Inhibitory effect of human TRIM5α on HIV-1 production

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Abstract

Tripartite motif-containing 5 isoform-α (TRIM5α), a host restriction factor, blocks infection of some retroviruses at a post-entry, pre-integration stage in a species-specific manner. A recent report by Sakuma et al. describes a second antiretroviral activity of rhesus macaque TRIM5α, which blocks HIV-1 production through rapid degradation of HIV-1 Gag poly-proteins. Here, we find that human TRIM5α limits HIV-1 production. Transient expression of TRIM5α decreased HIV-1 production, whereas knockdown of TRIM5α in human cells increased virion release. A single amino acid substitution (R437C) in the SPRY domain diminished the restriction effect. Moderate levels of human wild-type TRIM5α and a little amount of R437C mutant were incorporated into HIV-1 virions. The R437C mutant also lost restriction activity against N-tropic murine leukemia virus infection. However, the corresponding R to C mutation in rhesus macaque TRIM5α had no effect on the restriction ability. Our findings suggest human TRIM5α is an intrinsic immunity factor against HIV-1 infection. The importance of arginine at 437 aa in SPRY domain for the late restriction is species-specific.

Key words: human TRIM5α; HIV-1 production; restriction factor; intrinsic immunity; MLV.

1. Introduction

The intrinsic host defense system has recently come to light as a key player in restricting retroviral infection. Several intrinsic factors important in limiting HIV-1 infection have been identified. APOBEC3G [1] and APOBEC3F [2] interfere with the replication of HIV-1 and other retroviruses via their cytidine deaminase activity [3, 4]. The membrane protein Tetherin blocks the release of HIV-1 and other enveloped viruses by tethering them to the cellular membrane [5]. TRIM5α has been shown to be a post-entry restriction factor that confers resistance to HIV-1 in a species-specific manner. Even though these restriction factors are thought to be constitutively expressed and active before pathogen invasion [6, 7], they are induced by interferon and therefore may constitute innate immune defenses [5, 8, 9].

TRIM5α has been proposed to bind the incoming viral capsid and interfere with uncoating [10, 11]. It has also been reported that TRIM5α possesses E3 ubiquitin ligase activity and a mutation in its RING finger domain decreases its restriction ability [12-14]. Other reports suggest that TRIM5α prevents late RT product formation and inhibits viral cDNA nuclear import [15, 16].

Recently, another antiretroviral activity of TRIM5α of rhesus monkey, TRIM5α_{rh}, has been described, which is inhibition of HIV-1 production at a post-translational stage by degradation of the Gag protein [17]. SIV is resistant to this restriction. However, another group argued against this effect of TRIM5α_{rh} [18] although both showed that overexpression of TRIM5α_{rh} down-regulates HIV-1 production.

TRIM5α is a member of the tripartite motif protein family and contains RING, B-box 2, and coiled-coil domains, as well as a carboxy-terminal B30.2 (SPRY) domain. The contribution of each domain to HIV-1 post-entry restriction had been well documented. The RING domain contributes to the potency of restriction but is not absolutely essential. The B-box 2, coiled-coil and SPRY domains of TRIM5α are necessary for antiretroviral activity.

The coiled-coil domain is indispensable for TRIM5α oligomerization, which increases the avidity of TRIM5α for the retroviral capsid. Both the coiled-coil and SPRY domains are essential for TRIM5α to bind the virion core [19]. Variation in the SPRY domain is thought to be responsible for species-specific differences in retroviral restriction [19, 20]. During the study for the late-restriction, the RING structure and B-box 2, coiled-coil and ensuing linker 2 domains are all found to be responsible, while the N-teriminal region, RING and B-box2 domains have been shown to be essential for interaction between TRIM5α_{rh} and HIV-1 Gag. The coiled-coil domain and linker 2 region may play an effector function in late-restriction as well as the known effect on formation of the cytoplasmic body including TRIM5α_{rh} [21].

Human TRIM5α (TRIM5α_{hu}) had been considered to have no antiviral activity on HIV-1 production. However, during the course of our study comparing the TRIM5s of various species including primate TRIM5α and rodent TRIM5, we do found that TRIM5α_{hu} potently blocked the release of HIV-1 when expressed at the same level with TRIM5α_{rh}. Here, we provide the evidences that TRIM5α_{hu} inhibits production of HIV-1 progeny at physiological concentrations, suggesting it plays an important role in intrinsic defense against HIV-1.

2. Materials and Methods

2.1 Cell culture

Human 293T, HeLa, HT1080, TZM-bl [22] and Plat-gp cells [23] were maintained in DMEM containing 10% FCS and antibiotics. Jurkat E6-1 cells (ATCC number: TIB-152) were cultured in RPMI1640 containing 10% FCS.

2.2 Plasmids

The plasmid pRhT5α, which encodes C-terminal hemagglutinin (HA)-tagged TRIM5α_{rh}, was provided by Dr. Ikeda. The plasmid encoding HA-tagged Trim5α_{hu} was obtained from Dr. Shioda, and designated pHuT5αWT. HA-tagged TRIM5α_{hu} cDNA harboring the R437C

mutation was amplified by RT-PCR from human peripheral blood mononuclear cells (PBMC) with a previously described primer pair [17], and kindly provided by Dr. Y. Ikeda. It was then cloned into pcDNA3.1(Invitrogen), the same vector that was used to generate pRhT5α and pHuT5αWT. The resultant plasmid was designated pHuT5αR437C. For construction of pRhT5αR441C, a corresponding mutant of TRIM5α_{hu}R437C, the plasmid pRhT5α was used as a template for QuikChange site-directed mutagenesis (Stratagene). To generate TRIM5α encoding retroviruses, the retroviral vector pMX-puro [24] and Plat-gp packaging cells were used. The HA-tagged TRIM5α open reading frames (ORFs) described above were amplified by PCR using primers huT5α-F-EcoRI (5'-GCGAATTCCACCATGGCTTCTGGAATCCTG-3'; Underline shows EcoRI site.) and NotI-HA-R (5'-AGATAAGAATGCGGCCGCTCAAGCGTAATCTGGAACATCG-3'; Underline shows NotI site.) and then cloned into EcoRI and NotI sites of pMX-puro. The resulting plasmid was designated pMX-T5α-HA-puro. All of the TRIM5α proteins possess a carboxy-terminal HA tag to allow detection of the expression level. The HIV-1 molecular clone pNLΔpolEGFP was constructed by inserting the EGFP ORF in the Nef coding region of pNLΔpol [25] that lacks a 328 base pair fragment in the polymerase coding region of pNL4-3 [26]. The resultant plasmid produces the p55 Gag precursor protein that is processed to p24 capsid protein. The plasmid of pYK-JRCSF [27], pNL4-3, pSIVmac239 [28], pSA212, encoding full length genome of SIVagm [29] and p89.6 [30], were obtained from Dr. Y. Koyanagi, Dr. Adachi, Dr. Mori, Dr. Miura, and AIDS Research and Reference Reagent Program, respectively. The β-galactosidase (β-gal) expression plasmid pCDM-β-gal [31] was used.

2.3 Transfection and viral infection

Co-transfection of one of pNLΔpolEGFP, pYK-JRCSF, pNL4-3, p89.6, pSA212 and pSIVmac239 with TRIM5α expression plasmids (pRhT5α, pHuT5αWT, pHuT5αR437C) or

empty vector pcDNA3.1 into 293T cells was carried out using Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer's instructions. Two days later, the culture supernatants were harvested and the p24 concentration was measured with p24 Gag ELISA assay kit (Zeptmatrix). The p24 concentration was divided by intracellular β-gal activity evaluated by standard colorimetric methods. The value of control pcDNA3.1-transfected cells was set as 1, and compared with the value of various TRIM5α expression plasmid-transfected cells.

To measure the infectivity of progeny viruses, TZM-bl cells, the HeLa cell derivatives that express human CD4 and CCR5 and contain a luciferase construct under the control of the HIV-1 promoter, were seeded in 24-well plate (5×10^4/well). The next day, the medium was removed and the cells were incubated with 250μl of HIV-1 or SIV harboring culture media for 3.5 h followed by adding 750μl of fresh media. Forty-eight hours after infection, cells were washed and lysed, and then luciferase activity was measured using Promega's BrightGlo luciferase assay system. To calculate the production rate of infectious virus, the ratio of luciferase activities against the β-gal activities in HIV-1/SIV producing 293T cell lysates was calculated and the value of control pcDNA3.1-transfected sample was set as 1.

2.4 Preparation of viral like particle (VLP)

The culture supernatants (2 ml) were harvested 48h post-transfection, and cellular debris was removed by centrifugation at 2000 rpm (Himac CF 7D2, HITACHI) for 20 min, followed by filtration of the supernatants through 0.45-μm pore size syringe filters (Millipore). The supernatants (1ml) were then concentrated by ultracentrifugation through a 20% (w/v in PBS) sucrose cushion for 2 h at 45,000 rpm (TLA100.4 rotor; Beckman) at 4°C. The pellets were resuspended in 15 μl of lysis buffer (10mM Tris-HCl (pH 7.4), 1mM $MgCl_2$, 140mM NaCl, 0.5% NP-40, and protease inhibitors (Roche)) and analyzed by Western blotting.

2.5 Generation of cells stably expressing TRIM5α variants

Recombinant retroviruses encoding various TRIM5α were produced in Plat-gp cells by co-transfecting constructed retroviral vector plasmids which encoded a TRIM5α ORF (pMX-huT5αWT-HA-puro, pMX-huT5αR437C-HA-puro, pMX-rhT5α-HA-Puro, pMX-agmT5α-HA-Puro) and pMD.G [32], a plasmid encoding the vesicular stomatitis virus (VSV) G envelope glycoprotein. Forty-eight hour post-transfection, the culture supernatants were harvested and then transduced to 293T or HeLa cells (2×10^5) in the presence of 10μg/ml of polybrene. Cells were then selected in medium containing 2μg/ml puromycin (Sigma).

2.6 Production and infection of pseudoviruses

VSV-G-coated HIV-1 pseudovirus expressing Venus (HIV-1-Venus) was prepared by transfecting 293T cells with a combination of CSII-EF-MCS-IRES2-Venus, pMDLg/pRRE, pMD.G and pRSV-Rev [32]. One day after transfection, culture medium was replaced with fresh DMEM containing 10% FCS, 1% non-essential amino acid, 1mM sodium pyruvate, 10μM forskolin and antibiotics. Two days later, the culture medium was collected, filtered through 0.45μm filter membrane and frozen at -80 . N-tropic and B-tropic murine leukemia viruses (MLVs) were generated by co-transfecting 3 plasmids (pLNCX-GFP, pMD.G, and pCIGS-N or pCIGS-B) [33] into 293T cells. Forty-eight hours post transfection, the culture media was harvested and stored at -80 .

VSV-G-pseudotyped HIV-1-Venus was infected in the presence of 10μg/ml of polybrene to HeLa cells that had been transduced with various TRIM5α encoding retroviruses followed by selection in the presence of puromycin for 3 days. Forty-eight hours post-infection, the cells were harvested, washed once with PBS, fixed with PBS containing 1% formaldehyde and then subjected to fluorescence-activated cell sorting (FACS) analysis with a FACScalibur (Becton Dickinson).

2.7 Immunoblotting

The cells were harvested in the lysis buffer as described above. The primary antibody for detection of the HA tag was anti HA 3F10 (Roche). HIV-1 p55/p24 Gag protein was detected using a murine anti-p24 mAb (V107 [34], a kind gift of Dr. K. Ikuta). The amount of cell lysates analyzed by SDS-PAGE was normalized by β-gal activity. The bands were visualized by ECL western blotting system (GE healthcare). The intensity of bands was quantified using LAS 1000 mini (Fuji film) when necessary.

2.8 Reduction of Trim5α expression by siRNA

A mixture of siRNAs (B-bridge international, Inc) against TRIM5α_{hu} with the target sequences 5'-CCUGAGAACAUACGGCCUAdTdT-3', 5'-GAGAAAGCUUCCUGGAAGAdTdT-3' and 5'-CUGAAAAGCCUUACGAACUdTdT-3' were co-transfected into 5×10^4 HT1080 and 1×10^5 293T cells at a final concentration of 50nM together with 0.01μg of pNLΔpolEGFP using Lipofectamine2000 (Invitrogen). Jurkat E6-1 cells (2×10^6) were electroporated with 50 pmol of anti-TRIM5α_{hu} or control siRNA and 0.2 μg of pNLΔpolEGFP by nucleofection (Amaxa) using cell line nucleofector kit V and the program X-01.

2.9 RT-PCR

Total RNA from siRNA-transfected cells was isolated using the Absolutely RNA kit (Stratagene) according to the manufacturer's instructions. First-strand cDNA was synthesized using 0.5 μg total RNA in a 20 μl reaction volume with the ThermoScript First-Strand Synthesis System (Invitrogen), and the product was subjected to PCR using primers for TRIM5α (forward: 5'-TGGAAGACTCAAATACAGTATGACAA-3'; reverse: 5'-CATCTAGTTTCAGAGTTCGTAAG-3') and for human G3PDH (forward: 5'- AAA TGA GCT TGA CAA AGT GGT CG-3'; reverse: 5'- GTA TGA CAA CAG CCT CAA GAT CA-3') as an internal control. In parallel, the TRIM5α cDNA was quantified by lightCycler (Roche) realtime PCR.

3. Results

3.1 Effect of TRIM5α_{hu} on HIV-1 production

While investigating whether TRIM5α from various species exhibited intrinsic immunity against HIV-1 infection, we used two TRIM5α_{hu} clones as controls. Unexpectedly overexpression of one of TRIM5α_{hu} clone in human 293T cells reduced HIV-1 p24 Gag production and the other did not. After sequencing the two human TRIM5αs, we found a mutation from arginine to cysteine at amino acid (a.a.) 437 in the latter TRIM5α clone (Fig. 1A, plasmid pHuT5αR437C).

To directly compare the effect of wild type and mutant TRIM5α_{hu} and TRIM5α_{rh} on HIV-1 production, we transfected 293T cells with the HIV-1 molecular clone pNLΔpolEGFP together with varying amounts of C-terminal HA-tagged TRIM5α encoding plasmids (pHuT5αWT, pRhT5α and pHuT5αR437C). Then we evaluated the p24 levels in the culture supernatants. As shown in Fig. 1B, the production of p24 was not blocked by expression of the TRIM5α_{hu} R437C mutant, but was markedly suppressed by TRIM5α_{hu} wild type and TRIM5α_{rh} in a dose dependent manner. TRIM5α_{rh} looked more potent as an inhibitor than TRIM5α_{hu} wild type (Fig.1B). In parallel, intracellular expression of p24 was reduced as levels of pHuT5αWT and pRhT5α increased, but was unaltered in cells transfected with pHuT5αR437C (Fig. 1C). Since all TRIM5α proteins were expressed similarly at protein level in the transfected cells (Fig. 1C), these results suggest that TRIM5α_{hu} possesses the ability to restrict HIV-1 replication at late stage in its life cycle. Note that we used a half amount of pRhT5α plasmid for transfection since we found a half amount pRhT5α expressed an equal amount of TRIM5α-HA protein in 293T cells compared to pHuT5αWT and pHuT5αR437C. Since the vector for expression of TRIM5α_{hu} and TRIM5α_{rh} are the same, the difference of the expression level of TRIM5α was not due to the

difference of the promoter. Probably, TRIM5α_{rh} is more stable than TRIM5α_{hu} in 293T cells. To further confirm that TRIM5α_{hu} has restriction effect on HIV-1 production, we transfected 293T cells with infectious HIV-1 or SIV molecular clones together with a plasmid encoding various TRIM5α. We measured the p24 concentration in culture medium and also evaluated the viral titers by infection of progeny viruses to the TZM-bl indicator cells. As shown in Fig. 1D, TRIM5α_{hu} as well as TRIM5α_{rh} efficiently block the progeny virus production of JRCSF, NL4-3 and 89.6 in a dose dependent manner. In contrast, TRIM5α_{hu} R437C slightly limited the progeny viral production only when the highest amount of pHuT5αR437C was co-transfected. Meanwhile, neither TRIM5α_{hu}, TRIM5α_{rh} nor TRIM5α_{hu} R437C had significant effect on production of SIVmac239 and SIVagm (SA212) (Fig. 1D, lower panel). These observations clearly show the inhibitory effect of TRIM5α_{hu} on HIV-1 production.

3.2 Incorporation of TRIM5α into HIV-1 virions

Previous studies have shown that TRIM5α_{rh} associates with HIV-1 precursor Gag and is incorporated into HIV-1 virions [17]. Since our ELISA results indicated that p24 levels were significantly lower in the medium of TRIM5α_{rh}- as well as wild type TRIM5α_{hu}-transfected cells (see Fig. 1B), we analyzed the formation of virion like particles (VLPs) in the culture media and the incorporation of TRIM5α. As shown in Fig. 2, p24 levels in the VLP fraction were decreased in a dose dependent manner by TRIM5α_{hu} and TRIM5α_{rh} but not by TRIM5α_{hu} R437C, supporting the inhibitory effect of TRIM5α_{hu} on virus production and consistent with the results of the p24 ELISA (See Fig. 1B). However, while TRIM5α_{rh} was readily detected in VLP lysates and was inversely correlated with P24 levels, less amount of wild type TRIM5α_{hu} was detected in VLP fractions, and the mutant TRIM5$_{hu}$ was only marginally existent or even absent (Fig. 2A). These results suggest TRIM5α_{hu} has weaker

affinity for Gag than TRIM5α_{rh}, which is consistent with our finding that TRIM5α_{rh} inhibited HIV-1 more strongly than wild type TRIM5α_{hu} (Fig. 1B).

3.3 The corresponding mutation in TRIM5α_{rh} has no effect on its late restriction activity

An important question is that whether this arginine is also required for rhesus TRIM5α, since it had been reported that SPRY domain of TRIM5α_{rh} is dispensable to inhibit HIV-1 production [17, 21]. We mutated arginine residue to cysteine at 441a.a. of TRIM5α_{rh}, which corresponds 437a.a. of TRIM5α_{hu}, and selected a clone that can express the HA-tagged mutant TRIM5α_{rh} protein at the same level as the wild type. We co-transfected the TRIM5α_{rh} mutants with pNLΔpolEGFP to 293T cells, and then investigated the effect of the mutant on HIV-1 production and encapsidation into virions. As shown in Fig. 2B, R441C TRIM5α_{rh} mutant restricted HIV-1 production as severely as the wild type, and similar amounts of both wild type and mutant TRIM5α_{rh} proteins were incorporated into virions. These results are in consistence with Sakuma's report [17, 21].

3.4 Knockdown of endogenous TRIM5α restores HIV-1 production

To examine the inhibitory effect of TRIM5α_{hu} under physiological conditions, siRNA was used to suppress TRIM5α_{hu} expression. Human HT1080 or 293T cells were co-transfected with pNLΔpolEGFP and control siRNA or siRNA against TRIM5α_{hu}. As shown in Fig. 3A, p24 production was enhanced roughly 3 fold upon knockdown of TRIM5α_{hu} in HT1080 cells but was not altered in 293T cells in which TRIM5α_{hu} was expressed at low levels. In parallel, the amount of cellular TRIM5α_{hu} mRNA was markedly reduced in HT1080 cells, but much less in 293T cells (Fig. 3A left panel). It was noted that 293T cells produced over 170 times more p24 (58.68 ng/ml and 0.34 ng/ml, respectively) and expressed 110 times higher β-galactosidase activities (data not shown) than HT1080 cells, probably because of the better efficiency of transfection to 293T cells.

Next, we examined the effect of TRIM5α_{hu} knockdown on HIV-1 production in HIV-1 host cells. As a first step, we examined the level of expression of TRIM5α_{hu} in several T and macrophage cell lines (such as Jurkat, Molt4, MT-4, U937, and HL60) by quantitative RT-PCR and found that Jurkat E6-1 cells express the highest level of TRIM5α_{hu} mRNA (supplemental information Fig. 1). We then transfected pNLΔpolEGFP to these T cell lines by electroporation and evaluated the p24 production in the culture media. While JurkatE6-1, Molt4, and MT-4 showed similar transfection efficiency, MT-4 produced highest level of p24 (2.34 ng/ml) compared with Molt4 and JurkatE6-1 (250pg/ml and 114 pg/ml, respectively). This is reverse relation to the levels of Trim5α_{hu} mRNA (supplemental information Fig. 2). Next we examined the effect of TRIM5α knockdown on HIV-1 production in JurkatE6-1 cells. siRNA against TRIM5α_{hu} together with pNLΔpolEGFP were electroporated into the cells. Forty-eight hours later, the abundance of TRIM5α_{hu} mRNA was reduced to 35%, and concomitantly the amount of p24 Gag in the culture media was increased about 2.5 times compared to control siRNA (Fig. 3B). These results demonstrate that endogenous TRIM5α_{hu} is able to restrict HIV-1 progeny production.

3.5 Effect of the TRIM5α_{hu} R437C mutant on HIV-1 entry

The mutation R437C in TRIM5α_{hu} is located in the SPRY domain, in which alteration of a single amino acid has been reported to modulate TRIM5α restriction potency against HIV-1 infection at an early stage [35]. Therefore, we examined whether there was any difference between wild type TRIM5α_{hu} and the R437C mutant in restricting HIV-1 infection early in infection. At first we examined the effects of various Trim5αs, which were transiently expressed in 293T cells by transfection of pHuT5αWT, pRhT5α, and pHuT5αR437C, on the efficiency of infection of HIV-1-Venus that had been pseudotyped with VSV-G glycoprotein. The cells expressing TRIM5α_{hu}WT and TRIM5α_{hu}R437C showed slightly decreased rate of

HIV-1-Venus infection compared with the control vector pcDNA3.1-transfected cells, whereas TRIM5α_{rh}-expressing cells were markedly resistant (Fig. 4A). To confirm this result, using a retrovirus vector we established stable HeLa cells that express TRIM5α_{hu}WT, TRIM5α_{hu}R437C, and TRIM5α_{agm}. The cells were then infected with pesudotyped HIV-1-Venus as above. Similarly, TRIM5α_{agm}-expressing cells were markedly resistant to infection, By contrast, the marginal inhibitory effect on HIV-1 infection was observed for the cells expressing TRIM5α_{hu}WT and TRIM5α_{hu}R437C (Fig. 4C). Since all TRIM5αs were identically expressed (Fig. 4B and D), the difference in HIV-1 infection efficiency was not due to differences in TRIM5α levels. These results suggest that the mutation at residue 437 of TRIM5α_{hu} has subtle effect on HIV-1 infection at the post-entry stage.

3.6 Effect of TRIM5α_{hu} R437C on N- and B-MLV infection

TRIM5α_{hu} has been shown to restrict N-tropic but not B-tropic MLV infection [10]. Since the coiled-coil and the SPRY domains have been reported to be involved in this processes, we investigated whether the amino acid substitution at residue 437 in the SPRY domain alters the ability of TRIM5α_{hu} to inhibit N-tropic MLV infection. Wild type and mutant TRIM5α expressing 293T cells were established and infected with VSV-G-pseudotyped N-tropic and B-tropic MLV at various doses. In comparison with 293T cells transduced with the empty MX-puro vector, cells expressing wild type TRIM5α_{hu} or TRIM5α_{agm} were markedly resistant to infection with N-MLV but susceptible to B-tropic MLV, consistent with previous reports [36] (Fig. 5A). N-tropic MLV was infected to the R437C mutant expressing cells as efficiently as cells transduced with the empty MX-puro vector. Cells expressing TRIM5α_{rh} were susceptible as well, which is consistent with the published data [37]. These results indicate that the amino acid 437 in the SPRY domain is

involved in the ability of TRIM5α_{hu} to suppress both HIV-1 production and N-MLV infection.

4. Discussion

Sakuma et al. reported an inhibitory effect of TRIM5α_{rh} at a late phase of HIV-1 replication [17]. In this study we extended this finding to TRIM5α_{hu} and showed that this effect is specific, using a loss of function point mutant (TRIM5α_{hu}R437C). Although TRIM5α_{hu} has weaker ability to block the HIV-1 production than TRIM5α_{rh}, the knockdown experiment clearly showed that endogenous TRIM5α_{hu} do inhibit the HIV-1 production. The ability of TRIM5α_{hu} to reduce the production of HIV-1 some extent has also been reported previously [supplementary Fig.2 of 17, 18]. Accordingly our results suggest that endogenous TRIM5α_{hu} functions as an innate immunity molecule that reduces HIV-1 production.

The RING, B-box2 and coiled-coil motifs (RBCC) of TRIM5α_{rh} have been reported to be essential for blocking HIV-1 production. Particularly RING and B-box domains have been identified to regulate the interaction between TRIM5α_{rh} and HIV-1 Gag, while the coiled-coil domain determines the late restriction activity [21]. Our results suggest that the SPRY domain of TRIM5α_{hu} is also involved in this restriction and that arginine at reside 437 is important, since the arginine to cysteine mutation severely abolished HIV-1 inhibition (Fig. 1B and C). However, its importance is limited to TRIM5α_{hu}, as introduction of corresponding mutation into TRIM5α_{rh} did not alter the restriction activity of TRIM5α_{rh}. TRIM5α_{hu} specific effect of the Arg to Cys mutation is concordant with the reduction of affinity between TRIM5α and Gag, which was demonstrated by the encapsidation of TRIM5α into VLP. Since the affinity of TRIM5α_{hu} to Gag was naturally weaker than that of TRIM5α_{rh}, the effect of the mutation in TRIM5α_{hu}, but not TRIM5α_{rh}, may become phenotypically apparent.

The critical motif on the restriction at early stage of retroviral infection has been reported to lie between residues 332-340 of TRIM5α_{hu}, which shows the greatest sequence diversity among human, rhesus and African green monkey TRIM5αs [36, 38, 39]. For example, a single amino acid substitution (R332P) conferred the ability to restrict HIV-1 to TRIM5α_{hu} [39, 40]. A change of tyrosine 336 to alanine or lysine TRIM5α_{hu} enabled restriction of B-MLV, NB-tropic Moloney MLV and SIVmac [41, 42]. The arginine at residue 437 of TRIM5α_{hu} is located outside the motif and conserved among human, rhesus and rodent Trim5s (accession numbers: NM_001014023.1, NM_175677.4, and NP_001014045). Therefore, our results reveal that the C-terminal conserved region of the SPRY domain is also involved in the interaction of TRIM5α to HIV-1 Gag so as to play an important role in restriction of both HIV-1 production and human resistance to MLV infection. Since rodents are susceptible to MLV infection, the other portion besides Arg437a.a. of TRIM5α_{hu} should be also involved in the restriction effect on N-MLV infection.

Although Sakuma et al. reported that overexpression of TRIM5α_{rh} reduced both HIV-1 p55 and p24 levels, we observed reduction only of p24, while p55 levels remained constant (Fig. 1C). Instead, we noted TRIM5α_{hu} dependent reduction of p38, a processing intermediate of the HIV-1 Gag protein. This difference may be ascribed to more efficient Gag processing in our system, or insensitive detection of p55 by the anti Gag monoclonal antibody [34] used in this study. These results are not inconsistent with the proposed hypothesis that TRIM5α reduces HIV-1 production by degradation of HIV-1 Gag polyproteins. However, we cannot rule out the possibility that TRIM5α_{hu} may inhibit HIV-1 production by a different mechanism, because only low incorporation of TRIM5α_{hu} was observed in HIV-1 VLPs in contrast to the abundant incorporation of TRIM5α_{rh}. TRIM5α_{hu} might be involved in the HIV-1 Gag maturation rather than degradation of the polyproteins.

Although HIV-1 is known to replicate well in human cells, HIV-1 infection generally progresses to a latent stage that shows few or no symptoms and that can persist for decades. Adaptive immune responses such as those mediated by cytotoxic T cells (CTL) and the humoral system suppress viral replication during chronic infection. Aspects of the innate immune system can also contribute to viral suppression during chronic infection. For example, Apobec3G is induced in macrophages by IFN-α and reduces HIV-1 production even if the virus expresses the anti-Apobec3G factor Vif [9]. The ability of TRIM5α_{hu} to restrict HIV-1 production suggests that it may also constitute an innate immunity factor that functions to lower virus replication levels and elicit a long nonsymptom phase.

A considerable number of polymorphisms in TRIM5α_{hu} have been documented. Although the majority of them are not associated with susceptibility to HIV-1 infection [43, 44, 45], one common nonsynonymous single nucleotide polymorphism (SNP), R136Q, affects acquisition of HIV-1 infection [45]. A recent report by Torimiro et al. [46] revealed that about 4% of Baka pygmies in Cameroon were heterozygous for a truncation mutant of TRIM5α (R332X), which completely loses the ability to restrict HIV-1 infection. The Trim5α_{hu}R437C mutant in our study was amplified from the cDNA from peripheral blood mononuclear cells of an individual, suggesting the existence of another SNP related to retrovirus infection susceptibility although it cannot be ruled out that the mutation was introduced during PCR.

Taken together, our data provides evidence that endogenous human TRIM5α possesses suppressive activity at the step of HIV-1 progeny production, supporting the hypothesis that it comprises part of the innate immune system that limits HIV-1 replication. This notion is in line with data showing IFN-α treatment increases the levels of TRIM5α_{hu} mRNA and enhances antiviral activity against N-MLV infection [47]. IFN-α treatment also up-regulates TRIM5α mRNA in rhesus monkey cells, which correlates with enhanced TRIM5α-mediated pre- and post-integration restriction of HIV-1 replication [8]. Further investigation of the

mechanisms by which TRIM5α_{hu} prevents production of HIV-1 may provide valuable information for antiviral immune therapy.

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References

[1] A.M. Sheehy, N.C. Gaddis, J.D. Choi, M.H. Malim, Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein, Nature 418 (2002) 646-650.

[2] H.L. Wiegand, B.P. Doehle, H.P. Bogerd, B.R. Cullen, A second human antiretroviral factor, APOBEC3F, is suppressed by the HIV-1 and HIV-2 Vif proteins, EMBO J 23 (2004) 2451-2458.

[3] A. Takaori-Kondo, APOBEC family proteins: novel antiviral innate immunity, Int J Hematol 83 (2006) 213-216.

[4] R. Goila-Gaur, K. Strebel, HIV-1 Vif, APOBEC, and intrinsic immunity, Retrovirology 5 (2008) 51.

[5] S.J. Neil, T. Zang, P.D. Bieniasz, Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu, Nature 451 (2008) 425-430.

[6] P.D. Bieniasz, Intrinsic immunity: a front-line defense against viral attack, Nat Immunol 5 (2004) 1109-1115.

[7] M. Emerman, How TRIM5alpha defends against retroviral invasions, Proc Natl Acad Sci U S A 103 (2006) 5249-5250.

[8] R. Sakuma, A.A. Mael, Y. Ikeda, Alpha interferon enhances TRIM5alpha-mediated antiviral activities in human and rhesus monkey cells, J Virol 81 (2007) 10201-10206.

[9] G. Peng, K.J. Lei, W. Jin, T. Greenwell-Wild, S.M. Wahl, Induction of APOBEC3 family proteins, a defensive maneuver underlying interferon-induced anti-HIV-1 activity, J Exp Med 203 (2006) 41-46.

[10] M.J. Perron, M. Stremlau, M. Lee, H. Javanbakht, B. Song, J. Sodroski, The human TRIM5alpha restriction factor mediates accelerated uncoating of the N-tropic murine leukemia virus capsid, J Virol 81 (2007) 2138-2148.

[11] M. Stremlau, M. Perron, M. Lee, Y. Li, B. Song, H. Javanbakht, F. Diaz-Griffero, D.J. Anderson, W.I. Sundquist, J. Sodroski, Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor, Proc Natl Acad Sci U S A 103 (2006) 5514-5519.

[12] D. Perez-Caballero, T. Hatziioannou, F. Zhang, S. Cowan, P.D. Bieniasz, Restriction of human immunodeficiency virus type 1 by TRIM-CypA occurs with rapid kinetics

and independently of cytoplasmic bodies, ubiquitin, and proteasome activity, J Virol 79 (2005) 15567-15572.

[13] D. Perez-Caballero, T. Hatziioannou, A. Yang, S. Cowan, P.D. Bieniasz, Human tripartite motif 5alpha domains responsible for retrovirus restriction activity and specificity, J Virol 79 (2005) 8969-8978.

[14] T. Hatziioannou, D. Perez-Caballero, S. Cowan, P.D. Bieniasz, Cyclophilin interactions with incoming human immunodeficiency virus type 1 capsids with opposing effects on infectivity in human cells, J Virol 79 (2005) 176-183.

[15] L. Berthoux, S. Sebastian, E. Sokolskaja, J. Luban, Lv1 inhibition of human immunodeficiency virus type 1 is counteracted by factors that stimulate synthesis or nuclear translocation of viral cDNA, J Virol 78 (2004) 11739-11750.

[16] X. Wu, J.L. Anderson, E.M. Campbell, A.M. Joseph, T.J. Hope, Proteasome inhibitors uncouple rhesus TRIM5alpha restriction of HIV-1 reverse transcription and infection, Proc Natl Acad Sci U S A 103 (2006) 7465-7470.

[17] R. Sakuma, J.A. Noser, S. Ohmine, Y. Ikeda, Rhesus monkey TRIM5alpha restricts HIV-1 production through rapid degradation of viral Gag polyproteins, Nat Med 13 (2007) 631-635.

[18] F. Zhang, D. Perez-Caballero, T. Hatziioannou, P.D. Bieniasz, No effect of endogenous TRIM5alpha on HIV-1 production, Nat Med 14 (2008) 235-236; author reply 236-238.

[19] M. Stremlau, M. Perron, S. Welikala, J. Sodroski, Species-specific variation in the B30.2(SPRY) domain of TRIM5alpha determines the potency of human immunodeficiency virus restriction, J Virol 79 (2005) 3139-3145.

[20] B. Song, B. Gold, C. O'Huigin, H. Javanbakht, X. Li, M. Stremlau, C. Winkler, M. Dean, J. Sodroski, The B30.2(SPRY) domain of the retroviral restriction factor

TRIM5alpha exhibits lineage-specific length and sequence variation in primates, J Virol 79 (2005) 6111-6121.

[21] R. Sakuma, S. Ohmine, Y. Ikeda, Determinants for the rhesus monkey TRIM5{alpha}-mediated block of the late phase of HIV-1 replication, J Biol Chem (2009).

[22] X. Wei, J.M. Decker, H. Liu, Z. Zhang, R.B. Arani, J.M. Kilby, M.S. Saag, X. Wu, G.M. Shaw, J.C. Kappes, Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy, Antimicrob Agents Chemother 46 (2002) 1896-1905.

[23] S. Morita, T. Kojima, T. Kitamura, Plat-E: an efficient and stable system for transient packaging of retroviruses, Gene Ther 7 (2000) 1063-1066.

[24] M. Onishi, T. Nosaka, K. Misawa, A.L. Mui, D. Gorman, M. McMahon, A. Miyajima, T. Kitamura, Identification and characterization of a constitutively active STAT5 mutant that promotes cell proliferation, Mol Cell Biol 18 (1998) 3871-3879.

[25] Y. Iwakura, T. Shioda, M. Tosu, E. Yoshida, M. Hayashi, T. Nagata, H. Shibuta, The induction of cataracts by HIV-1 in transgenic mice, AIDS 6 (1992) 1069-1075.

[26] A. Adachi, H.E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, M.A. Martin, Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone, J Virol 59 (1986) 284-291.

[27] Y. Koyanagi, S. Miles, R.T. Mitsuyasu, J.E. Merrill, H.V. Vinters, I.S. Chen, Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms, Science 236 (1987) 819-822.