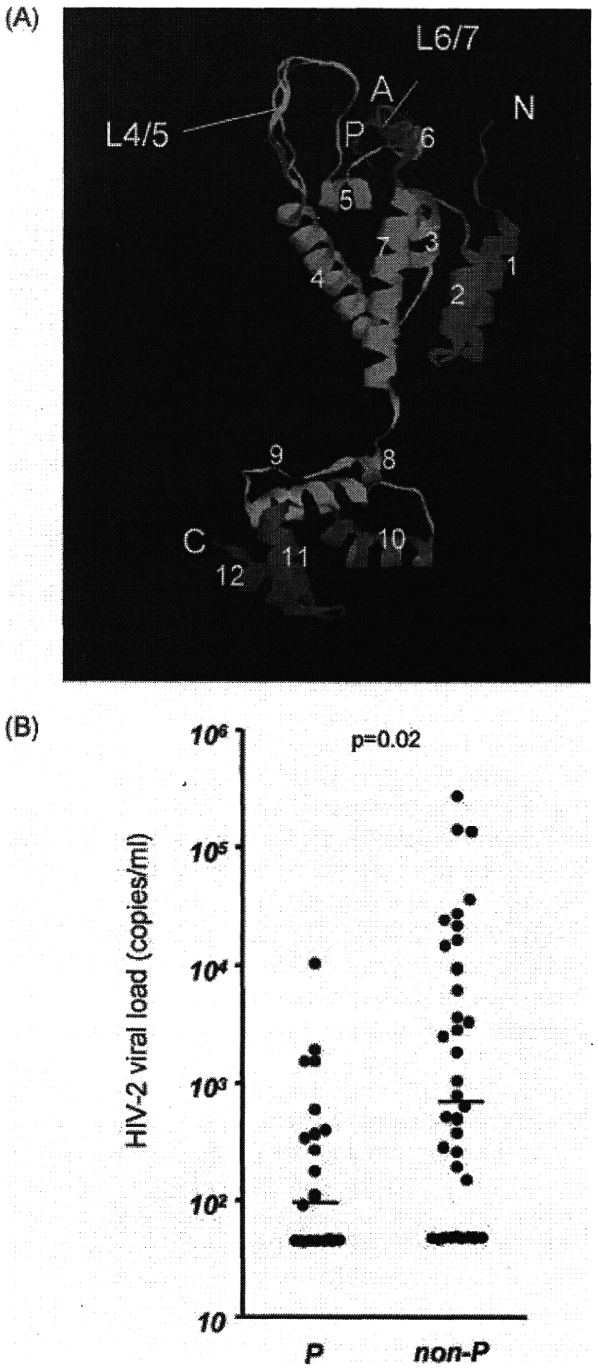

in Guinea Bissau comprising both high- and low-VL patients demonstrated that CA from viruses in low-VL patients had proline residues at the 119th or 120th position, but in patients with higher VL, the 119th or 120th position was frequently occupied by non-proline residues. Stratification of the subjects according to the presence or absence of proline at the 119th or 120th position showed a 3-fold difference in the median VL of the two groups (Figure 5B). These results demonstrate that HIV-2 replication in infected individuals can be linked to CA variation and human TRIM5α sensitivity [68].

In a case of Rh TRIM5α, Ylinen *et al.* mapped one of the determinants of TRIM5α sensitivity in a loop between α-helices 4 and 5 (L4/5) of HIV-2 [69]. They replaced the L4/5 of the SIVmac CA (CA 78 to CA97) with that of HIV-2 CA (CA78 to CA98) in the SIVmac backbone and found that the resultant mutant virus showed impaired growth ability in Rh cells compared with the parental SIVmac239. Although we could confirm this finding, the reciprocal virus with SIVmac239 CA L4/5 in the HIV-2 backbone did not gain resistance against Rh TRIM5α. However, we recently found that multiple components including L4/5 and L6/7 in the N-terminal half of SIVmac CA contributed to the escape of SIVmac from Rh TRIM5α (Kono *et al.* manuscript in preparation). Lin and Emerman also reported that SIVagmTAN with both HIV-1 L4/5 and L6/7 was susceptible to Rh TRIM5α restriction [70].

How do these regions in CA interact with specific regions in SPRY domain of TRIM5α? For this, it is necessary to obtain detailed and accurate structural information of the complex between viral CA

Figure 5. A single amino acid of HIV-2 CA affects 3-D structure of CA and its replication in infected individuals. (A) The 3-D models of six HIV-2 CAs were constructed with the homology-modeling technique. N and C indicate the amino termini and carboxyl termini, respectively. The ribbons represent the backbones of CA, and the 12 colour-coded-helices are numbered. P and A indicate a loop between α-helices 6 and 7 (L6/7) with a proline residue (in red) and an alanine residue (in blue), respectively, at position 119. (B) HIV-2 viral load (VL) correlates with amino acids variation at position 119 or 120 of CA. The differences in numbers were caused by the 1 amino acid insertion at the N-terminal part of CA in some HIV-2 isolates. Log-transformed VL for each patient in a West African community cohort is plotted. The patients were stratified according to the presence or absence of proline at position 119 or 120 (119P or non-P). Median VL of each group is indicated by a horizontal bar

and TRIM5α SPRY. Structural data on the binding surface between HIV-1 CA and TRIM5α SPRY of various primate species would also help to develop small molecular compounds that enhance anti-HIV-1 activity of human TRIM5α, and such compounds could be used as new therapeutic measures in HIV-1 infection.

ROLE OF CYCLOPHILIN A (CYPA)

The hydrophobic pocket of CypA makes direct contact with the proline residue at the 90th position and adjacent residues on the L4/5 of HIV-1 CA [71–73]. This interaction can be disrupted experimentally by mutations that alter CA proline 90 or adjacent residues [72], and by competitive inhibitors of the interaction including cyclosporine A (CsA) [74–76]. Disruption of CypA-CA interaction reduces HIV-1 susceptibility in human cells [72,74,76–80], with the block occurring early, at the time of reverse transcription [74]. These data have led to the hypothesis that, by binding to CA, CypA protects HIV-1 against antiviral restriction activity in human cells [81,82].

In OWM cell lines, however, CsA treatment has been reported to increase HIV-1 replication [15,83,84]. Concerning the relationship between CypA and TRIM5α, two groups reported that the simultaneous knock-down of CypA and TRIM5 in OWM cells caused minimal additional increase of HIV-1 infection compared with knock-down of TRIM5α alone, suggesting that CypA acts in trans to promote TRIM5α-mediated restriction of HIV-1 [85,86]. However, we demonstrated that exogenous expression of CypA suppresses HIV-1 infection in OWM cells in the absence of functional TRIM5α and that the inhibitory activity of CypA depends upon the interaction of CypA moiety with HIV-1 CA, while disruption of CypA-CA interaction by CsA treatment enhanced the HIV-1 susceptibility of OWM cells even in the absence of functional TRIM5α [41]. It is noteworthy that one of the two groups mentioned earlier observed slightly higher HIV-1 infection after simultaneous knock-down of CypA and TRIM5 than after knock-down of TRIM5 alone at the low-dose virus infection [85], suggesting that there is a TRIM5α-independent anti-HIV-1 activity mediated by CypA in OWM cells.

As already mentioned, L4/5 of SIVmac and HIV-2 CA is one of the determinants for Rh TRIM5α restriction, but it should be noted that SIVmac and HIV-2 CA L4/5s, unlike HIV-1 CA L4/5, do not bind to CypA [72].

CHIMERIC VIRUSES BETWEEN SIVMAC AND HIV-1 (SHIV)

In order to establish a monkey model of HIV-1/AIDS study, various SHIVs have been constructed and tested for their replicative capability in simian cells. The first SHIV was generated with a genetic backgbone of SIVmac containing HIV-1 *tat*, *rev*, *vpu* and *env* genes [11]. Although such an SHIV is useful for the analysis of humoral immune responses against Env protein [87–89], SHIVs containing other HIV-1 structural proteins, especially the Gag-Pol protein, were urgently needed since it is well known that the cellular immune response against Gag is important for disease control [90–92].

Construction of an SHIV with a minimal segment of SIVmac was reported by Kamada *et al.* [93]. This virus (NL-ScaVR, Figure 6A) contains the L4/5 of CA and the entire *vif* segment of SIVmac and was designed to escape from restrictions mediated by ApoB mRNA editing catalytic sub unit (APOBEC) 3G and CypA in OWM cells. APOBEC3G modifies the minus strand viral DNA during reverse transcription, resulting in impairment of viral replication [94–96], but this activity could be counteracted with the viral protein Vif [97–99]. Although HIV-1 Vif can potently suppress human APOBEC3G, it is not effective against Rh APOBEC3G, which explains at least partly the restriction of HIV-1 replication in monkey cells. As mentioned above, CypA directly binds to L4/5 of HIV-1 CA but not to SIVmac CA and augments HIV-1 infection in human cells but inhibits its replication in OWM cells [15,84,100].

NL-ScaVR could replicate in pig-tailed monkey primary CD4+ T cells as well as in the CM T cell line HSC-F. In both HSC-F and primary CD4+ T cells, NL-ScaVR grew to lower titers than did SIVmac [93], and inoculation into pig-tailed monkey with this SHIV did not cause CD4+ T cell depletion or any clinical symptoms [101]. In contrast, stHIV-1, another SHIV carrying 202 amino acid residues of SIVmac CA and *vif* generated by Hatziioannou *et al.*, could replicate more efficiently in Rh cells [102] and contains more of the SIVmac-derived CA segment than the virus reported by Kamada *et al.*, suggesting the presence outside of L4/5 of another determinant of intrinsic restriction factor(s) in CA.

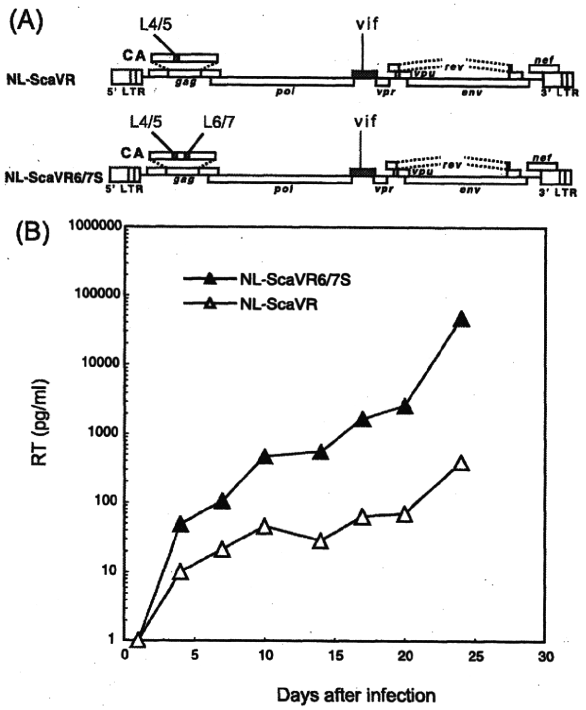

Figure 6. Modification of L6/7 of CA improves replication in CM cells of HIV-1 derivative. (A) Structure of chimeric viruses between simian immunodeficiency virus isolated from macaque (SIVmac) and HIV-1. White bars denote HIV-1 (NL4-3) and gray bars SIVmac239 sequences. (B) Equal amounts of NL-ScaVR (white triangles: virus with SIVmac L4/5 and *vif*), or NL-ScaVR6/7S (black triangles: virus with SIVmac L4/5, L6/7, and *vif*) were inoculated into CM CD4-positive T cell line HSC-F, and culture supernatants were collected periodically. The levels of reverse transcriptase (RT) in culture supernatants were measured by ELISA

As mentioned earlier, a single amino acid in L6/7 of HIV-2 CA was identified as a determinant of the susceptibility of HIV-2 to CM TRIM5α. Introduction of the entire SIVmac L6/7 of CA into the previously constructed version of HIV-1 derivatives with SIVmac L4/5 of CA and *vif* (NL-ScaVR6/7S, Figure 5A) caused only four amino acid changes in CA but showed improvement of replication capability of HIV-1 in CM cell line HSC-F (Figure 6B) and primary CD4+ T cells. While the high-dose inoculation of particles of wild type HIV-1 into monkey cells saturated endogenous TRIM5α and enhanced the subsequent infection with HIV-1, the introduction of both L4/5 and L6/7 of SIVmac greatly impaired the ability of HIV-1 particles to saturate TRIM5α [103]. These observations support the notion that TRIM5α recognises the overall structures composed of L4/5 and L6/7 of HIV-1 CA.

PROTEASOME-DEPENDENT AND PROTEASOME-INDEPENDENT MECHANISMS OF RESTRICTION

The RING domain containing proteins possess E3 ubiquitin ligase activity [33]. In fact, Rh TRIM5α was found to be poly-ubiquitinated and degraded rapidly via the ubiquitin-proteasome pathway [104], while disruption of the RING domain eliminated its auto-ubiquitination [104]. Furthermore, it was demonstrated that TRIM5α is degraded via the ubiquitin-proteasome pathway during HIV-1 restriction [105]. As for the antiviral activity of TRIM5α, however, deletion of the RING domain only partially attenuated its anti-HIV-1 activity [34,106], and modulation of E1 ubiquitin-activating enzyme expression did not affect TRIM5α-mediated restriction activity in a temperature dependent cell line [106]. Moreover, proteasome inhibitors did not affect TRIM5α mediated HIV-1 restriction but caused HIV-1 to generate late reverse transcripts during TRIM5α mediated restriction [107,108]. Proteasome-dependent and -independent pathways may therefore be involved in HIV-1 restriction by Rh TRIM5α (Figure 7). A proteasome-dependent HIV-1 restriction would mean co-degradation of TRIM5α with incoming viral capsids in proteasomes but 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 composed of recombinant HIV-1 CA-NC fusion protein with the purified 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 [38]. 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.

We recently found that the contribution of the RING domain to retrovirus restriction by TRIM5α differed among viral species. SIVmac completely escaped from attacks by RING mutants of AGM TRIM5α that could still moderately restrict HIV-1 infection. Addition of proteasome inhibitor MG132 had no effect at all on the anti-HIV-1 activity of AGM TRIM5α, but disrupted at

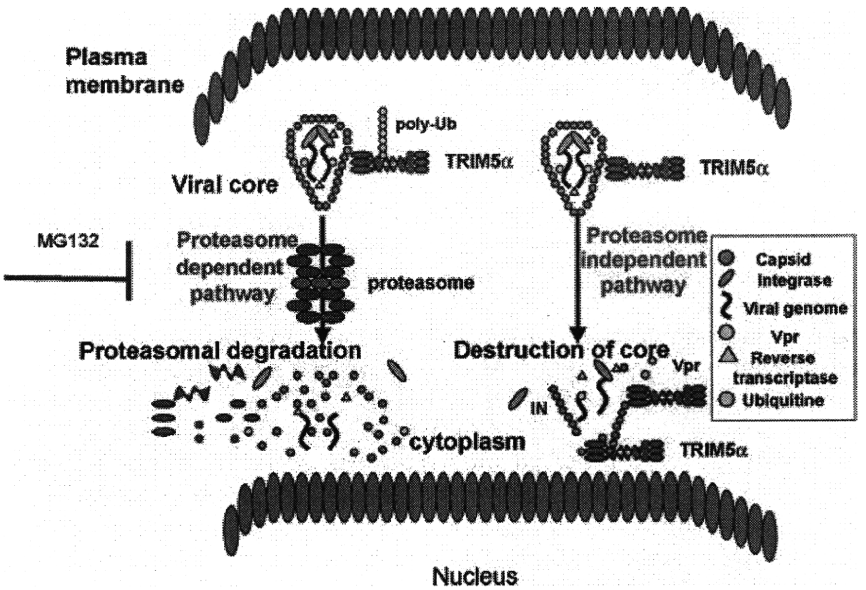

Figure 7. Current model of TRIM5α restriction pathways. (Left) A proteasome-dependent pathway. Oligomerized TRIM5α recognizes the incoming viral core through its SPRY domain and is poly-ubiquitinated in a RING-dependent manner. Ubiquitinated TRIM5α and the viral core complex are degraded in proteasome, while MG132 inhibits the activity of proteasome. (Right) A proteasome-independent pathway. Although RING mutant TRIM5α cannot ubiquitinate, HIV-1 CA and Rh or CM TRIM5α binding via the SPRY domain causes destruction of the viral core without any other cellular factors

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 CM TRIM5α is both proteasome-dependent and -independent (Maegawa *et al.* submitted).

POLYMORPHISMS IN HUMAN TRIM5 GENE

HIV-1 infection in humans is generally characterised by a long-term, chronic disease course gradually progressing to AIDS. Polymorphisms in human *CCR5* and other genes reportedly affect the susceptibility to HIV-1 transmission and/or the rate of disease progression to AIDS [109,110]. Sawyer *et al.* reported a common histidine-to-tyrosine polymorphism at the 43rd amino acid residue (H43Y) of the human *TRIM5α* gene[111]. This single nucleotide polymorphism (SNP) is located in the RING domain (Figure 2) and was shown to greatly reduce the ability of TRIM5α to restrict N-MLV [111]. Several studies have reported that the anti-HIV-1 activity of TRIM5α with 43Y was lower than that with 43H *in vitro* [111,112] which was confirmed in a previous study of ours, although the difference in anti-HIV-1 activity was very small [113].

Associations of H43Y with the rate of progression to AIDS have been tested in several studies, but with inconsistent results [111–115]. Previously, we compared 43Y frequencies of HIV-1 infected long-term non-progresssors and standard progressors in France and Japan and failed to find any differences in 43Y frequency between the two groups in either country, indicating that this SNP does not affect disease progression [113]. In spite of the lower anti-HIV-1 activity of TRIM5α with 43Y, Javanbakht *et al.* have found a paradoxical protective effect of TRIM5α with 43Y against HIV-1 transmission in African-American [111,112]. Interestingly, we also found that the 43Y-allele was found less frequently in Japanese and Indian HIV-1 infected subjects than in the ethnic-matched controls [116]. The reasons for the discrepancy between the epidemiological and functional effects of H43Y remain unclear at present and further studies are required to clarify the impact of H43Y on susceptibility to HIV-1 transmission and/or rate of progression to AIDS.

In contrast to HIV-1, HIV-2 replication was moderately inhibited by the wild type human TRIM5α [59], but not at all by human TRIM5α with 43Y (Maegawa *et al.* submitted). This suggests that H43Y polymorphism has a stronger impact on HIV-2 than on HIV-1 in infected individuals. It would thus be of interest to investigate whether this allele affects the rate of disease progression in HIV-2-infected individuals.

In the B-box domain, we recently found in Japan a novel and rare glycine-to-arginine substitution at the 110th position of TRIM5α (G110R) (Figure 2) and this 110R allele was observed more frequently in HIV-1-infected subjects than in controls. As was observed epidemiologically, this substitution weakened the anti-HIV-1 and anti-HIV-2 activity *in vitro* [116]. These findings together indicate that anti-HIV-1 activity of human TRIM5α can hardly protect human beings from an HIV-1 pandemic, but it may affect HIV-1 transmission.

EVOLUTION OF *TRIM5* GENE

TRIM5 homologs have been found in the genomes of primates, mouse, rat, dog, cow and pig, but not in chicken [30,117]. *TRIM5* homolog genes are found in large numbers in cow, rat and mouse, but the human genome contains only a single *TRIM5* gene, and the dog's homolog was inactivated by a transposon [118]. No antiviral activity against eight retroviruses has been reported for the TRIM5 homologs of mouse (TRIM12 and TRIM30) [117] and mouse TRIM30 targets TAB2 (TAK1-binding protein 2) for degradation [119]. It is speculated that human TRIM5α also interact with a molecule of certain signal transduction cascade.

The *TRIM5* gene varies considerably among primate species. The distribution of positively selected amino acid site is located in the SPRY domain and coiled-coil domains [44,120,121]. It is thus not surprising that the very beginning of SPRY domain (V1) is highly variable since TRIM5α interacts with the retroviral core through this region as discussed above, while the main pressure for positive selection may be endogenous retroviruses [122]. In contrast to that for SPRY polymorphisms, the hypothesis to explain positive selection operating on the coiled-coil domain is too speculative, since the well-established function of the coiled-coil domains is only to promote TRIM5α oligomerisation [35]. Interestingly, in Rh, there is a 339th-TFP-341st to Q polymorphism in TRIM5α (Figure 2) [121], which reduces the anti-HIV-2 activity [45]. Position 332 in human TRIM5α is arginine (R) and there is no polymorphism in the human genome, but P/Q was found in Rh and R/P/Q polymorphism in sooty mangabeys [121,123] (Figure 2). H43Y polymorphism was frequently found in human but not in monkey species [118]. We recently found that H43Y mutation in AGM TRIM5α failed to affect its antiviral activity (Nakayama *et al.*). Since H43Y substitution seems to be neutral in monkey TRIM5α, there is no advantage in its accumulation in monkey population.

Among New World monkeys, owl monkeys possess CypA as a fusion protein with TRIM5 (TRIMCyp) as a result of LINE-1-mediated retrotranspositional insertion [124,125]. CypA can bind to the CA of HIV-1, so that the TRIMCyp expressed in owl monkey cells recognises the HIV-1 core and shows an anti-HIV-1 effect. Retrotransposition of *CypA* into the *TRIM5* gene also occurred independently in OWM Rh and pig-tailed monkeys [22,126–129]. Rh TRIMCyp restricts infection of HIV-2 and feline immunodeficiency virus but not HIV-1 [129], and TRIMCyp expressed in pig-tailed monkey also lacks anti-HIV-1 activity [22,126,127]. A lack of functional TRIM5α expression in pig-tailed monkey enabled Hatziioannou *et al.* to construct an SHIV strain that differs from HIV-1 only in the *vif* gene and can efficiently replicate in pig-tailed monkeys [130].

CONCLUSION

Within 5 years, many studies have emphasised the impact of TRIM5α on retroviral infection. The variable regions of the SPRY domain of TRIM5α evolved under the pressure of viral infection, and surface exposed loops of viral CA played a critical role in its escape from restriction of TRIM5α. Detailed and accurate structural analysis of the binding surface between viral CA and TRIM5α SPRY is thus needed for the development of new antiretroviral drugs that enhance anti-HIV-1 activity of human TRIM5α.

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Contribution of RING domain to retrovirus restriction by TRIM5α depends on combination of host and virus

Hikoichiro Maegawa, Tadashi Miyamoto, Jun-ichi Sakuragi, Tatsuo Shioda, Emi E. Nakayama *

Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita-shi, Osaka 565-0871, Japan

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ABSTRACT

The anti-retroviral restriction factor TRIM5α contains the RING domain, which is frequently observed in E3 ubiquitin ligases. It was previously proposed that TRIM5α restricts human immunodeficiency virus type 1 (HIV-1) via proteasome-dependent and -independent pathways. Here we examined the effects of RING domain mutations on retrovirus restriction by TRIM5α in various combinations of virus and host species. Simian immunodeficiency virus isolated from macaque (SIVmac) successfully avoided attacks by RING mutants of African green monkey (AGM)-TRIM5α that could still restrict HIV-1. Addition of proteasome inhibitor did not affect the anti-HIV-1 activity of AGM-TRIM5α, whereas it disrupted at least partly its anti-SIVmac activity. In the case of mutant human TRIM5α carrying proline at the position 332, however, both HIV-1 and SIVmac restrictions were eliminated as a result of RING domain mutations. These results suggested that the mechanisms of retrovirus restriction by TRIM5α vary depending on the combination of host and virus.

Introduction

Replication of retroviruses is influenced by several factors in host cells. Tripartite motif protein (TRIM) 5α has been identified as a restriction factor of human immunodeficiency virus type 1 (HIV-1) in rhesus monkey (Rh) cells (Stremlau et al., 2004). Rh TRIM5α potently restricts HIV-1 but only weakly does so simian immunodeficiency virus isolated from macaque (SIVmac) (Stremlau et al., 2004; Song et al., 2005), whereas African green monkey (AGM) TRIM5α can potently restrict both HIV-1 and SIVmac (Nakayama et al., 2005; Song et al., 2005). TRIM5α consists of the RING, B-box 2, coiled-coil, and SPRY (B30.2) domains (Reymond et al., 2001). Differences in the amino acid sequences in the SPRY domain of TRIM5α of different monkey species were shown to affect the species-specific restriction of retrovirus infection (Perez-Caballero et al., 2005a; Nakayama et al., 2005; Sawyer et al., 2005; Stremlau et al., 2005; Yap et al., 2005). In addition, biochemical studies have shown that TRIM5α associates with retroviral capsid (CA) protein in detergent-stripped virions or with an artificially constituted core structure composed of capsid–nucleocapsid (CA–NC) fusion protein in a SPRY domain-dependent manner (Sebastian and Luban, 2005; Stremlau et al., 2006a). The SPRY domain is thus thought to recognize viral core. The coiled-coil domain of TRIM5α is important for the formation of homo-oligomers (Mische et al., 2005) and is essential for antiviral activity (Javanbakht et al., 2006). The intact B-box 2 domain is also required for TRIM5α mediated antiviral activity, since the restrictive activity of TRIM5α is diminished by amino acid substitutions in the B-box 2 domain (Javanbakht et al., 2005). RING containing proteins were frequently found to possess E3 ubiquitin ligase activity (Jackson et al., 2000). Indeed, Rh TRIM5α was poly-ubiquitinated and degraded rapidly via the ubiquitin-proteasome pathway, while disruption of the RING domain eliminated its auto-ubiquitination (Diaz-Griffero et al., 2006). Furthermore, it was demonstrated that TRIM5α is degraded via the ubiquitin-proteasome pathway during HIV-1 restriction (Rold and Aiken, 2008). However, deletion of the RING domain in TRIM5α only partially attenuates anti-HIV-1 activity (Javanbakht et al., 2005; Perez-Caballero et al., 2005b). Moreover, modulation of E1 ubiquitin-activating enzyme expression did not affect TRIM5α-mediated restriction activity in a temperature-dependent cell line (Perez-Caballero et al., 2005b) and finally, proteasome inhibitors did not affect TRIM5α mediated HIV-1 restriction (Anderson et al., 2006; Perez-Caballero et al., 2005b; Rold and Aiken, 2008; Stremlau et al., 2006a; Wu et al., 2006) even though they allowed HIV-1 to generate viral late reverse transcripts under TRIM5α mediated HIV-1 restriction (Anderson et al., 2006; Wu et al., 2006). The exact role of the TRIM5α RING domain in retrovirus restriction thus remains unclear.

In the study presented here, we investigated the effects of RING domain mutations on HIV-1 and SIVmac restrictions by TRIM5α and report that TRIM5α restricts HIV-1 and SIVmac differently.

* Corresponding author. Fax: +81 6 6879 8347.
E-mail address: emien@biken.osaka-u.ac.jp (E.E. Nakayama).

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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

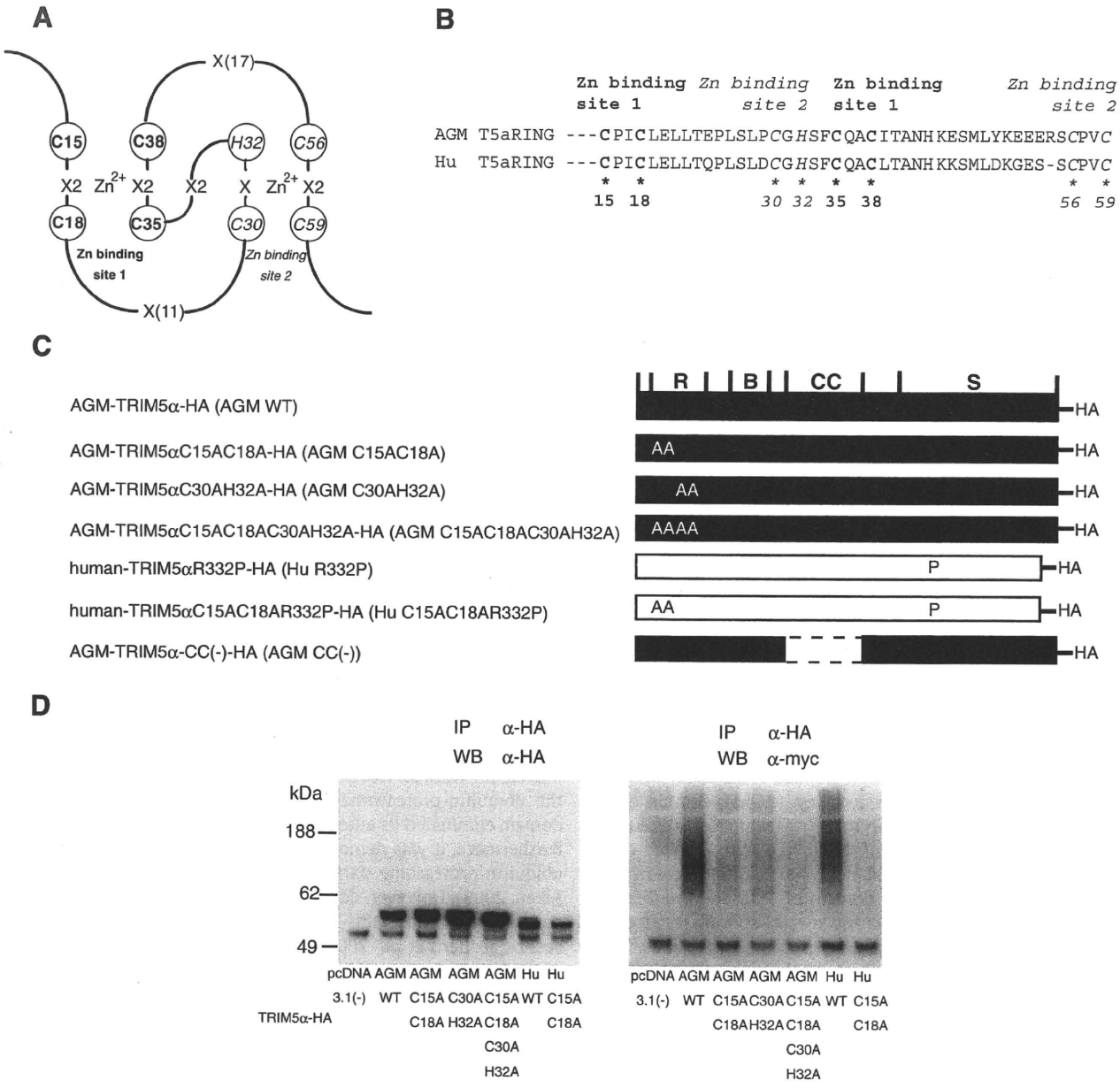


Fig. 1. Auto poly-ubiquitination of TRIM5α was impaired by RING domain mutations. (A) The RING finger zinc binding motif. The numbered AGM TRIM5α zinc-binding ligands are shown in circles. Each zinc atom is coordinated tetrahedrally by four ligands. Zinc-binding site 1 (**bold**) and site 2 (*italic*) 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

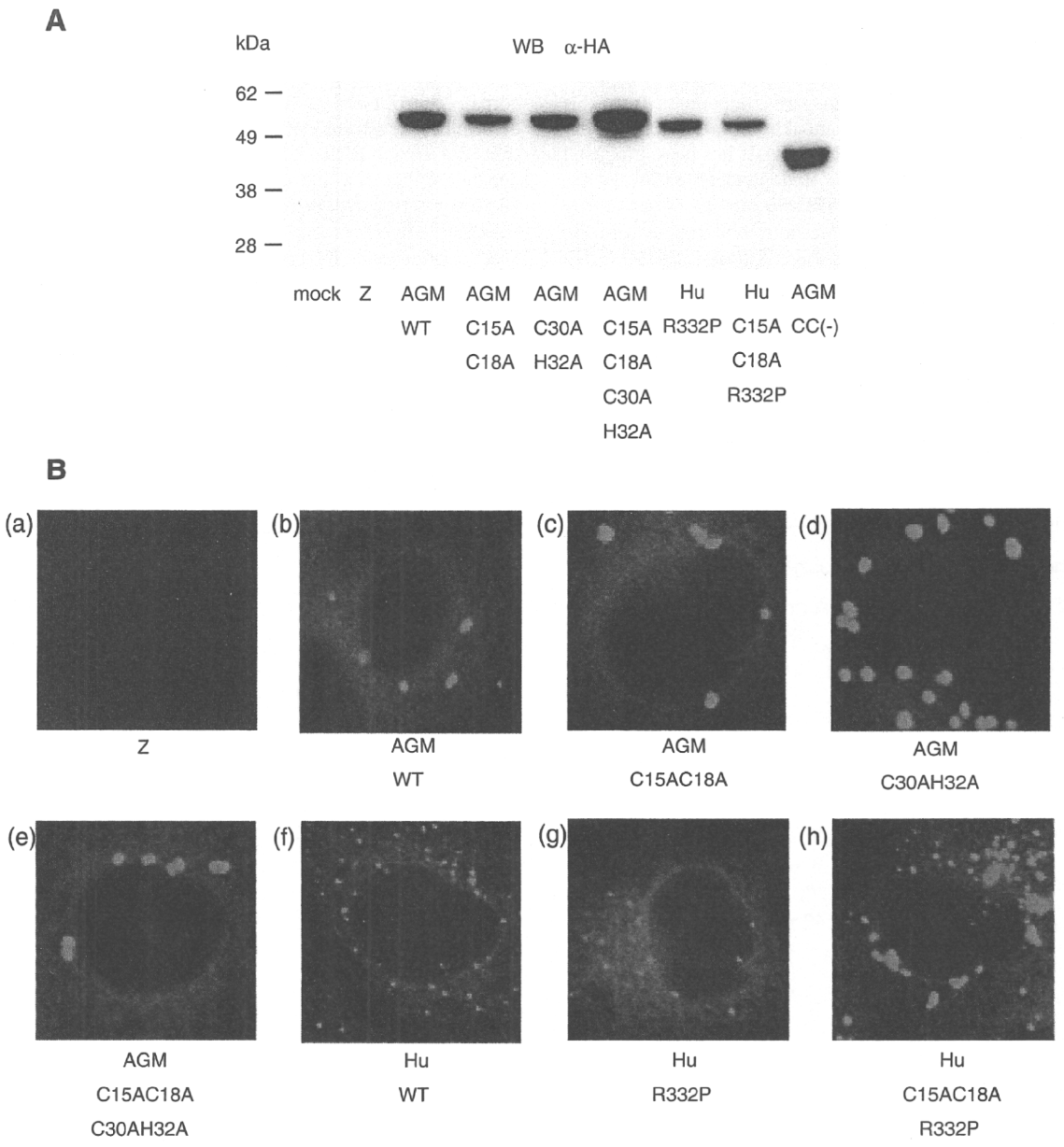


Fig. 2. Expression of RING mutant TRIM5α proteins. (A) Expression of various TRIM5αs. TRIM5α proteins in MT4 cells mock infected (mock) or infected with parental Z strain of SeV (Z), SeVs expressing AGM TRIM5α (AGM WT), AGM TRIM5α C15AC18A (AGM C15AC18A), AGM TRIM5α C30AH32A (AGM C30AH32A), AGM TRIM5α 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 conforcal 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.

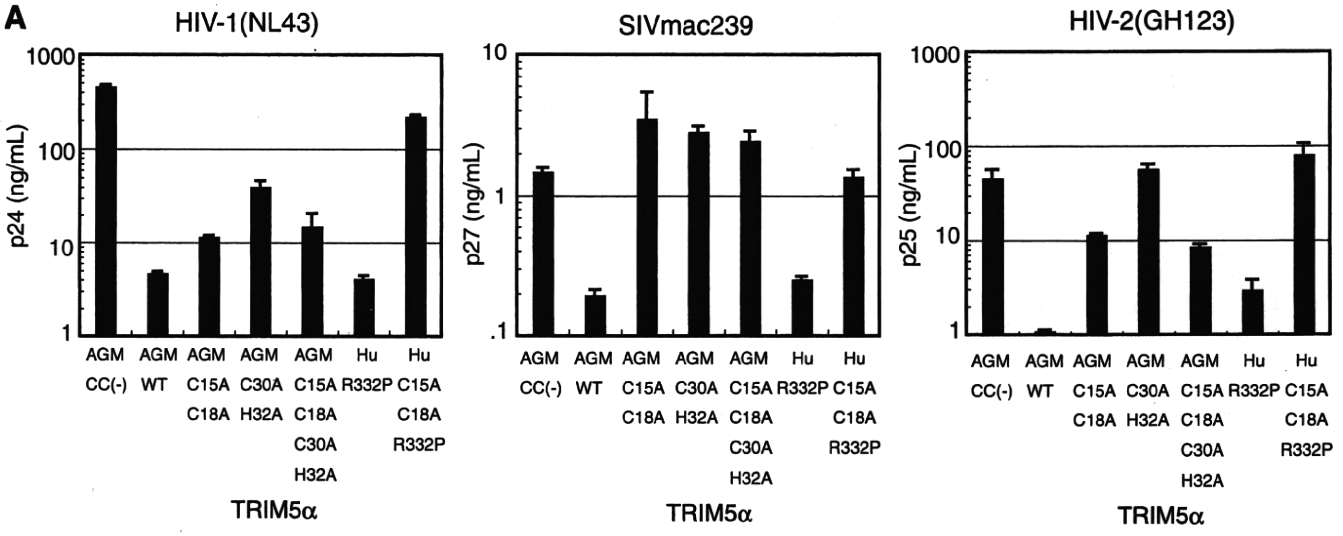
Contribution of RING domain to retrovirus restriction by human TRIM5α with arginine-to-proline substitution at the 332nd position

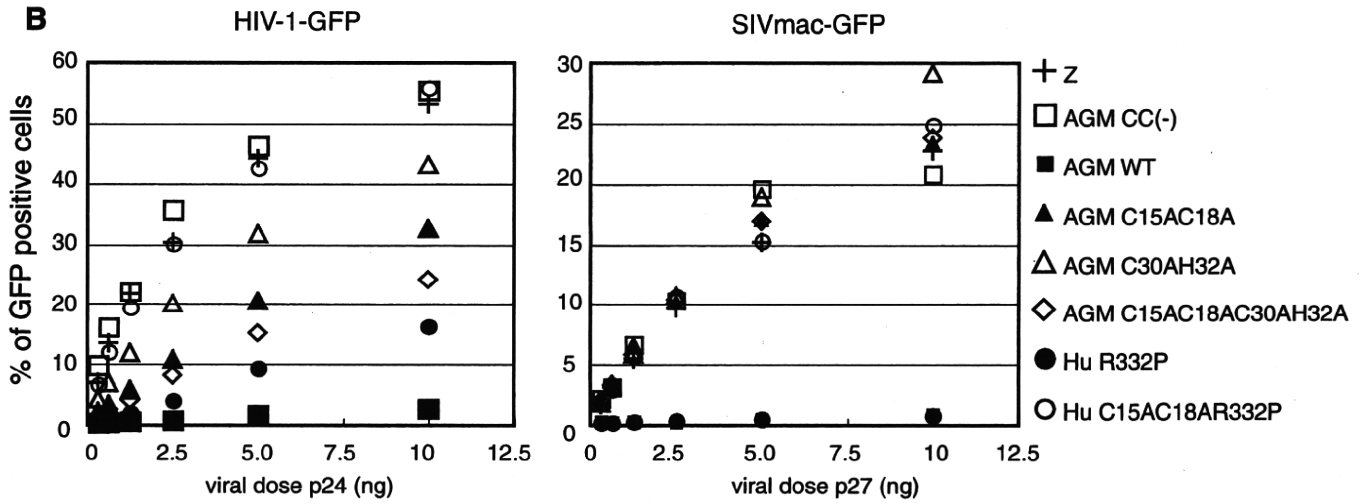
An arginine-to-proline substitution at the 332nd position (R332P) in the SPRY domain reportedly conferred strong anti-HIV-1 and anti-SIVmac activities to human TRIM5α (Stremlau et al., 2005; Yap et al., 2005). To determine whether cystein residue substitutions in the RING domain of human TRIM5α with 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.

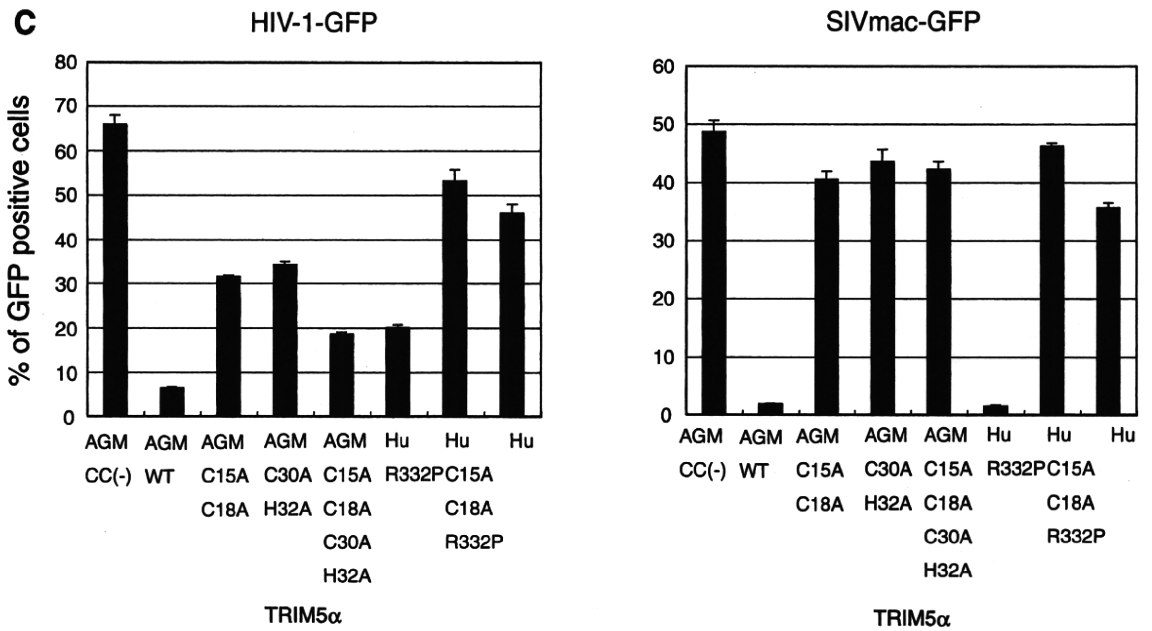
Effect of proteasome inhibition on antiviral activity of TRIM5α

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)
1000
100
10
1
.1
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

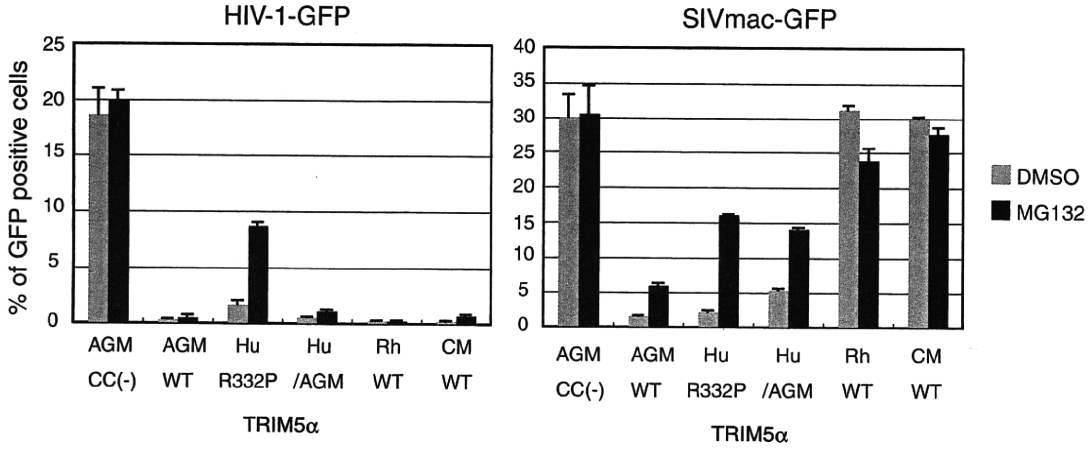


Fig. 4. The effect of proteasome inhibition on antiviral activity of TRIM5α depends on combinations of TRIM5α and viruses. MT4 cells were infected with SeVs expressing AGM CC (−), AGM WT, Hu R332P, Hu/AGM, Rh WT or CM WT. Cells were then superinfected with HIV-1-GFP or SIVmac-GFP in the presence of 10 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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