation of T cell activation state (for review, see reference [6]). These functions are well conserved among primate lentiviruses, but the ability to down-regulate CD3 is lost in viruses of the HIV-1 lineage. It has been proposed that the inability of HIV-1 Nef to down-regulate CD3 leads to increased sensitivity of T cells for immune activation and to programmed cell death (for review, see references [6,7]). Functional contribution of each activity of HIV-1 Nef described above to HIV-1 pathogenesis *in vivo* remains to be determined.

Here, we focus on our current knowledge of Nef-mediated enhancement of virion infectivity in indicator cell lines (artificially generated target cells) and stimulation of viral replication in PBMCs and PBLs (natural target cells), which would affect virus spread and pathogenesis *in vivo*.

2. Characteristics of HIV-1 Nef

Nef is a membrane-associated phosphoprotein that is synthesized abundantly in early stage of viral infection. Nef is incorporated into virions and associates with viral core. Numbers of reports have shown that HIV-1 Nef interacts with various cellular proteins via distinct domains (Fig. 1). Of

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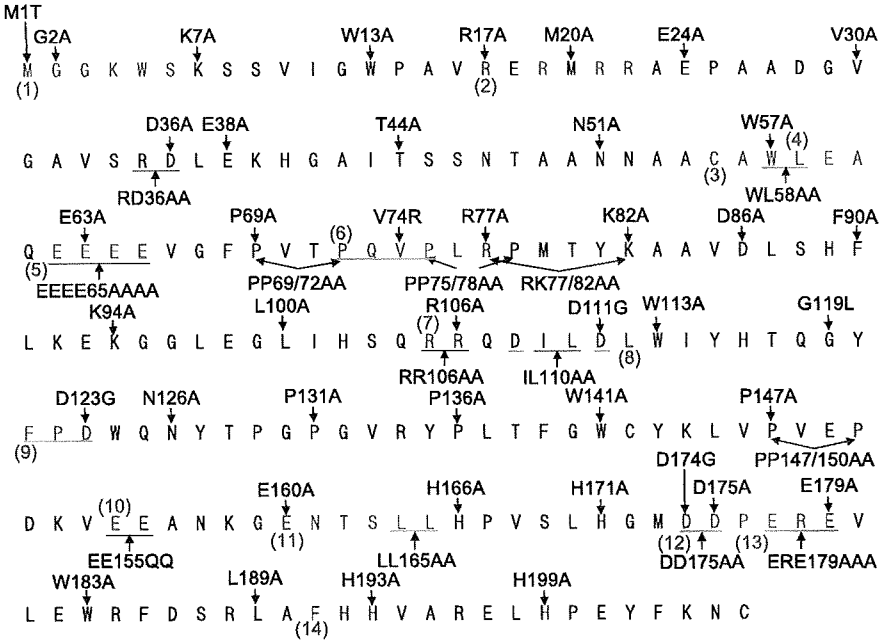

Fig. 1. Amino acids in HIV-1 Nef important for its functionality. Amino acid sequence of HIV-1 Nef from NL4-3 (GenBank accession no. AF324493) and the functionally important regions are shown. Position of site-specific mutations and designation of the mutants are also shown by arrows. Green underlines indicate the regions already reported to be involved in infectivity enhancement of virions and/or stimulation of viral replication in PBMCs and PBLs. Red colored amino acids and numbers represent the motifs of Nef that interact with or important for: (1) N-myristoyl transferase and Dynamin2, MGxxxS; (2) membrane targeting, R17/19/21/22; (3) HIV-1 protease, CAW↓LEA (cleavage site); (4) cytoplasmic tail of CD4, WL; (5) PACS-1 (Phosphofurin acidic cluster sorting protein-1), EEEE; (6) SH3 domains of Src family kinases, PxxPxR; (7) Pak1/2 (p21-activated kinase-1/2) and Nef multimerization, RR; (8) Pak2 and Nef multimerization, L; (9) human thioesterase and Dynamin2, FPD; (10) β-COP (β-coat protein), EE; (11) adaptor proteins AP-1/2/3, ExxxLL; (12) V1H (catalytic subunit H of vacuolar ATPase), DD; (13) c-Raf1 kinase, DDPxxE; (14) Pak2, F. X indicates any amino acids.

various functional roles assigned for Nef, the membrane-binding ability, which depends on its N-terminal myristoylation, is critical for all activities such as CD4 down-regulation and enhancement of virion infectivity. Mutational analysis of HIV-1 NL4-3 and SF2 *nef* alleles in the proviral construct has revealed that some regions are required for infectivity enhancement of virions in indicator cells and/or for stimulation of viral replication in PBMCs and PBLs [6,8–13]. These include $WL_{57,\ 58}$, $P_{72}xxP_{75}$, $D_{108}D_{111}$, $FPD_{121,\ 122,\ 123}$, $LL_{164,\ 165}$, $DD_{174,\ 175}$, and $ERE_{177,\ 178,\ 179}$ (Fig. 1). The domains involved in the other functions of HIV-1 Nef are summarized in references [6,11,13]. In order to give a general picture of the contribution of Nef to viral infectivity *in vitro*, we ourselves have generated a series of site-directed mutants throughout the *nef* gene in the context of HIV-1 NL4-3 proviral genome (Fig. 1). The resultant 55 mutants with single or multiple amino acid alterations were then monitored for viral infectivity in indicator cells. As shown in Fig. 2, overall, the mutational effects were rather small but distinct for some variants, confirming the previously published results. Even the mutants carrying multiple amino acid substitutions (such as EEEE65AAAA and ERE177AAA) showed a considerable infectivity. In addition, there appeared to be no large distinct regions or domains affecting much the viral infectivity *in vitro*. However, we have noticed that some point mutants (M1T, G2A, V30A, D111G, G119L, P136A, W183A, and L189A) show a low infectivity similar to that of the *nef*-minus virus (NL–Xh). These mutants were found to grow poorly in PBMCs in agreement with the results in indicator cells (Fig. 2), indicating the importance of the original wild-type amino acids in Nef for viral replication *in vitro*. Needless to say, the N-terminal myristoylation (M1T and G2A) and central multimerization (D111G) signals of Nef are critical for its activity (Figs. 1 and 2).

3. Enhancement of virion infectivity and stimulation of viral replication by HIV-1 Nef

As mentioned above, it has been suggested that Nef is an important factor for pathogenesis *in vivo* according to data from the macaque/*nef*-deleted SIVmac model and from analysis of human non-progressors infected with *nef*-deleted HIV-1. In contrast with the *in vivo* studies, initial works *in vitro* have labeled *nef* a negative regulatory factor, based on the observation that Nef down-regulates viral replication by suppressing transcription from HIV-1 LTR [14–16]. However, it has been well established now that Nef does promote modestly or affects little the viral spread in cultured cells. Nef shows positive effect on HIV-1 replication in PBLs particularly when the cells are infected with HIV-1 prior to cell activation [17,18].

In single-cycle replication assays, Nef modestly enhances virion infectivity [17,18]. Enhancement of the infectivity requires expression of Nef in virus-producer cells [19–21], suggesting that Nef may somehow modify HIV-1 virions

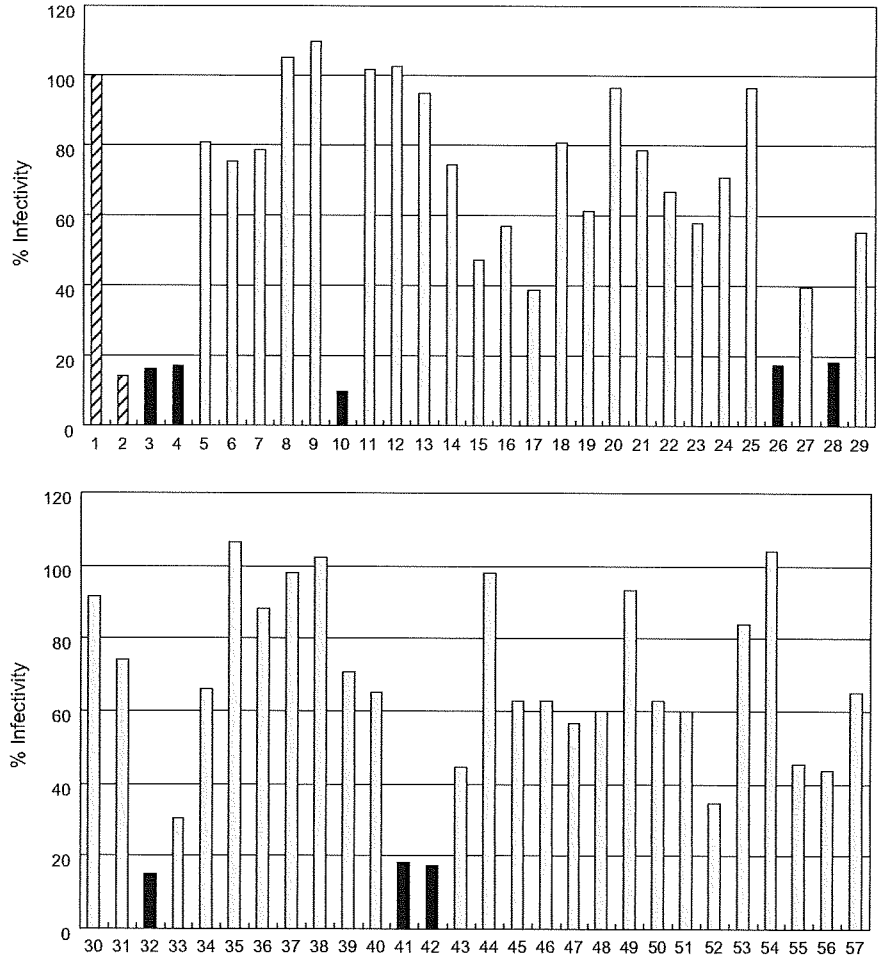

Fig. 2. Infectivity of mutant virions in indicator cells. Mutants produced in transfected cells were assayed for their infectivity in MAGI indicator cells [45], and titers relative to that of wild-type virus are shown. Diagonally striped, black, and grey bars reperesent control samples (positive and negative), mutants with less than 20% infectivity relative to wild-type virus, and the other mutants, respectively. Virus samples are as follows: 1, NL4-3 (wild-type virus); 2, NL-Xh (δNef virus); 3, M1T; 4, G2A; 5, K7A; 6, W13A; 7, R17A; 8, M20A; 9, E24A; 10, V30A; 11, D36A; 12, E38A; 13, T44A; 14, N51A; 15, W57A; 16, E63A; 17, P69A; 18, V74R; 19, R77A; 20, K82A; 21, D86A; 22, F90A; 23, K94A; 24, L100A; 25, R106A; 26, D111G; 27, W113A; 28, G119L; 29, D123G; 30, N126A; 31, P131A; 32, P136A; 33, W141A; 34, P147A; 35, E160A; 36, H166A; 37, H171A; 38, D174G; 39, D175A; 40, E179A; 41, W183A; 42, L189A; 43, H193A; 44, H199A; 45, RD36AA; 46, WL58AA; 47, EEEE65AAAA; 48, PP69/72AA; 49, PP75/78AA; 50, RK77/82AA; 51, RR106AA; 52, IL110AA; 53, PP147/150AA; 54, EE155QQ; 55, LL165AA; 56, DD175AA; 57, ERE177AAA.

(Fig. 3). HIV-1 virions produced in the absence of Nef fail to accomplish efficient reverse transcription of viral RNA genome, indicating that Nef functions at a post-entry step of viral replication [20,22,23]. On the other hand, it has been shown that infectivity enhancement by Nef depends on the route of virus entry. Nef is not required for infectivity enhancement of the HIV-1 virions pseudotyped with the Env that mediates pH-dependent virus entry through endocytic compartments [24,25]. Recently, however, it has been reported that Nef can enhance infectivity of HIV-1 virions pseudotyped with Tva (a receptor for subgroup A Rous sarcoma virus) even if viral entry process is pH-dependent [26].

Since Nef is a core-associated protein and a small quantity of Nef exists in virions (around 10 molecules) [27], virion Nef may modulate a post-entry step(s) for viral replication in target cells such as uncoating and/or core trafficking (Fig. 3). However, whether virion-associated Nef is required for enhancement of virion infectivity is controversial. Virion-associated Nef has been reported to be dispensable for infectivity enhancement, because a mutant Nef, that is poorly incorporated into virions, still has the ability to promote viral replication in PBLs [13]. In contrast, it has been observed that virion incorporation of Nef correlates with infectivity enhancement as revealed by experiments utilizing the cyclophillin A-Nef fusion protein and cyclospolin A [28].

In total, major proposed mechanisms for Nef-mediated infectivity enhancement are as follows (Fig. 3). (a) Nef enhances HIV-1 cytoplasmic entry by fusion at the plasma membrane [29]. This argues against the observations that Nef enhances proviral DNA synthesis without its effect on virus entry [19,20,23] and that Nef does not affect HIV-1 fusion with target cells [30,31]. Since HIV-1 virions can enter into target cells in two ways (fusion and endocytosis), virion endocytosis may have masked the positive effect of Nef on

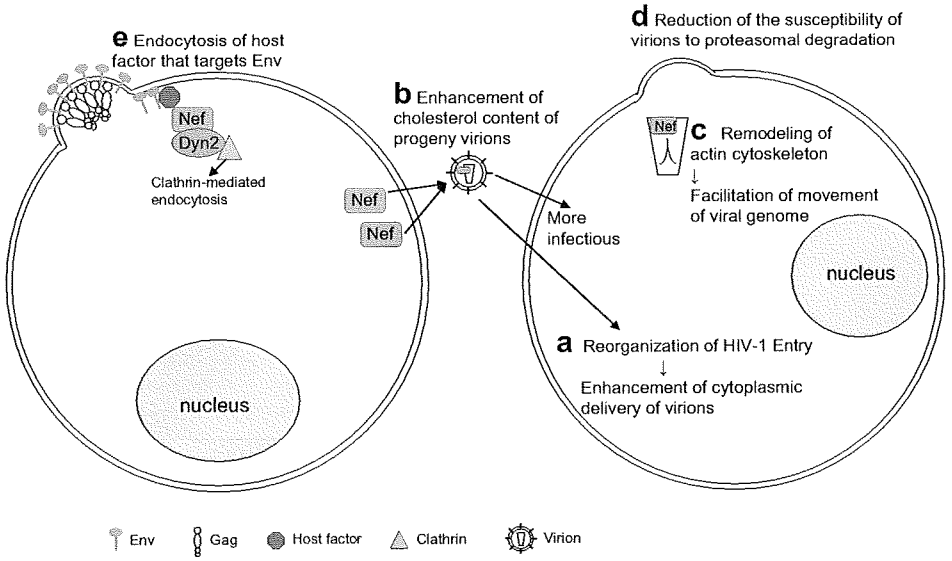

Fig. 3. Schematic representation of mechanism for infectivity enhancement of HIV-1 particles by Nef. Major proposed mechanisms for augmentation of virion infectivity by Nef are summarized. (a) Nef functions as an entry factor and enhances cytoplasmic delivery of virions. (b) Nef increases the biosynthesis of cholesterol and enhances cholesterol content of progeny virions, leading to production of virions more infectious than Nef-deficient virions. (c) Nef alters actin cytoskeleton of host cells and facilitates the movement of the HIV-1 genome through the cortical actin network. (d) Nef reduces the susceptibility of virions to proteasomal degradation in target cells by modifying virions during particle assembly. (e) Nef down-regulates a host factor other than CD4, which targets Env in a Dynamin2- and clathrin-dependent manner.

HIV-1 entry by fusion at plasma membrane [29]. More recently, it has been suggested that the entry of HIV-1 through endocytosis is facilitated by Nef [26]. (b) Nef increases synthesis and transport of cholesterol, enhances cholesterol content of virions, and thus makes virions more infectious [32]. The positive effect of cholesterol on virion infectivity is controversial, because several groups have reported that Nef does not influence cholesterol content of HIV-1 virions [33,34]. (c) Following fusion at plasma membrane, virion-associated Nef alters actin cytoskeleton and facilitates the trafficking of HIV-1 viral genome [31]. (d) Nef reduces susceptibility of HIV-1 virions to proteasomal degradation in target cells, because treatment with proteasome inhibitors relieves infectivity enhancement of Nef-defective HIV-1 [35]. However, proteasome inhibitors may affect viral infectivity via indirect effect on the cell cycle or increased expression of a host cell factor that facilitates infection, not by the inhibition of degradation of incoming virions [36]. (e) Nef associates with Dynamin 2, a regulator of vesicular trafficking, and Nef may down-regulate a host factor other than CD4 that targets Env in a Dynamin2- and clathrin-dependent manner [34].

Although Nef-mediated enhancement of virion infectivity in indicator cells and promotion of viral replication in PBMCs and PBLs are fundamental properties of primate lentiviruses [37], it has been noticed that Nef enhances HIV-1 infectivity more strongly in P4-CCR5 cells than in TZM-bl cells and PBMCs [38]. Both indicator cell lines (P4-CCR5 and TZM-bl) are derived from HeLa cells, and the factor that accounts for the observation described above would be the difference in their susceptibility to viruses. TZM-bl cells are more susceptible for HIV-1 infection and express higher level of CD4 than P4-CCR5 cells [38]. On the other hand, it also has been suggested that the efficiency of viral replication in PBLs correlates with the ability of Nef to down-regulate CD4 but not with that to enhance viral infectivity [12,39]. The claims described in (a) to (e) are sometimes controversial and inconsistent, and the mechanism for infectivity enhancement by Nef remains to be definitively elucidated. Because most studies on virion infectivity to date have been done in HeLa-derived indicator cell lines, and furthermore, requirement for Nef activity may be influenced by activation status and types of target cells [38], it is necessary to analyze the functional role of Nef in natural target cells or equivalents relevant for infection *in vivo* [6,38].

4. Conclusions

Genetic analysis to elucidate the functional role for Nef in viral life cycle is difficult because: (1) An apparent multi-functional protein like Nef interacts with various cellular proteins via different domains. Therefore, mutations introduced would generate pleiotropic effects; (2) Requirement of Nef for enhancement of viral infectivity depends on virus strains [40] and its biological activity encoded by various *nef* alleles differs significantly [13,38,41]. Nonetheless, it is important to clarify the molecular biological basis for up-regulation of *in vitro* infectivity by Nef to understand *in vivo* activity of Nef. In this regard, it is intriguing to note the most recent developments that Nef may be associated with species-specific pathogenic potentials of SIV/HIVs [42–44]. Most studies on Nef reported to date have been done with various Nef proteins in diverse cell types for purposes of every kind. Future research should be performed in the direction of understanding *in vivo* role of multi-functional Nef.

Acknowledgements

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Short communication

Status of APOBEC3G/F in cells and progeny virions modulated by Vif determines HIV-1 infectivity

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Abstract

We examined various HIV-1 Vif mutants for interaction with APOBEC3 proteins (A3G/A3F). All replication-defective proviral mutants were found to carry A3G/A3F in virions, and of these, a replication-defective mutant with Vif that binds to A3G in cells but not in virions was noted. Furthermore, a mutant Vif protein that suppresses A3F activity but does not exclude A3F from virions was identified. We also showed that incorporation of Vif into virions is dependent on its interaction with A3G/A3F. Taken together, we concluded that functional binding of Vif to A3G/A3F in cells and/or virions is critical for viral infectivity.

Keywords: HIV-1; Vif; APOBEC3G; APOBEC3F; Virion-incorporation

1. Introduction

Vif protein of HIV-1 efficiently suppresses the anti-retroviral effect imposed by A3G/A3F in natural target cells [1]. Extensive studies have revealed that HIV-1 Vif consists of two major domains with various functional motifs [2,3]. HIV-1 Vif interacts with its substrates A3G/A3F by N-terminal region, and suppresses their antiviral function by targeting them to proteasomal degradation through C-terminal region. There are several motifs for binding to A3G and/or A3F in the N-terminal region [2–9], and are those for forming an E3 ubiquitin ligase complex in the C-terminal region (Fig. 1A) [2,3,10–13]. HIV-1 Vif is believed to overcome the antiviral activity of A3G and A3F predominantly by mediating their degradation via ubiquitin–proteasome pathway, thereby inhibiting their incorporation into progeny virions [14–17]. Consistently, our replication-defective proviral *vif* mutants carry a higl level of A3G and/or A3F in virions [3]. On the other hand, whether the Vif packaged into progeny virions is biologically significant remains unclear.

In this study, to clarify virological basis of Vif to neutralize A3G and A3F, we examined our replication-defective proviral mutants for the level and status of A3G/A3F in cells and in virions by immunoblotting/immunoprecipitation analysis (Fig. 1). We demonstrate here that virion incorporation of Vif depends upon its binding to A3G/A3F. Moreover, while we confirmed that all replication-defective proviral mutants contain a relatively high level of A3G/A3F in progeny virions, a mutant Vif protein (Y40A in Fig. 1) that can suppress A3F activity but does not exclude A3F from virions was noted. In addition, we found a mutant Vif protein (H108A in Fig. 1) from a replication-defective proviral clone that binds to A3G in cells but does not in virions. Together with our results published before [3], we can conclude that functional binding of Vif to A3G/A3F in cells or virions are critical for viral infectivity.

2. Materials and methods

2.1. Plasmid DNAs

Proviral HIV-1 *vif* mutant clones, and Flag-tagged human A3G- and A3F-expression vectors have been previously described [3].

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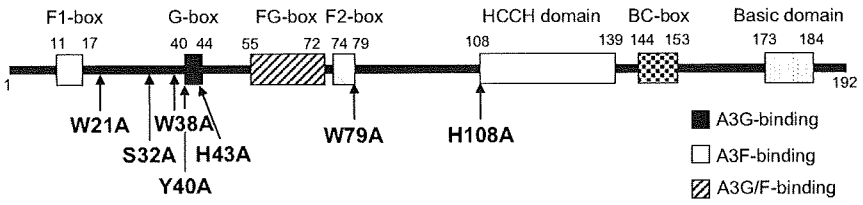
A



B

Mutant	Neutralization		Suppression of expression in cells		Binding in cells		Suppression of virion-incorporation	
	A3G	A3F	A3G	A3F	A3G	A3F	A3G	A3F
W21A	–	–	–	–	–	–	–	–
S32A	–	–	–	–	–	–	–	–
W38A	–	–	–	–	–	–	–	–
Y40A	–	+	–	–	–	+	–	–
H43A	–	+	–	+	–	+	–	–
W79A	+	–	+	–	+	–	+	–
H108A	–	–	–	–	+	+	–	–

Fig. 1. Seven HIV-1 *vif* mutants used in this study. (A) Positions and designations of the mutants. All mutants are derived from HIV-1 NL4-3 clone [20] (GenBank accession no. AF324493). Functionally important domains or motifs [2,3] are indicated. (B) Ability of the seven Vif mutants to act against A3G and A3F. Activity similar to that of WT Vif is scored as (+), and poor or no activity relative to WT Vif is indicated by (−). Results are summarized from this study and our previous report [3].

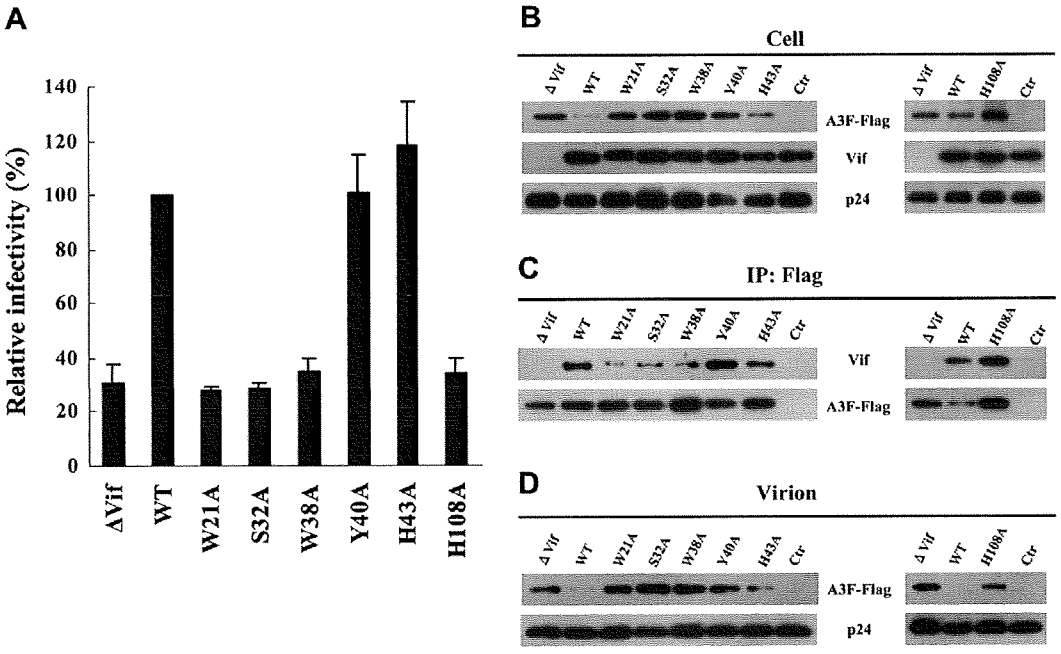


Fig. 2. Activity of *vif* mutants against A3F. (A) Effect of A3F on single-cycle infectivity of the mutants. Viral samples were prepared from 293T cells co-transfected with each proviral clone (15 μg) and an A3F-expression vector (1 μg), and their infectivity was determined in MAGI cells [19]. Infectivity relative to that of WT virus is shown. (B) Expression level of A3F in virus-producing cells. 293T cells were co-transfected as in (A), and cell lysates for immunoblot analysis were prepared two days later. Two independent experiments are shown. Ctr, no A3F-expression vector. (C) Ability of WT and mutant Vif proteins to bind to A3F in cells. Lysates of 293T cells co-transfected with each proviral clone and an A3F-expression vector as above were prepared, and used for immunoprecipitation (IP) of A3G-Flag by anti-Flag-M2 agarose. Interaction of various Vif proteins and A3F was then analyzed by immunoblotting using anti-Vif or anti-Flag antibody. Ctr, mock-transfection. (D) Pakaging of A3F into mutant virions. Virions prepared from 293T cells co-transfected with each proviral clone and an A3F-expression vector were analyzed by immunoblotting using antibodies indicated. Ctr, no A3F-expression vector.

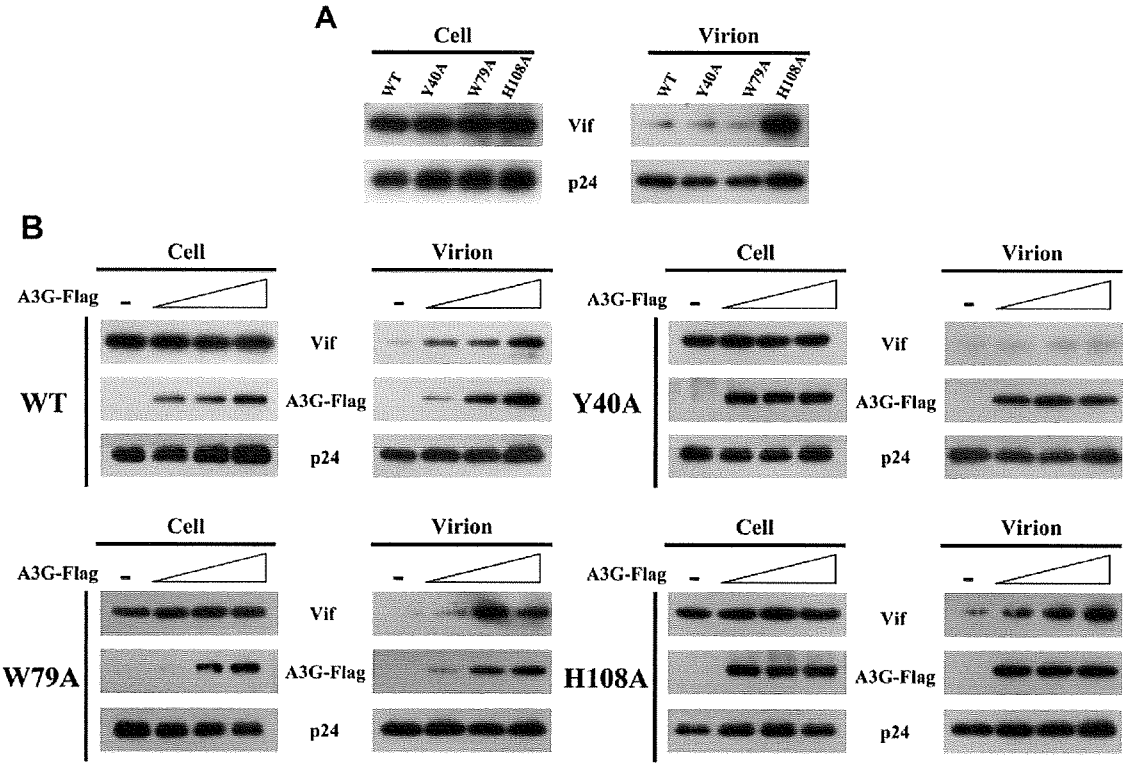


Fig. 3. Virion incorporation of mutant Vif proteins in the presence of A3G. (A) Comparison of Vif level in cells and virions prepared by a regular transfection; 293 T cells were co-transfected with each proviral clone (15 μg) and an A3G-expression vector (1 μg). (B) Vif and A3G levels in cells and virions with increasing amounts of A3G. 293T cells were co-transfected with a constant amount of proviral clone (15 μg) and increasing amounts of A3G-expression vector (0, 1, 2, and 3 μg), and levels of Vif, A3G, and Gag-p24 were monitored by immunoblotting.

2.2. Cells

293T [18] and MAGI [19] cell lines were maintained in Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum.

2.3. Transfection and virion preparation

Transfection of 293T cells was carried out by calcium–phosphate co-precipitation method [20]. To prepare virion samples, culture fluids were harvested from transfected 293T cells at 48 h post-transfection, filtered through 0.45-μm filters, and viral particles were concentrated by ultracentrifugation through 25% sucrose for 2 h at 80,000× *g* using SW41 as previously described [21].

2.4. Viral infectivity determined by MAGI assays

To determine single-cycle replication potential of the mutants, viral samples were prepared from 293T cells co-transfected with each proviral clone and an expression vector of A3G or A3F at 48 h post-transfection. Viral infectivity was then determined by MAGI assay as previously described [19].

2.5. Immunoblotting/immunoprecipitation

Cell and virion fractions were prepared from transfected 293T cells, and lysed in a lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA and 1% protease inhibitor cocktail (Sigma)). Lysates were then resolved on SDS-PAGE, followed by electrophoretic transfer to polyvinilidene fluoride membranes. Membranes were treated with anti-FLAG (Sigma), anti-Vif (NIH AIDS Research and References Reagent Program) or anti-Ga-p24 [21] antibodies, and visualized by the ECL plus Western blotting detection system (Amersham Pharmasia Biotech Inc.). For immunoprecipitation (IP) analysis, cell and virion lysates were mixed with anti-FLAG M2 agarose (Sigma), and incubated at 4 °C for 3 h. Reaction mixture was then washed three times with TBS buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl) and eluted by the addition of 3 × FLAG peptides (Sigma). After centrifugation, the supernatants were analyzed by immunoblotting.

3. Results

3.1. Biological and biochemical characterization of vif mutants

Recent studies have shown that various amino acids in the N-terminal region of HIV-1 Vif is important for its binding to A3G/A3F [2–9], and the results can be summarized as shown in Fig. 1A [2,3]. We have recently constructed thirty site-directed proviral point mutants from the NL4-3 clone encoding Vif of 192 amino acids [3]. Of the seventeen and thirteen

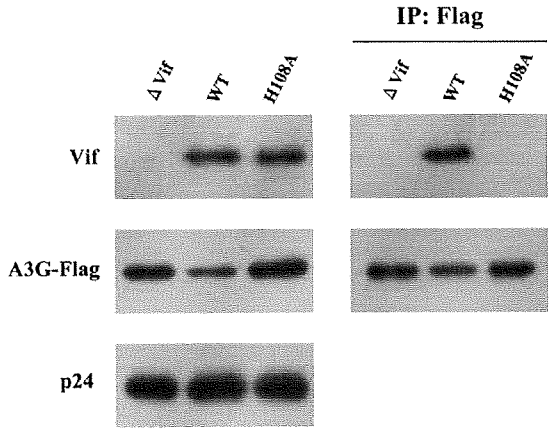


Fig. 4. Analysis of a mutant Vif protein in H108A virions. Virions were prepared from 293T cells co-transfected with each proviral clone (15 μg) and an A3G-expression vector (3 μg). Lysates of virions were used for direct immunoblotting (left) and for immunoprecipitation (IP) of A3G-Flag by anti-Flag-M2 agarose followed by immunoblotting (right). Anti-Vif, anti-Flag, or anti-Gag-p24 antibody was used for detection.

mutants in the N-terminal and C-terminal portions, respectively, eight (N-terminal) and seven (C-terminal) were found to be defective for virus growth in non-permissive H9 cells [3]. Replication-defective mutants designated W21A, S32A, W38QA, Y40A, H43A, W79, and H108A (Fig. 1A) were selected for analysis in this study. Mutants W21A, S32A, W38QA, Y40A, and H43A could not or poorly bind to A3G and were unable to neutralize its activity efficiently (Fig. 1B) [3], and no detailed reports on them other than ours have been published. Mutant W79A did bind to A3G but not to A3F, and could suppress A3G activity but not A3F activity (Fig. 1B) [3]. Another mutant H108A, a mutant in the HCCH motif of C-terminal domain (Fig. 1A), could bind to A3G but was replication-defective (Fig. 1B) [3].

First of all, we evaluated the activity of various mutants described above against A3F. As shown in Fig. 2A, out of six mutants tested, only two (Y40A and H43A) were able to suppress A3F activity, showing single-cycle replication similar to that of wild-type (WT) clone. Level of A3F in virus-producing cells were then monitored (Fig. 2B). As expected, WT Vif reduced A3F expression much relative to that by various mutants including ΔVif clone. Unexpected results were those for Y40A and H43A. Y40A behaved like ΔVif in this analysis, and H43A reduced A3F expression more than ΔVif although less efficiently than WT. When the ability to bind to A3F was examined by IP (Fig. 2C), while mutants W21A, S32A, and W38A were clearly defective, Y40A, H43A, and H108A (a mutant of C-terminal domain) were found to retain this activity. Finally, we checked packaging of

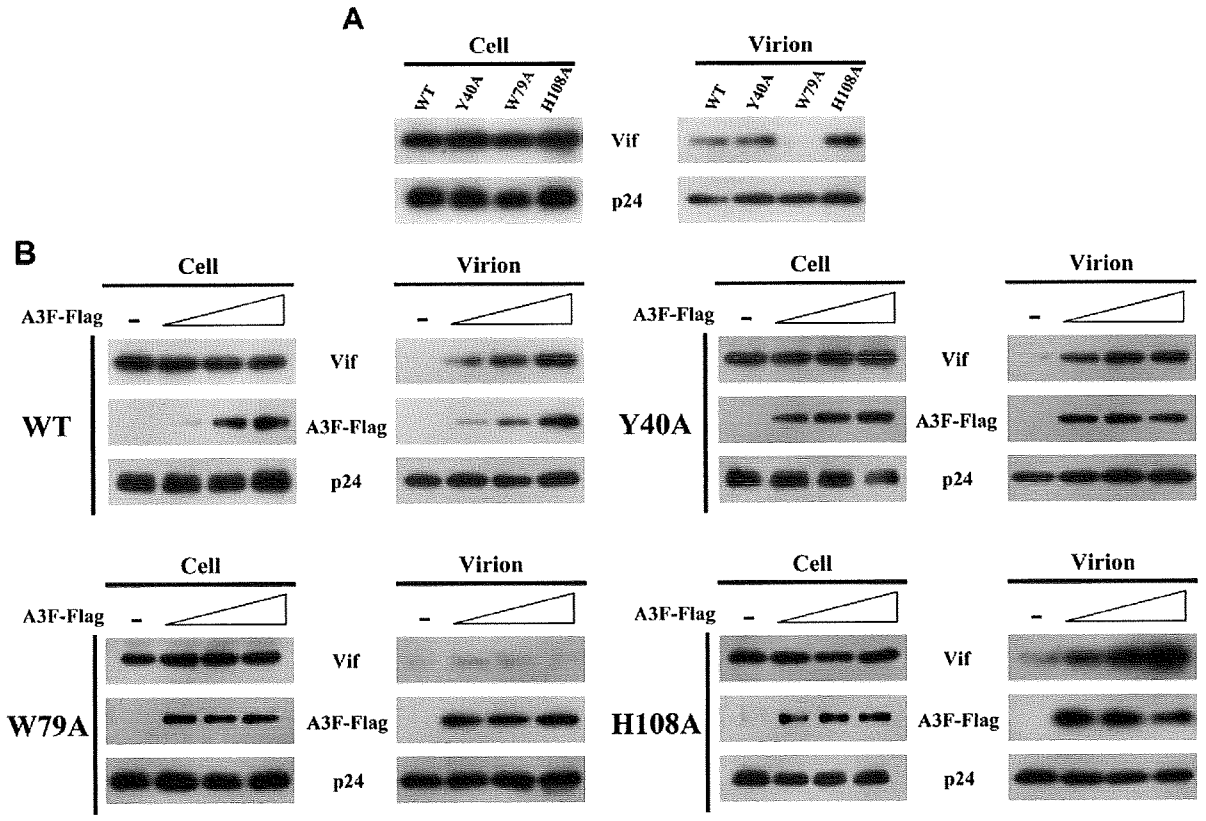


Fig. 5. Virion incorporation of mutant Vif proteins in the presence of A3F. (A) Comparison of Vif level in cells and virions prepared by a regular transfection; 293 T cells were co-transfected with each proviral clone (15 μg) and an A3F-expression vector (1 μg). (B) Vif and A3F levels in cells and virions with increasing amounts of A3F. 293T cells were co-transfected with a constant amount of proviral clone (15 μg) and increasing amounts of A3F-expression vector (0, 1, 2, and 3 μg), and levels of Vif, A3F, and Gag-p24 were monitored by immunoblotting.

A3F into virions of these mutants. As shown in Fig. 2D, incorporation of A3F into virions was efficiently suppressed by WT Vif but not by the other mutant Vif proteins. In summary, mutants W21A, S32A, and W38A did not bind to A3F, being unable to counteract the activity of A3F (Fig. 1B). Mutant Y40A was able to bind to A3F and suppress its anti-viral activity (Fig. 1B). This mutant was notable because it poorly reduce A3F expression in cells and its progeny virions carried a high level of A3F (Fig. 2). Mutant H43A showed a phenotype similar to that of Y40A but in a less effective manner (Fig. 1B).

3.2. Virion-incorporation of mutant Vif proteins

It has been reported that HIV-1 Vif is incorporated into virions through an interaction with viral RNA and associates with a nucleoprotein complex [22,23]. We were interested in determining whether interaction of Vif and A3G/A3F affects the virion-incorporation of Vif.

As shown in Fig. 3A, in the presence of A3G, a similarly low level of Vif was observed in virions of WT, Y40A, and W79A on a regular transfection condition. Virions of H108A, carrying a mutation within the degradation domain for A3G/A3F, contained a large amount of Vif. We next examined dose-dependent effect of A3G on Vif packaging (Fig. 3B). While expression level in cells of WT and mutant Vif proteins is A3G dose-independent, A3G enhanced the virion-packaging of Vif from WT, W79A, and H108A, which can bind to A3G. Mutant virions of Y40A, which lacks the binding ability, did not contain Vif. Furthermore, WT and W79A virions similarly had A3G in a dose-dependent manner, and those of Y40A and H108A contained a constantly high level of A3G. Based on a unique feature of H108A in Fig. 3, we examined the status of Vif/A3G in virions. As shown in Fig. 4, although WT and H108A virions both carry Vif and A3G, no complex between the two molecules was detected in those of H108A.

Virion-packaging of Vif in the presence of A3F is also monitored. As shown in Fig. 5A, Vif level in cells and virions was quite similar to that in Fig. 3A. Results for A3F dose-dependency of Vif level in cells and virions (Fig. 5B) were also as predicted from those in Figs. 2 and 3. Virions from W79A, which cannot bind to A3F [3], contained no Vif. Y40A and H108A showed a similar phenotype here but only the former neutralized A3F activity (Fig. 2A). Of interest, Y40A virions carried a constant level of A3F, being different from WT virions (Fig. 5B). In summary, all replication-defective mutants studied here contained A3G/A3F in their virions (Fig. 1B).

4. Discussion

In this study, we newly found that W21, S32, and W38 of HIV-1 Vif are important for its binding to A3F (Fig. 2), in addition to A3G reported by us [3]. Therefore, another FG-box, which acts as a binding motif for both A3G/A3F, may exist in neighboring place of G-box in the N-terminal domain (Fig. 1A). Our mutants of Y40 and H43 in the G-box (Y40A and H43A) behaved uniquely against A3G/A3F (Figs. 2, 3, and 5). While both mutants bind poorly to A3G and can not efficiently neutralize A3G activity [3], they did bind to A3F and suppress A3F activity. However, notably, Y40A and H43A virions contained a high level of A3F. Whether Y40A and H43A bind to A3F in virions remains to be determined. The results described above would represent a novel issue: amino acids or motifs around the G-box (Fig. 1A) may be critical for neutralization of A3G/A3F by unknown mechanism. Detailed studies on this area of HIV-1 Vif is required to have a definitive answer.

Another point of this study is whether incorporation of Vif into progeny virions is dependent on its binding to A3G/A3F. Since all replication-defective proviral point mutants examined so far in our studies (W21A, S32A, W38A, Y40A, H43A, Y69A, E76A, W79A, and H108A in reference [3] and this report) contain A3G/A3F in their virions, extensive analysis of the interaction between Vif and A3G/A3F in cells and virions would be critical to clarify virological basis for anti- A3G/A3F activity of Vif. Our results here clearly showed that mutant Vif proteins incapable of binding to A3G/A3F do not reduce the expression of A3G/A3F in cells, and that the mutant virions contain A3G/A3F (Figs. 3 and 5, and reference [3]). Results in Figs. 3 and 5 also indicated that virion packaging of Vif is dependent on its binding to A3G/A3F. Moreover, even mutant H108A, which can bind to A3G in cells and is unable to exclude it from virions (Fig. 3 and reference [3]), did not bind to A3G in virions.

In total, it can be concluded that exclusion of A3G/A3F from virions through degradation of A3G/A3F in cells by Vif and/or functional binding of Vif and A3G/A3F in virions is crucial for counteraction against antiviral activity of A3G/A3F (Fig. 1B).

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