Role of TRIM5 α RING Domain E3 Ubiquitin Ligase Activity in Capsid Disassembly, Reverse Transcription Blockade, and Restriction of Simian Immunodeficiency Virus $\nabla^{\dagger}$

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The mammalian tripartite motif protein, TRIM5 α , recognizes retroviral capsids entering the cytoplasm and blocks virus infection. Depending on the particular TRIM5 α protein and retrovirus, complete disruption of the TRIM5 α RING domain decreases virus-restricting activity to various degrees. TRIM5 α exhibits RING domain-dependent E3 ubiquitin ligase activity, but the specific role of this activity in viral restriction is unknown. We created a panel of African green monkey TRIM5 α (TRIM5 α_{AGM}) mutants, many of which are specifically altered in RING domain E3 ubiquitin ligase function, and characterized the phenotypes of these mutants with respect to restriction of simian and human immunodeficiency viruses (SIV_{mac} and HIV-1, respectively). TRIM5 α_{AGM} ubiquitin ligase activity was essential for both the accelerated disassembly of SIV_{mac} capsids and the disruption of reverse transcription. The levels of SIV_{mac} particulate capsids in the cytosol of target cells expressing the TRIM5 α variants strongly correlated with the levels of viral late reverse transcripts. RING-mediated ubiquitylation and B30.2(SPRY) domain-determined capsid binding independently contributed to the potency of SIV_{mac} restriction by TRIM5 α_{AGM} . In contrast, TRIM5 α proteins attenuated in RING ubiquitin ligase function still accelerated HIV-1 capsid disassembly, inhibited reverse transcription, and blocked infection. Replacement of the helix-4/5 loop in the SIV_{mac} capsid with the corresponding region of the HIV-1 capsid diminished the dependence of restriction on TRIM5 α RING function. Thus, ubiquitylation mediated by the RING domain of TRIM5 α_{AGM} is essential for blocking SIV_{mac} infection at the stage of capsid uncoating.

Following entry into the cells of certain mammalian species, some retroviruses encounter blocks prior to reverse transcription (5, 22, 25, 52, 71, 79). For example, human immunodeficiency virus type 1 (HIV-1) infection is blocked at this stage in the cells of Old World monkeys (25, 52, 71). The simian immunodeficiency virus of macaques (SIV_{mac}) encounters similar early blocks in the cells of New World monkeys and African green monkeys (25). These early postentry blocks are mediated by TRIM5 α , and interspecies variation in TRIM5 α accounts for species-specific patterns of viral restriction (23, 32, 62, 73, 75, 88). Differences in susceptibility to TRIM5 α are determined by the retroviral capsid protein (8, 58).

TRIM5 α is a member of the tripartite motif (TRIM) family of proteins, which contain RING, B-box, and coiled-coil (RBCC) domains (50, 65). The α isoform of TRIM5 also has a carboxy-terminal B30.2(SPRY) domain, which mediates specific capsid recognition and is important for antiretroviral activity (10, 41, 53, 57, 60, 63, 67, 74, 76, 89). The coiled-coil and B-box2 domains contribute to the avidity of TRIM5 α for the retroviral capsid by mediating the dimerization and higher-order association, respectively, of TRIM5 α proteins (11, 27, 31, 38, 43, 44, 51). Some primate species express a TRIMCyp

protein, in which the RBCC domains of TRIM5 are fused to cyclophilin A (6, 7, 45, 54, 56, 68, 81, 83). TRIMCyp proteins block the infection of a subset of retroviruses whose capsid proteins bind cyclophilin A (6, 7, 12, 37, 45, 46, 54, 56, 68, 69, 80, 81, 83). In cells expressing potentially restricting TRIM5 α or TRIMCyp proteins, particulate retroviral capsids are converted to soluble capsid proteins more rapidly than in control cells (10, 11, 13, 28, 64, 77). Thus, potent TRIM5 α and TRIMCyp proteins promote premature, accelerated capsid disassembly.

The zinc-binding RING finger domain is the signature of a class of E3 ubiquitin ligases involved in proteasome-mediated protein degradation and other aspects of protein regulation (3, 18, 26, 50). Ubiquitin (Ub) ligation has been shown to be important for the ability of some TRIM proteins to mediate their effects on a target protein; in some cases, this leads to the proteasome-dependent turnover of the targeted protein, but in other cases, modulation of protein function or even stabilization of the protein results (19, 30, 39, 50, 55). TRIM5 α possesses E3 ubiquitin ligase activity and can ubiquitylate itself (87). Deletion of the TRIM5 α /TRIMCyp RING domain or the disruption of RING domain folding by alteration of the zinc-binding cysteine residues results in various effects on retroviral inhibition, depending on the TRIM5 protein and the restricted virus (12, 29, 48, 60, 75). The SIV_{mac}-restricting activity of African green monkey TRIM5 α (TRIM5 α_{AGM}) is severely attenuated by alteration of RING domain cysteines (48). However, because RING domain changes can affect TRIM5 α steady-state levels, intracellular localization, higher-order self-

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association, or capsid binding (14, 29, 34; X. Li and J. Sodroski, unpublished observations), and because alteration of RING domain cysteines likely disrupts multiple functions, mechanistic interpretation of this observation is complicated. Nonetheless, these cysteine changes in the TRIM5 α_{AGM} RING domain or complete deletion of the rhesus macaque TRIM5 α (TRIM5 α_{rh}) RING domain only partially attenuated HIV-1-inhibiting activity (29, 60). Moreover, TRIM5 α_{AGM} and TRIM5 α_{rh} efficiently restrict HIV-1 infection in the presence of proteasome inhibitors (2, 61, 66, 71, 84). Furthermore, owl monkey TRIMCyp blocks HIV-1 infection and human TRIM5 α blocks N-tropic murine leukemia virus (N-MLV) infection in cells expressing a temperature-sensitive ubiquitin-activating (E1) enzyme, even at the non-permissive temperature (61). Thus, while the integrity of the RING domain is important for TRIM5 α -mediated restriction of some retroviruses (48), the precise mechanistic role of the RING domain, ubiquitylation, or the proteasome in retroviral restriction is still uncertain.

Here, we investigate the basis for the dramatic difference in SIV $_{mac}$ restriction mediated by the TRIM5 α proteins from two African green monkey subspecies, both of which restrict HIV-1 infection. A RING domain alteration was found to be responsible for the difference in SIV $_{mac}$ restriction potency. Subsequent study of SIV $_{mac}$ restriction by a panel of RING mutants implicated E3 ubiquitin ligase activity in TRIM5 α_{AGM} -mediated premature disassembly of the SIV $_{mac}$ capsid and disruption of reverse transcription. TRIM5 α RING-mediated ubiquitin ligase function and B30.2(SPRY) domain-mediated capsid binding independently contributed to SIV $_{mac}$ restriction. In contrast, the E3 ubiquitin ligase activity of the RING domain exerted little impact on the effect of TRIM5 α_{AGM} on HIV-1 capsid disassembly, reverse transcription, or infection. Replacement of the helix-4/5 loop of the SIV $_{mac}$ capsid with the corresponding region from the HIV-1 capsid diminished the requirement for TRIM5 α_{AGM} ubiquitin ligase activity in the restriction process.

MATERIALS AND METHODS

Cell lines and cloning. HeLa, HEK293T, and Cf2Th cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. To generate stable cell lines expressing TRIM5 α variants, recombinant retrovirus vectors were produced, using the pLPCX vector plasmid and a packaging system (pVPack-GP and pVPack-VSV-G; Stratagene), as described previously (75). The retroviral vectors were used to transduce either HeLa or Cf2Th cells, followed by selection in either 1 μ g/ml or 5 μ g/ml puromycin, respectively. Once the cell lines were generated, the level of TRIM5 α expressed stably in the cells was determined by Western blotting with an antibody (Roche) directed against the hemagglutinin (HA) epitope tag on the TRIM5 α proteins (see below).

The TRIM5 genes from the *tantalus* and *pygerythrus* subspecies of African green monkeys were originally cloned by PCR amplification from COS-1 and Vero cells, respectively, as previously reported (73). All TRIM5 genes were cloned by using the EcoRI and ClaI sites in pLPCX; the encoded TRIM5 α proteins have an HA tag at their C termini. All mutations were introduced by using overlap PCR and were confirmed by sequencing at the Dana-Farber Cancer Institute (DFCI) Molecular Biology Core Facility. The chimeras between TRIM5 $\alpha_{AGM}(\text{Tan})$ and TRIM5 $\alpha_{AGM}(\text{Pyg})$ or between TRIM5 $\alpha_{AGM}(\text{Tan})$, TRIM5 $\alpha_{AGM}(\text{Pyg})$, and TRIM5 α_{rh} were made by swapping the corresponding gene fragments generated by combinations of EcoRI, BstXI, SphI, BamHI, and ClaI.

SIV $_{mac}$ (HIV 4/5) contains an SIV $_{mac}$ capsid with the helix-4/5 loop replaced by that of HIV-1. To generate the SIV $_{mac}$ (HIV 4/5) proviral clone, a PstI-SpeI

fragment from the HIV-1 *gag-pol* gene was used to replace the corresponding fragment of the SIV $_{mac}$ - Δ Nef Δ Env-GFP plasmid.

Single-round infection assay. Recombinant green fluorescent protein (GFP)-expressing viruses [SIV $_{mac}$ -GFP, HIV-1-GFP, and SIV $_{mac}$ (HIV-1 H4/5)-GFP] pseudotyped with the vesicular stomatitis virus (VSV) G glycoprotein were generated by transient transfection of 293T cells, using either the calcium phosphate method or Lipofectamine 2000, as described previously (25, 73, 75). For the single-round infection assay, virus stocks were serially diluted 2-fold and used to infect 24-well plates seeded with 2×10^4 cells the day before. After 48 to 60 h of incubation at 37°C, each well was washed with phosphate-buffered saline (PBS), and the cells were detached and fixed with 3.7% paraformaldehyde. The percentage of GFP-positive cells was analyzed by fluorescence-activated cell sorting (FACS).

The relative levels of SIV $_{mac}$ infection in cells expressing different TRIM5 α variants were calculated using the data from three independent single-round infections. In making the comparison, we used the lowest dose of virus that yielded >99% GFP positivity in the control target cells that had been transduced with the empty LPCX vector. At this virus dose, the percentage of GFP-positive cells observed in the target cells expressing a particular TRIM5 α variant was divided by the percentage of GFP-positive cells observed in the control LPCX-transduced cells to derive the relative SIV $_{mac}$ infection level.

TRIM5 α oligomerization and half-life. TRIM5 α -expressing Cf2Th cell lines were seeded in 6-well plates a day in advance. Cells were lysed with MPER lysis buffer (Pierce) containing 1 $\times$ protease inhibitor cocktail (Roche). After centrifugation at $15,000 \times g$ for 10 min at 4°C, the soluble fractions were retrieved, and a Bradford assay (Bio-Rad) was used to measure the concentration of total protein in the lysates.

For cross-linking TRIM5 α , the same amount of protein from each lysate was mixed with increasing amounts (0, 0.5, and 2 mM) of ethylene glycol bis-[sulfo-succinimidylsuccinate] (sulfo-EGS) (Pierce) and incubated at room temperature for 30 min. The reactions were stopped with 1 M Tris-HCl, pH 8.0. The cross-linked TRIM5 α proteins were analyzed by Western blotting with an anti-HA antibody.

To measure the half-lives of TRIM5 α variants, TRIM5 α -expressing Cf2Th cells were incubated in medium containing cycloheximide at a final concentration of 100 μ g/ml. Cells were harvested at regular intervals, washed with PBS, and kept on ice until the final batch of cells was harvested. The cells were then lysed in MPER lysis buffer, as described above. The same amount of protein from each lysate was analyzed by SDS-PAGE and Western blotting to detect the HA-tagged TRIM5 α protein.

TRIM5 α binding to HIV-1 and SIV $_{mac}$ capsids. The HIV-1 and SIV $_{mac}$ capsid-nucleocapsid (CA-NC) fusion proteins were purified from IPTG (isopropyl- β -D-thiogalactopyranoside)-induced *Escherichia coli* as previously described (20). The assay measuring the binding of TRIM5 α variants to the assembled HIV-1 CA-NC complexes was performed as previously described (77). To assemble SIV $_{mac}$ CA-NC complexes, 0.6 mM purified protein was mixed with 16 μ M (TG) $_{50}$ DNA oligonucleotide in 50 mM Tris-HCl, pH 7.0, and 1 M NaCl and incubated at 37°C for more than 1 day. The resulting SIV $_{mac}$ CA-NC complexes were negatively stained and examined under the electron microscope; these studies confirmed that the diameters and lengths of the cylindrical CA-NC complexes are similar to those of previously reported HIV-1 CA-NC complexes (20) (see Fig. S3 in the supplemental material).

Approximately 2×10^6 HEK293T cells were transfected to express the different TRIM5 α proteins transiently and then harvested 24 h later. The cells were lysed in 0.2 ml of hypotonic lysis buffer (10 mM Tris-HCl, pH 7.0, 10 mM KCl, and 1 mM EDTA), and a concentrated NaCl solution was added to achieve a final concentration of 200 mM. After a brief centrifugation at 4°C to remove large cell debris, the supernatant was spun at $110,000 \times g$ for 1 h at 4°C in a Beckman ultracentrifuge. After this preclearing spin, the retrieved supernatant was brought up to a final volume of 200 μ l, with the supernatant prepared in parallel from cells transfected with the empty pLPCX plasmid (if necessary). To these mixtures, the same amount of SIV $_{mac}$ CA-NC complexes was added and incubated at 30°C for 1 h. After incubation, 20 μ l of the mixture was saved for further analysis ("input"). The rest of the mixture was layered onto a 60% (wt/vol) sucrose cushion (prepared in 1 $\times$ PBS) and centrifuged at $110,000 \times g$ for 1 h at 4°C in a swinging-bucket rotor. The pellet was resuspended in SDS sample buffer and subjected to SDS-PAGE. The gels were Western blotted for the bound TRIM5 α proteins with an anti-HA antibody and stained with Coomassie brilliant blue to visualize the SIV $_{mac}$ CA-NC proteins. Control experiments indicated that, in the absence of added SIV $_{mac}$ CA-NC complexes, no TRIM5 α proteins were detected in the pellets (data not shown).

In vitro E3 ubiquitin ligase activity. The E3 ubiquitin ligase activity of a TRIM5 α variant was assessed by measuring self-ubiquitylation *in vitro*. Either

FIG. 1. A single RING domain residue determines the potency of SIV_{mac} restriction by TRIM5_αAGM variants. (A) The domains of TRIM5_α are depicted above, with the locations of the major variable regions (v1, v2, and v3) of the B30.2(SPRY) domain represented. The amino acid residues that differ between TRIM5_αAGM(Tan) (Tan) and TRIM5_αAGM(Pyg) (Pyg) are shown in single-letter code below. The B30.2(SPRY) region chimeras are depicted. B, B-box2 domain. (B) The steady-state expression levels of the TRIM5_α variants were determined by Western blotting with an antibody directed against the C-terminal HA epitope tag. As a reference control, the Western blots were probed with an antibody against

transfected HEK293T cells transiently expressing TRIM5 α or Cf2Th cells stably expressing TRIM5 α were used in this assay. Cells were harvested, washed with PBS, and then lysed in either RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 $\times$ protease inhibitor cocktail) or NP-40 buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP-40, and 1 $\times$ protease inhibitor cocktail). After cell debris was removed by brief centrifugation, 5 μ g of anti-HA antibody (Sigma) was added to the soluble fraction and incubated at 4°C for 1 h or overnight. Magnetic protein G beads (Invitrogen), preequilibrated with the lysis buffer, were used to precipitate the complex of TRIM5 α and anti-HA antibody, washed twice with the same lysis buffer and once with HEPES buffer (50 mM HEPES, pH 8.0, 200 mM NaCl, and 0.5 mM dithiothreitol [DTT]), and resuspended in 10 μ l HEPES buffer. The protein G-bead suspension was mixed with 0.44 μ g E1, 0.34 μ g UbcH5a, and 20 nM myc-ubiquitin (final concentration) in either the presence or absence of the Energy regeneration system (1 $\times$ Energy regeneration solution and 1 mM ATP, final concentration) in a reaction volume of 20 μ l. UbcH5a was used as the E2 enzyme for all *in vitro* ubiquitylation assays, except when the different E2 enzymes were screened in the experiments shown in Fig. S2C in the supplemental material. All of the components were purchased from Boston Biochem. The mixture was incubated on a 37°C shaker for different lengths of time. The reactions were stopped by adding SDS sample buffer, and the proteins were resolved on SDS-polyacrylamide gels. The unmodified, monoubiquitylated, and polyubiquitylated TRIM5 α proteins were detected using an anti-HA antibody. Control experiments demonstrated that the ratio of polyubiquitylated TRIM5 α protein to the unmodified form was a reliable indicator of TRIM5 RING E3 ubiquitin ligase activity (data not shown). The anti-myc Western blot was used to verify that the *in vitro* ubiquitylation reaction was successful. Because the anti-myc Western blot detects the ubiquitin-charged E1 enzyme and other potential cellular contaminants, the anti-HA Western blot was used for quantification of the E3 ubiquitin ligase activity of the TRIM5 α protein of interest. In two or three independent experiments, the amounts of unmodified and polyubiquitylated TRIM5 α proteins were measured using a densitometer. After subtracting the background polyubiquitylated TRIM5 α signal observed in the absence of the added Energy regeneration mixture, the ratio of polyubiquitylated to unmodified TRIM5 α protein for each TRIM5 α variant was normalized to that observed for wild-type TRIM5 $\alpha_{AGM(Tan)}$. The relative E3 ubiquitin ligase activity is expressed as a percentage of the activity of wild-type TRIM5 $\alpha_{AGM(Tan)}$, which is set at 100%.

Measurement of viral reverse transcription products. One day prior to infection, 5×10^5 Cf2Th cells were seeded in 6-well plates. The next day, the cell medium was replaced by 1 ml fresh medium containing recombinant viruses, prepared as described above. Each virus stock used for measurement of viral cDNA production was quantitated by an *in vitro* reverse transcriptase (RT) assay described previously (75), and 5×10^4 cpm of RT activity was used for infection. The virus stocks were all pretreated with 60 units of turbo-DNase I (Ambion) at 37°C for 2 h prior to incubation with cells. As a negative control, cells were treated with 25 μ M azidothymidine (AZT) for 1 h at 37°C prior to and during infection. For measurement of the early reverse transcripts (minus-strand strong-stop cDNA), cells were incubated with virus for 3 h; for measurement of the late reverse transcripts, a 10- to 12-hour incubation was used. After 37°C incubation in cell culture media with viruses, total DNA was extracted with the DNeasy kit (Qiagen). One-hundred nanograms of total DNA was used as a template for the PCRs, which were conducted in a final volume of 50 μ l under the following conditions: 95°C for 3 min, followed by 32 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 30 s. The reaction products were analyzed on a 1.5% agarose gel. The primer pairs were as follows: for minus-strand strong-stop cDNA, forward, 5'-AGTCGCTCTGCGGAGAGGCTG-3', and reverse, 5'-TGCTAGGGATTTCCTGCTTCGGTTT-3'; for late reverse transcripts, forward, 5'-CACTAGCA

GGTAGAGCCTGGGTGT-3', and reverse, 5'-GTCATCCCACTGGGAAGTTTGAGC-3' (modified from reference 21). The band intensities were measured with Fluor-Chem FC2 (Alpha Innotech). The relative level of late reverse transcripts was expressed as a percentage of the level observed in control cells transduced with the empty LPCX vector.

Measurement of the fate of the SIV_{mac} capsid. The Cf2Th cells expressing different TRIM5 α variants were seeded in T75 flasks. On the following day, the flask was incubated at 4°C for 15 min, and the cell medium was replaced by 6 ml cold cell culture medium containing 5×10^6 RT cpm units of recombinant, VSV G-pseudotyped viruses. In some experiments, viruses prepared without the VSV G glycoprotein were studied as controls. After incubation at 4°C for 20 min, the cell-virus mixture was shifted to 37°C. After incubation at 37°C for 6 to 10 h, the cells were washed three times with cold PBS and then treated with pronase (7 mg/ml in Dulbecco's modified Eagle's medium [DMEM]; Roche) for 5 min on ice. The cells were harvested, washed three times with cold PBS, resuspended in 250 μ l hypolysis buffer (10 mM Tris-Cl, pH 8.0, 10 mM KCl₂, 1 mM EDTA), and lysed with a motorized pestle (Sigma) for 1 min. After centrifugation at $1,500 \times g$ for 3 min, the supernatant was transferred to a new prechilled tube and mixed with 250 μ l cold hypolysis buffer. Fifty microliters of this mixture was saved as "input" for analysis by Western blotting. The remaining mixture was adjusted to 300 mM NaCl, incubated at room temperature for 15 min, and then loaded onto a 50% (wt/vol) sucrose cushion in PBS. The particulate viral capsids were pelleted by centrifugation at 30,000 rpm for 2 h in a Beckman SW41 rotor at 4°C. From the top of each tube, 100 μ l of supernatant was saved for analysis, and the pellet was resuspended in 60 μ l 1 $\times$ sample buffer. Equal volumes of the input, supernatant, and pellet samples were analyzed by Western blotting with an anti-SIV serum (NIH AIDS Research and Reference Reagent Program) and peroxidase-conjugated anti-human antibody (Pierce). Western blots were exposed to film for time periods that allowed detection of signals but avoided saturation of the film. The intensity of each p27 capsid band was measured by densitometry. The relative amount of pelletable capsid was expressed as a percentage of that seen in the control target cells transduced with the empty LPCX vector.

Statistical analysis. Correlations between measurements of relative TRIM5 α E3 ubiquitin ligase activity, the levels of late reverse transcription products, the amount of pelletable capsid, and the level of SIV_{mac} infection were tested using Prism software (GraphPad Software, Inc.). Spearman rank correlation analysis and linear regression were used to obtain the Spearman rank correlation coefficient (r_s) and the P value.

The TRIM5 α variants included in each analysis were as follows: (i) for the relative level of SIV_{mac} infection versus E3 ubiquitin ligase activity, Tan, Pyg, E24N, E24D, E24Q, P29G, P29F, L19A, E20A, and L26A; (ii) for late reverse transcripts versus E3 ubiquitin ligase activity, Tan, Pyg, E24N, E24D, E24Q, P29G, P29F, L19A, E20A, L26A, RtVp, RpVp, RtVt, and RpVt; and (iii) for pelletable capsid versus late reverse transcripts and for E3 ubiquitin ligase activity versus pelletable capsids, Tan, Pyg, E24Q, P29F, L19A, E20A, L26A, RtVp, RpVp, RtVt, and RpVt.

RESULTS

Determinants of restriction potency in TRIM5 α proteins from African green monkey subspecies. The TRIM5 α proteins, TRIM5 $\alpha_{AGM(Tan)}$ and TRIM5 $\alpha_{AGM(Pyg)}$, from the *tantalus* and *pygerythrus* subspecies of African green monkeys, respectively, both restrict HIV-1 infection potently (10). However, only TRIM5 $\alpha_{AGM(Tan)}$ efficiently inhibits SIV_{mac} infec-

β -actin. (C and D) HeLa cells expressing the TRIM5 α_{AGM} variants or transduced with the empty LPCX vector were incubated with the indicated volume of recombinant VSV G-pseudotyped SIV_{mac}-GFP or HIV-1-GFP virus stocks. After 72 h, GFP-positive cells were analyzed by FACS. The results shown are representative of those obtained in three independent experiments. (E) The TRIM5 α proteins in the lysates of stably expressing Cf2Th cells were incubated with sulfo-EGS at the indicated concentrations. The cross-linked TRIM5 α proteins were analyzed by SDS-PAGE and Western blotting with an antibody directed against the C-terminal HA epitope tag. The positions of monomeric and dimeric TRIM5 α proteins are indicated. (F) The abilities of the TRIM5 α variants to bind HIV-1 capsid complexes were tested by incubating the input amounts of TRIM5 α proteins with HIV-1 CA-NC complexes assembled *in vitro*. After centrifugation through a 70% sucrose cushion, the pellet was analyzed by Western blotting for the bound TRIM5 α protein. The CA-NC protein was detected by Coomassie brilliant blue staining of the gel. (G) The half-lives of the indicated TRIM5 α variants were investigated by incubating Cf2Th cells expressing the TRIM5 α proteins at 37°C in cycloheximide (CHX)-containing medium for the indicated times. The cells were then placed on ice and lysed. Equal amounts of protein from the cell lysates were Western blotted with an antibody directed against the HA epitope tag.

tion. There are eight amino acid differences between TRIM5 $\alpha_{AGM(Tan)}$ and TRIM5 $\alpha_{AGM(Pyg)}$; five of these involve amino acid residues in the B30.2(SPRY) domain (Fig. 1A). Chimeras between these TRIM5 α_{AGM} proteins were created and tested for SIV $_{mac}$ -restricting ability (Fig. 1A to C). These studies revealed that the TRIM5 $\alpha_{AGM(Tan)}$ B30.2(SPRY) domain did not determine the potency of TRIM5 $\alpha_{AGM(Tan)}$ in restricting SIV $_{mac}$. Therefore, a subset of the 3 amino acid residues in the RING, coiled-coil, and linker 2 regions that differ between TRIM5 $\alpha_{AGM(Tan)}$ and TRIM5 $\alpha_{AGM(Pyg)}$ must contribute to SIV $_{mac}$ restriction potency. Each of these residues was individually changed in both TRIM5 α_{AGM} proteins to the amino acid found in the other TRIM5 α_{AGM} protein. HeLa cells stably expressing these TRIM5 α_{AGM} variants were challenged with SIV $_{mac}$ and HIV-1 (Fig. 1D; see Fig. S1B in the supplemental material). The results indicated that phenylalanine 34 in the RING domain is necessary and sufficient to account for the potent SIV $_{mac}$ -restricting ability of TRIM5 $\alpha_{AGM(Tan)}$. Leucine 34 in TRIM5 $\alpha_{AGM(Pyg)}$, which is less commonly found in TRIM5 or its close relatives (74), compromises the ability of the protein to restrict SIV $_{mac}$ infection. The parental TRIM5 $\alpha_{AGM(Pyg)}$ protein and all the mutants containing leucine 34 were efficiently expressed (Fig. 1B) and potently blocked HIV-1 infection (Fig. 1D; see Fig. S1B in the supplemental material). We conclude that the TRIM5 $\alpha_{AGM(Pyg)}$ protein fails to restrict SIV $_{mac}$ infection because of the identity of RING domain residue 34.

To assess whether leucine 34 influences the global conformation of the TRIM5 α_{AGM} protein, other known properties of TRIM5 proteins were examined. The parental TRIM5 α_{AGM} proteins and residue 34 mutants were similar with respect to dimerization efficiency and binding to HIV-1 CA-NC complexes (Fig. 1E and F). TRIM5 $\alpha_{AGM(Pyg)}$ exhibited a lower turnover rate than TRIM5 $\alpha_{AGM(Tan)}$, but leucine 34 in the RING domain contributed only slightly to this difference (Fig. 1G). The ability to dimerize, bind HIV-1 capsids, and restrict HIV-1 infection supports the structural and functional integrity of these TRIM5 α_{AGM} variants.

Replacement of TRIM5 α_{AGM} RING domain loops with those from other TRIM5 α proteins. The structure of the human TRIM5 RING domain has been solved (1); like other RING/PHD-like/U-box domains, the TRIM5 RING domain coordinates two zinc ions by virtue of a cross-brace motif (1, 85, 94, 95). Two loops (loop 1 and loop 2) that flank the central α -helix contribute to the putative site of interaction with the E2 ubiquitin-conjugating enzyme (1, 85, 94, 95). Residue 34 is buried beneath the interface of these loops, and changes in residue 34 could potentially alter the conformation of either loop. Although the RING domain amino acid residues predicted to contact E2 are invariant in TRIM5 proteins, there are species-specific differences among the loop 1 and loop 2 sequences; loop 2 has more divergent sequences than loop 1 (Fig. 2A) (74). To investigate the possible functional implications of these differences, the loop 1 and loop 2 sequences of TRIM5 $\alpha_{AGM(Tan)}$ were replaced by those of TRIM5 α proteins from other primates. Replacement of the TRIM5 $\alpha_{AGM(Tan)}$ loop 1 with those of TRIM5 α from humans, rhesus macaques, or squirrel monkeys resulted in severe decreases in SIV $_{mac}$ restriction, despite efficient expression of all three mutant proteins (Fig. 2B; see Fig. S1A in the supplemental material).

These mutant proteins all potently restricted HIV-1 infection (see Fig. S1C in the supplemental material). In contrast to the phenotypes observed for loop1-substituted TRIM5 $\alpha_{AGM(Tan)}$ proteins, TRIM5 $\alpha_{AGM(Tan)}$ with the human TRIM5 loop 2 inhibited SIV $_{mac}$ infection as efficiently as the parental TRIM5 $\alpha_{AGM(Tan)}$ protein (Fig. 2C). Apparently, restriction of SIV $_{mac}$ infection by TRIM5 $\alpha_{AGM(Tan)}$ is sensitive to changes in loop 1 of the RING domain.

Alteration of the TRIM5 $\alpha_{AGM(Tan)}$ RING domain loop 1. The loop 1 regions of human and AGM TRIM5 α RING domains differ in sequence only at residues 24 and 29 (Fig. 2A), both of which are surface exposed in the TRIM5 RING structure (1). Each of these residues in TRIM5 $\alpha_{AGM(Tan)}$ was altered to multiple other amino acids. Cells expressing these mutant proteins were challenged with SIV $_{mac}$. The SIV $_{mac}$ -restricting ability of TRIM5 $\alpha_{AGM(Tan)}$ tolerated conservative changes in glutamic acid 24 but was completely eliminated by less conservative substitutions at this position (Fig. 2D). None of the TRIM5 $\alpha_{AGM(Tan)}$ proteins with changes in proline 29 restricted SIV $_{mac}$ infection (Fig. 2E). The sensitivity of TRIM5 α_{AGM} proline 29 to alteration may result from its proximity to the zinc-coordinating cysteine 30. These results support the importance of loop 1 of the RING domain to the SIV $_{mac}$ -restricting ability of TRIM5 $\alpha_{AGM(Tan)}$.

Amino acid residues in loop 1 that are conserved in the TRIM5 proteins of different species are predicted to contribute to binding the E2 ubiquitin-conjugating enzyme, based on analogy with other RING/PHD-like/U-box domains (1, 85, 93, 94). To investigate the potential importance of E2 interaction for TRIM5 $\alpha_{AGM(Tan)}$ function, two loop 1 residues, leucine 19 and glutamic acid 20, which are predicted to reside within or near, respectively, the E2-binding site (1, 84, 85, 93), were altered to alanine. As a control, leucine 26, which is in loop 1 but is not predicted to reside near the E2-binding site (1, 85, 94, 95), was also changed to alanine. Cells expressing the wild-type and mutant TRIM5 $\alpha_{AGM(Tan)}$ proteins, as well as TRIM5 $\alpha_{AGM(Pyg)}$, were challenged with SIV $_{mac}$ and HIV-1. The TRIM5 $\alpha_{AGM(Tan)}$ L19A and E20A proteins exhibited minimal anti-SIV $_{mac}$ activity (Fig. 2F and Table 1). The TRIM5 $\alpha_{AGM(Tan)}$ L26A mutant was nearly as effective as the wild-type TRIM5 $\alpha_{AGM(Tan)}$ protein in restricting SIV $_{mac}$ infection (Fig. 2F). However, all three loop 1 TRIM5 $\alpha_{AGM(Tan)}$ mutants efficiently restricted HIV-1 infection (see Fig. S1C in the supplemental material). Apparently, specific residues in loop 1 of the TRIM5 $\alpha_{AGM(Tan)}$ RING domain are more critical for restriction of SIV $_{mac}$ than for restriction of HIV-1.

E3 ubiquitin ligase activity of TRIM5 variants. Purified rhesus monkey TRIM5 α protein has been shown to exhibit E3 ubiquitin ligase activity *in vitro*, resulting in self-ubiquitylation (58). We established an *in vitro* assay to examine the E3 ubiquitin ligase activities of TRIM5 α variants. In this assay, the transiently expressed HA-tagged TRIM5 α protein was immunoprecipitated by an anti-HA antibody and subsequently mixed with purified recombinant E1, E2, and myc-ubiquitin. As the E3-interacting regions of the E2 ubiquitin-conjugating enzymes are well conserved across vertebrate species (91), human reagents were employed in these assays. With increasing times of incubation at 37°C, more slowly migrating forms of TRIM5 $\alpha_{AGM(Tan)}$ were observed; concomitant incorporation of the myc-ubiquitin into proteins is consistent with

FIG. 2. Specific sequences in loop 1 of the TRIM5 α _{AGM(Tan)} RING domain are critical for SIV_{mac} restriction. (A) The sequences of the RING domains of TRIM5 α proteins from African green monkeys (*tan* [Tan] subspecies), humans, rhesus monkeys, and squirrel monkeys are aligned. The amino acid residue numbers are based on those of TRIM5 α _{AGM(Tan)}. Areas where sequence differences exist are highlighted with gray boxes. The dots above the sequence indicate cysteine and histidine residues involved in zinc coordination. The locations of loops 1 and 2 are shown. (B to F) HeLa (B to E) or Cf2Th (F) cells, either transduced with the empty LPCX vector or expressing TRIM5 α _{AGM(Pyg)} or the indicated TRIM5 α _{AGM(Tan)} variants, were incubated with different amounts of recombinant VSV G-pseudotyped SIV_{mac}-GFP virus stocks. Different preparations of the SIV_{mac}-GFP virus stock were used in the experiments. After 72 h, GFP-positive cells were analyzed by FACS. The results shown are representative of those obtained in three independent experiments.

TRIM5 α _{AGM(Tan)} polyubiquitylation (see Fig. S2A in the supplemental material). To examine the possibility that other co-precipitated cellular E3 ligase activities might mediate this ubiquitylation activity, we washed the immunoprecipitated TRIM5 α _{AGM(Tan)} with buffers containing increasing concentrations (from 0 to 1 M) of salt and then performed the *in vitro* ubiquitylation assay (see Fig. S2B in the supplemental material). The lack of apparent effect of salt washing on polyubiq-

uitylation activity is consistent with the observed E3 ligase activity being mediated by TRIM5 α _{AGM(Tan)} itself. This interpretation is strengthened by our observation that changes in the TRIM5 α _{AGM(Tan)} RING domain dramatically influenced the degree of observed self-ubiquitylation (see below). Of the different E2 enzymes tested in the *in vitro* ubiquitylation assay, only UbcH5 functioned as an E2 ubiquitin-conjugating enzyme in support of both TRIM5 α _{rh} and TRIM5 α _{AGM(Tan)} polyubiq-

TABLE 1. Phenotypes of TRIM5 α variants

TRIM5 α variant	SIV _{mac} restriction ^a	Ubiquitin ligase activity ^b	Late SIV _{mac} reverse transcripts ^c	Binding to SIV _{mac} CA-NC complexes ^d	Particulate SIV _{mac} cytoplasmic capsids ^e
Rh	+	+/++++	+/++++	+	+++
Tan	++++	+++	—	+	—/+
Pyg	—/+	+	+++	++++	+++
Tan P29F	+	+	+++	+	++
Tan E24Q	++++	+++	—	ND	+
Tan L19A	+	+	+/++++	+	+++
Tan E20A	+	+	+/++++	+	ND
Tan L26A	++++	+++	—	ND	+
Rh-Rt	+++	+++	ND	ND	ND
Rh-Rp	—/+	+	ND	ND	ND
Rh-loop1(Tan)	+++	+++	+	ND	ND
Rh-loop2(Tan)	+	+	+++	ND	ND
Rh-RtVp	++++	+++	—	+/++++	—/+
Rh-RpVp	+/++++	+	++++	+/++++	++++
Rh-RtVt	+/++++	+++	—	+	+
Rh-RpVt	+++	+	+++	+	+++

^a The relative SIV_{mac} infection level in Cf2Th cells expressing the given TRIM5 α variant was determined as described in Materials and Methods. The level of SIV_{mac} restriction represents the complement of the relative infection level (++++, more than 90% of the wild-type TRIM5 α _{AGM(Tan)} level; +++, 50 to 80%; ++, 25 to 50%; +, 0 to 25%; —, not detected).

^b The ubiquitin ligase activity of each TRIM5 α variant was determined in the *in vitro* autoubiquitylation assay described in Materials and Methods. The E3 ubiquitin ligase activity of each TRIM5 α variant relative to that of wild-type TRIM5 α _{AGM(Tan)} is indicated: +++, 80 to 100% of the wild-type TRIM5 α _{AGM(Tan)} E3 ligase activity; ++, 40 to 80%; +, less than 40%.

^c Late SIV_{mac} reverse transcripts were measured as described in Materials and Methods. The level relative to that observed following SIV_{mac} infection of cells transduced with the empty LPCX vector is reported: +++++, greater than 120% of the level in LPCX-transduced; +++, 80 to 100%; ++, 40 to 80%; +, 10 to 40%; —, not detected; ND, not determined.

^d Binding of the TRIM5 α variant to SIV_{mac} CA-NC complexes was measured as described in Materials and Methods. The binding relative to that of the wild-type TRIM5 α _{AGM(Pyg)} is reported: +++++, 90 to 100% of the binding observed for the TRIM5 α _{AGM(Pyg)} protein; +++, 60 to 80%; ++, 30 to 60%; +, less than 25%; ND, not determined.

^e The amount of particulate SIV_{mac} capsid in the cytosol of cells expressing the TRIM5 α variant was determined as described in Materials and Methods. The amount of particulate capsid relative to that in cells transduced with the empty LPCX vector is reported: +++++, more than 100% of the amount of particulate capsid seen in LPCX-transduced cells; +++++, 80 to 100%; +++, 50 to 70%; ++, 30 to 50%; +, less than 30%; —, not detected; ND, not determined.

ubiquitylation (data not shown; see Fig. S2C in the supplemental material). No TRIM5 α polyubiquitylation was observed in the absence of added E2 enzyme or an energy source. These results are consistent with those previously reported for TRIM5 self-ubiquitylation (87). In additional studies using wild-type and mutant ubiquitin proteins, TRIM5 α _{rh} polyubiquitylation occurred regardless of the position of the available lysine on the ubiquitin molecule (see Fig. S2D in the supplemental material). In addition, TRIM5 α _{rh} was found to have at least 5 sites of autoubiquitylation (see Fig. S2E in the supplemental material).

The E3 ubiquitin ligase activities of the TRIM5 α variants studied here were compared in the above-mentioned *in vitro* assay. Of note, wild-type TRIM5 α _{AGM(Tan)} functioned as an efficient E3 ubiquitin ligase, whereas the wild-type TRIM5 α _{AGM(Pyg)} exhibited slower kinetics and a lower efficiency of polyubiquitylation (Fig. 3A). As expected from the predicted E2-interacting residues of TRIM5, L19A and E20A changes both greatly reduced the E3 ligase activity of TRIM5 α _{AGM(Tan)}, but the E24Q and L26A mutants exhibited E3 ubiquitin ligase activities comparable to that of wild-type TRIM5 α _{AGM(Tan)} (Fig. 3B and C). Substantially less self-ubiquitylation was observed for the TRIM5 α _{AGM(Tan)} P29F and F34L mutants (Fig. 3C and data not shown). For the TRIM5 α _{AGM(Tan)} variants mentioned above, a strong correlation between *in vitro* E3 ubiquitin ligase activity and SIV_{mac} restriction potency was observed (Fig. 3D) ($r_s = 0.9893$; two-tailed $P < 0.0001$).

Capsid-binding abilities of TRIM5 α _{AGM} RING mutants. To determine if any of the TRIM5 α _{AGM} RING changes affected

recognition of the SIV_{mac} capsid, we assembled SIV_{mac} CA-NC complexes *in vitro* (see Fig. S3 in the supplemental material, electron micrograph), and measured the binding of the TRIM5 α proteins to the complexes. TRIM5 α _{AGM(Tan)} bound SIV_{mac} CA-NC complexes with an efficiency comparable to that of TRIM5 α _{rh} (Table 1). Unexpectedly, the binding of the TRIM5 α _{AGM(Pyg)} protein to SIV_{mac} CA-NC complexes was significantly better than that of the TRIM5 α _{AGM(Tan)} protein (Fig. 4 and Table 1). The binding of the TRIM5 α _{AGM(Tan)} F34L, P29F, L19A, and E20A mutants to the CA-NC complexes was comparable to that of the wild-type TRIM5 α _{AGM(Tan)} protein. The TRIM5 α _{AGM(Pyg)} L34F mutant bound CA-NC complexes comparably to the wild-type TRIM5 α _{AGM(Pyg)} protein. Thus, these changes in the TRIM5 α _{AGM} RING domain, which exert dramatic effects on both restriction of SIV_{mac} infection and E3 ligase activity, do not detectably affect the interaction of the mutant TRIM5 α _{AGM} proteins with the SIV_{mac} capsid.

Contribution of RING and capsid-binding functions to TRIM5 α restriction. To assess the relative contributions of RING-mediated ubiquitin ligase activity and capsid binding to TRIM5 α restriction, we sought a system in which each of these components could be individually modulated. Because TRIM5 α _{rh} is a weak inhibitor of SIV_{mac} infection, we used TRIM5 α _{rh} as a backbone to modify ubiquitin ligase activity (by RING substitution) and SIV_{mac} capsid-binding ability [by B30.2(SPRY) substitution]. Thus, segments of the TRIM5 α _{rh} RING domain and/or the B30.2(SPRY) domain were replaced by the equivalent regions from TRIM5 α _{AGM(Tan)} or

FIG. 3. Restriction of SIV_{mac} infection by TRIM5α RING variants correlates with E3 ubiquitin ligase activity. (A) The abilities of HA-tagged TRIM5α_{AGM(Tan)} and TRIM5α_{AGM(Pyg)} proteins precipitated from the lysates of stably expressing cells to mediate autoubiquitylation *in vitro* are shown. After the indicated times of incubation at 37°C, the reaction products were subjected to Western blotting with an anti-HA epitope tag. Polyubiquitylated forms of TRIM5α having more than 5 conjugated ubiquitins are indicated. The numbers beneath the lanes represent the percent conversion of TRIM5α protein to polyubiquitylated forms. (B and C) The assay for *in vitro* autoubiquitylation of wild-type (wt) TRIM5α_{AGM(Tan)}, the indicated TRIM5α_{AGM(Tan)} mutants, and wild-type TRIM5α_{rh} (Rh) or TRIM5α_{rh} with the indicated RING substitutions was performed for 2 hours at 37°C in the absence (–) or presence (+) of the energy regeneration system (ERS). The numbers beneath the lanes represent the percent conversion of TRIM5α protein to polyubiquitylated forms. (D) Relationship between the *in vitro* E3 ubiquitin ligase activities of TRIM5α variants and the relative levels of SIV_{mac} infection observed in cells expressing those TRIM5α proteins. The r_s and P value are shown.

TRIM5α_{AGM(Pyg)} (Fig. 5A). In the Rh-Rt and Rh-Rp chimeras, the RING domain of TRIM5α_{rh} is replaced by the RING domains of TRIM5α_{AGM(Tan)} and TRIM5α_{AGM(Pyg)}, respectively. In another pair of constructs [Rh-loop 1(Tan) and Rh-loop 2(Tan)], we replaced loop 1 and loop 2, respectively, of the TRIM5α_{rh} RING domain with the equivalent loop from TRIM5α_{AGM(Tan)}. To modulate capsid binding, some of the

RING domain chimeras were further modified by the replacement of the TRIM5α_{rh} B30.2(SPRY) domain v1 variable region with that of TRIM5α_{AGM(Tan)} or TRIM5α_{AGM(Pyg)} (Rh-RtVp, Rh-RtVt, Rh-RpVp, and Rh-RpVt in Fig. 5A).

The chimeric proteins and the parental TRIM5α proteins were stably expressed in canine Cf2Th cells, which were challenged with SIV_{mac}. The Rh-Rt protein with the

FIG. 4. RING domain changes in TRIM5 α_{AGM} affecting SIV $_{mac}$ restriction do not alter capsid binding. The abilities of the TRIM5 α variants to bind SIV $_{mac}$ capsid complexes were tested by incubating the input amounts of TRIM5 α proteins with SIV $_{mac}$ CA-NC complexes assembled *in vitro*. After centrifugation through a 60% sucrose cushion, the pellet was analyzed by Western blotting for the bound TRIM5 α protein. The gel was stained with Coomassie brilliant blue to detect the CA-NC protein.

TRIM5 $\alpha_{AGM(Tan)}$ RING domain inhibited SIV $_{mac}$ infection better than either wild-type TRIM5 α_{rh} or the Rh-Rp protein with the TRIM5 $\alpha_{AGM(Pyg)}$ RING domain (Fig. 5B, left, and Table 1). However, all three TRIM5 α variants restricted HIV-1 infection equivalently (Fig. 5B, right). These results imply that the TRIM5 α_{rh} and TRIM5 $\alpha_{AGM(Pyg)}$ RING domains are not as functionally active as the TRIM5 $\alpha_{AGM(Tan)}$ RING domain and that TRIM5 α RING function is more important for restriction of SIV $_{mac}$ than of HIV-1. Replacement of the TRIM5 α_{rh} RING domain loop 1 sequence with that of TRIM5 $\alpha_{AGM(Tan)}$ was sufficient to achieve enhanced restriction of SIV $_{mac}$ infection [Rh-loop 1(Tan) in Table 1]. As only amino acid residue 29 differs between the loop 1 sequences of TRIM5 α_{rh} and TRIM5 $\alpha_{AGM(Tan)}$, we conclude that the gain in SIV $_{mac}$ -restricting activity results from the replacement of histidine 29 in TRIM5 α_{rh} with proline. In contrast, the Rh-loop 2(Tan) protein, in which loop 2 of TRIM5 α_{rh} was replaced by that of TRIM5 $\alpha_{AGM(Tan)}$, did not efficiently inhibit SIV $_{mac}$ infection (Table 1). The E3 ubiquitin ligase activity of the TRIM5 α_{rh} -TRIM5 α_{AGM} chimeras was examined using the *in vitro* self-ubiquitylation assay. Of note, the Rh-Rt protein exhibited much greater efficiency than the Rh-Rp protein in this assay (Fig. 3C), supporting the correlation between E3 ligase activity and SIV $_{mac}$ restriction. Also consistent with this correlation is the observation that the potently restricting Rh-loop 1(Tan) chimera, with only the TRIM5 $\alpha_{AGM(Tan)}$ RING loop 1 region, exhibited more ubiquitin ligase activity than the poorly restricting Rh-loop 2(Tan) protein (Fig. 3C). No significant difference in the turnover rates of the TRIM5 α_{rh} , Rh-Rp, and Rh-Rt proteins was observed (see Fig. S4 in the supplemental material). These results support a role for the E3 ubiquitin ligase activity of the TRIM5 α RING domain in restricting SIV $_{mac}$ infection.

The Rh-RtVp, Rh-RtVt, Rh-RpVp, and Rh-RpVt chimeras contain RING domains and B30.2(SPRY) domain v1 regions from TRIM5 $\alpha_{AGM(Tan)}$ or TRIM5 $\alpha_{AGM(Pyg)}$ (Fig. 5A). All of these chimeric proteins inhibited SIV $_{mac}$ infection more efficiently than TRIM5 $\alpha_{AGM(Pyg)}$ and TRIM5 α_{rh} (Fig. 5C and Table 1). Several independent experiments resulted in the same order of anti-SIV $_{mac}$ activity: Rh-RtVp > Rh-RtVt = Rh-RpVp > Rh-RpVt. For these four chimeras, both the RING domain and B30.2(SPRY) domain sequences contribute to the potency of SIV $_{mac}$ -restricting ability. The Rh-RtVp

and Rh-RtVt chimeras with the TRIM5 $\alpha_{AGM(Tan)}$ RING domain inhibited SIV $_{mac}$ infection more efficiently than the matched proteins (Rh-RpVp and Rh-RpVt, respectively) with the RING domain of TRIM5 $\alpha_{AGM(Pyg)}$. Thus, we tested the hypothesis that the greater SIV $_{mac}$ restriction potency of the chimeric proteins with the TRIM5 $\alpha_{AGM(Tan)}$ RING domain was associated with higher E3 ubiquitin ligase activity. The Rh-RtVp and the Rh-RtVt chimeras containing the TRIM5 $\alpha_{AGM(Tan)}$ RING domain exhibited dramatically more self-ubiquitylation in the *in vitro* assay than the Rh-RpVp and Rh-RpVt chimeras with the TRIM5 $\alpha_{AGM(Pyg)}$ RING domain (Fig. 5D). TRIM5 α E3 ubiquitin ligase activity was not apparently influenced by the sequence of the B30.2(SPRY) v1 region (Fig. 5D, compare Rh-RtVp with Rh-RtVt, and also Rh-RpVp with Rh-RpVt). These results are consistent with our hypothesis that superior E3 ubiquitin ligase activity contributes to the potency of SIV $_{mac}$ restriction by the chimeric TRIM5 α proteins with the RING domain of TRIM5 $\alpha_{AGM(Tan)}$.

The B30.2(SPRY) domain v1 variable region is longer in TRIM5 α_{AGM} than in TRIM5 α_{rh} due to a tandem duplication of 20 amino acid residues (73, 74). In the Rh-RtVp, Rh-RtVt, Rh-RpVp, and Rh-RpVt chimeras, the v1 region from either TRIM5 $\alpha_{AGM(Tan)}$ or TRIM5 $\alpha_{AGM(Pyg)}$ substitutes for that of TRIM5 α_{rh} . The v1 sequences of TRIM5 $\alpha_{AGM(Tan)}$ differ by 5 amino acid residues from those of TRIM5 $\alpha_{AGM(Pyg)}$ (Fig. 1A). The Rh-RtVp and Rh-RpVp chimeras with the B30.2(SPRY) v1 region from TRIM5 $\alpha_{AGM(Pyg)}$ inhibited SIV $_{mac}$ infection more efficiently than the matched proteins (Rh-RtVt and Rh-RpVt, respectively) with the TRIM5 $\alpha_{AGM(Tan)}$ B30.2(SPRY) v1 region (Fig. 5C). We tested the hypothesis that differences in the efficiency of capsid binding determined the observed differences in SIV $_{mac}$ restriction potency by measuring the binding of these TRIM5 α variants to SIV $_{mac}$ CA-NC complexes. As described above, the binding of the TRIM5 $\alpha_{AGM(Pyg)}$ protein to SIV $_{mac}$ capsid complexes was significantly better than that of the TRIM5 $\alpha_{AGM(Tan)}$ protein (Fig. 5E and Table 1). Both chimeric proteins (Rh-RtVp and Rh-RpVp) with the B30.2(SPRY) v1 region from TRIM5 $\alpha_{AGM(Pyg)}$ bound SIV $_{mac}$ capsid complexes more efficiently than the chimeric proteins (Rh-RtVt and Rh-RpVt) with the TRIM5 $\alpha_{AGM(Tan)}$ v1 region (Fig. 5E and Table 1). The SIV $_{mac}$ capsid-binding abilities of the Rh-RtVp and Rh-RpVp chimeras were almost equivalent to that of TRIM5 $\alpha_{AGM(Pyg)}$. Differences in the RING domains of the TRIM5 α chimeras had no apparent effect on the efficiency of binding to the SIV $_{mac}$ CA-NC complexes. Thus, the v1 variable region of the B30.2(SPRY) domain is the major determinant of differences in SIV $_{mac}$ capsid-binding ability among these TRIM5 α variants.

From these data, we conclude that both the RING domain, with associated E3 ubiquitin ligase activity, and the B30.2 v1 region, which determines capsid-binding affinity, independently contribute to the potency of SIV $_{mac}$ restriction. Thus, the Rh-RtVt chimera, which has potent E3 ubiquitin ligase activity but poor capsid-binding ability, achieved a level of SIV $_{mac}$ restriction similar to that of the Rh-RpVp chimera, which has poor E3 ubiquitin ligase activity but potent capsid-binding ability (Fig. 5C and Table 1).

SIV $_{mac}$ reverse transcription in TRIM5 α -expressing cells. The TRIM5 α variants studied here exhibited defined differences in RING-mediated E3 ubiquitin ligase activity and, in

FIG. 5. RING domain-mediated ubiquitin ligase activity and B30.2(SPRY)-mediated capsid binding independently determine restriction potency. (A) Wild-type TRIM5 α_{rh} and chimeric proteins in which the TRIM5 α_{AGM} RING domain and, in some cases, the B30.2(SPRY) v1 region were replaced by the corresponding regions of TRIM5 α_{AGM} (Tan) or TRIM5 α_{AGM} (Pyg). (B and C) Cf2Th cells expressing the indicated TRIM5 α_{AGM} variants or transduced with the empty LPCX vector were incubated with the indicated volume of recombinant VSV G-pseudotyped SIV $_{mac}$ -GFP or HIV-1-GFP virus stock. After 72 h, GFP-positive cells were analyzed by FACS. The results shown are representative of those obtained in three independent experiments. (D) *In vitro* autoubiquitylation of the TRIM5 α_{AGM} (Tan), TRIM5 α_{AGM} (Pyg), and TRIM5 α_{rh} chimeric proteins after a 2-h incubation at 37°C in the absence (–) or presence (+) of the ERS. (E) The abilities of the TRIM5 α variants to bind SIV $_{mac}$ capsid complexes were tested by incubating the input amounts of TRIM5 α proteins with SIV $_{mac}$ CA-NC complexes assembled *in vitro*. After centrifugation through a 60% sucrose cushion, the pellet was analyzed by Western blotting for the bound TRIM5 α protein. The gel was stained with Coomassie brilliant blue to detect the CA-NC protein.

some cases, in capsid-binding affinity, as well. This afforded an opportunity to examine the impacts of changes in TRIM5 α ubiquitylation capacity and capsid binding on the reverse transcription process and the fate of the retroviral capsid in in-

fectected cells. The level of late SIV $_{mac}$ reverse transcripts following the infection of cells expressing TRIM5 α_{AGM} (Pyg) was comparable to that measured in cells transduced with the empty LPCX vector control (Fig. 6A). In contrast, the levels of

FIG. 6. TRIM5 α RING-mediated ubiquitylation is essential for disruption of SIV_{mac} reverse transcription. Cf2Th cells transduced with the empty LPCX vector or expressing the TRIM5 α _{AGM}(Tan), TRIM5 α _{AGM}(Pyg), and TRIM5 α _{rh}-TRIM5 α _{AGM} chimeras were incubated with 7.5×10^4 RT cpm units of SIV_{mac}-GFP, and 100 ng of total DNA extracted from these cells was subjected to PCR with primers specific for GFP (A), minus-strand strong-stop DNA (B, D, E, and F), or late reverse transcripts (B). In addition, cells transduced with the empty LPCX vector were pretreated with AZT as a control. (C) Correlation is shown between the E3 ubiquitin ligase activities of TRIM5 α proteins and the levels of late reverse transcripts detected in cells expressing the TRIM5 α proteins following incubation with SIV_{mac}-GFP. The r_s and P value are shown. (D) Cf2Th cells expressing the Rh-RpVp or Rh-RtVp TRIM5 α protein or transduced with the empty LPCX vector were pretreated for 1 h with the proteasome inhibitor MG132 (3 and 10 μ M) or ALLN (2.5 and 5 μ M) or with the solvent DMSO (dimethyl sulfoxide) as a control. SIV_{mac}-GFP was added to the cells, and the virus-cell mixture was incubated at 37°C for 2 h in the presence of the proteasome inhibitors. The medium was then

SIV_{mac} late reverse transcripts were dramatically reduced in target cells expressing TRIM5 $\alpha_{\text{AGM(Tan)}}$ or the functionally active TRIM5 $\alpha_{\text{AGM(Tan)}}$ E24Q and L26A mutants (Fig. 6A and Table 1). Efficient SIV_{mac} reverse transcription was observed in cells expressing the TRIM5 $\alpha_{\text{AGM(Tan)}}$ L19A, E20A, and P29F mutants, which are defective in E3 ligase activity and SIV_{mac} restriction (Fig. 2 and 3 and Table 1). Thus, for this group of TRIM5 α_{AGM} variants, both RING-mediated ubiquitylation and inhibition of viral reverse transcription correlated with SIV_{mac} restriction potency.

The levels of SIV_{mac} late reverse transcripts were also examined in target cells expressing the TRIM5 α_{rh} -TRIM5 α_{AGM} chimeric proteins (Fig. 6A and Table 1). The levels of SIV_{mac} cDNA were lowest in the target cells expressing the chimeric proteins (Rh-RtVp and Rh-RtVt) containing the TRIM5 $\alpha_{\text{AGM(Tan)}}$ RING domain, which specifies robust E3 ubiquitin ligase activity. SIV_{mac} reverse transcript levels were highest in cells expressing the Rh-RpVp protein and only slightly lower in cells expressing the Rh-RpVt protein. Of note, although the levels of SIV_{mac} infection were nearly identical in target cells expressing the Rh-RtVt and Rh-RpVp proteins (Fig. 5C), which contain counterbalancing RING and B30.2(SPRY) domain functions, the levels of SIV_{mac} reverse transcripts were markedly greater in the cells expressing the Rh-RpVp protein. Examination of minus-strand strong-stop cDNA corroborated the results observed for the late SIV_{mac} reverse transcripts (Fig. 6B and data not shown), indicating that the TRIM5 α -mediated blocks occur prior to or at the earliest stages of reverse transcription. For the entire panel of TRIM5 α variants, inhibition of viral reverse transcription strongly correlated with the E3 ubiquitin ligase efficiency of the RING domain (Fig. 6C) ($r_s = 0.9510$; two-tailed $P < 0.0001$).

Effects of proteasome inhibition on TRIM5 α -mediated restriction and disruption of reverse transcription. A previous study suggested that approximately 10% of the TRIM5 α_{AGM} restriction of SIV_{mac} infection could be relieved by treatment of the target cells with the proteasome inhibitor MG132 (48). We investigated the effects of inhibiting the proteasome on the reverse transcription block to SIV_{mac} infection mediated by the TRIM5 α_{rh} -TRIM5 α_{AGM} chimeras. Late SIV_{mac} reverse transcripts in target cells expressing the Rh-RtVp protein increased after treatment of the target cells with the proteasome inhibitor MG132 or ALLN (Fig. 6D). The proteasome inhibitor-mediated increase in Rh-RtVp late reverse transcripts was evident by 1 h after infection (Fig. 6E). Treatment with proteasome inhibitors minimally affected the levels of SIV_{mac} late reverse transcripts in target cells expressing the Rh-RpVp protein or control cells transduced with the empty LPCX vector

(Fig. 6D). By titrating the amount of input DNA in the assay, ALLN treatment was shown to result in only a 3-fold increase in the levels of SIV_{mac} reverse transcripts in the Rh-RpVp-expressing or LPCX-transduced cells (Fig. 6F). Thus, proteasome inhibition specifically diminishes the ability of a TRIM5 α protein with potent RING-associated E3 ubiquitin ligase activity to mediate decreases in SIV_{mac} cDNA following infection.

The effects of proteasome inhibition on the restriction of SIV_{mac} infection by the Rh-RtVp and Rh-RpVp chimeric proteins were examined. Treatment of the control LPCX-transduced cells with the proteasome inhibitor MG132 resulted in a modest decrease in SIV_{mac} infection, perhaps due to the effects of the treatment on cell viability (Fig. 6G). MG132 treatment increased the efficiency with which SIV_{mac} infected cells expressing the Rh-RtVp protein but did not affect SIV_{mac} infection of the Rh-RpVp-expressing cells. Thus, proteasome inhibition partially relieves SIV_{mac} restriction by a TRIM5 α protein with potent RING-associated E3 ubiquitin ligase activity, but not by a matched TRIM5 α protein with poor RING E3 ubiquitin ligase function.

Fate of the SIV_{mac} capsid in cells expressing the TRIM5 α variants. To investigate the impact of TRIM5 RING function on capsid disassembly, the amount of particulate SIV_{mac} capsids in the cytoplasm of SIV_{mac}-challenged cells expressing the TRIM5 α variants was measured (77). Minimal amounts of SIV_{mac} capsid proteins were detected in the cytosol of cells exposed to a recombinant SIV_{mac} without envelope glycoproteins (Fig. 7A). The amount of particulate SIV_{mac} capsids detected in the cytosol of cells expressing a TRIM5 α protein from spider monkeys (a New World monkey) was less than that detected in cells expressing TRIM5 $\alpha_{\text{AGM(Tan)}}$, which in turn was less than that seen in TRIM5 α_{rh} -expressing cells (Fig. 7A). This order corresponds to the level of SIV_{mac} infection observed in the cells expressing the TRIM5 α proteins from these three monkey species (17) (Table 1). The level of cytosolic particulate SIV_{mac} capsids was lower in infected cells expressing TRIM5 $\alpha_{\text{AGM(Tan)}}$ than in TRIM5 $\alpha_{\text{AGM(Pyg)}}$ -expressing cells (Fig. 7B). The level of cytosolic particulate SIV_{mac} capsids was lower in cells expressing the restricting TRIM5 $\alpha_{\text{AGM(Tan)}}$ L26A mutant than in cells expressing the poorly restricting L19A mutant (Fig. 7B).

The levels of cytosolic particulate SIV_{mac} capsids in infected cells expressing the TRIM5 α_{rh} -TRIM5 α_{AGM} chimeric proteins were also examined. A high level of particulate SIV_{mac} capsids was observed in the cells expressing the Rh-RpVp and Rh-RpVt proteins (Fig. 7C). Lower levels of particulate SIV_{mac} capsids, comparable to those seen in TRIM5 $\alpha_{\text{AGM(Tan)}}$ -ex-

replaced with fresh medium without proteasome inhibitors, and the cells were cultured at 37°C for 3 h and then harvested. Total DNA was extracted and analyzed by PCR for minus-strand strong-stop DNA. (E) Cf2Th cells expressing the Rh-RpVp or Rh-RtVp TRIM5 α proteins were treated with proteasome inhibitors or DMSO solvent and exposed to SIV_{mac}-GFP as for panel D. At the indicated times, cells were harvested and used for DNA extraction and analysis of minus-strand strong-stop DNA. At the bottom is a longer exposure (exp) of the middle blot. (F) Semiquantitative PCR was performed with 2-fold dilutions of the total DNA extracted from the LPCX-transduced control cells and the Rh-RpVp-expressing cells exposed to SIV_{mac}-GFP. The density of each band was measured with the FluorChem FC2 System (Alpha Innotech). (G) Cf2Th cells expressing the Rh-RtVp and Rh-RpVp TRIM5 α proteins or transduced with the empty LPCX vector were pretreated with either DMSO (solid symbols) or MG132 (open symbols) for 1 h. The cells were then incubated with SIV_{mac}-GFP in the presence of 5 $\mu\text{g/ml}$ Polybrene for 2 h in the continued presence of DMSO or MG132. The cells were then washed and returned to culture for 48 h until FACS analysis was performed.

FIG. 7. TRIM5 α RING-mediated ubiquitylation is important for the accelerated disassembly of SIV_{mac} capsids. (A to D) The fate of the SIV_{mac} capsid was examined in C2Th cells expressing the indicated TRIM5 α variants or transduced with the empty LPCX vector. The cells were incubated for 6 to 8 h with SIV_{mac}-GFP that was pseudotyped with the VSV G glycoprotein (+Env) or that was produced in the absence of the VSV G glycoprotein (-Env). The input, supernatant, and pellet fractions were obtained by sucrose density gradient centrifugation, as described in Materials and Methods. (D) Target cells were treated with MG132 (+) or DMSO (-) prior to and during addition of SIV_{mac}-GFP, as described in the legend to Fig. 6. The experiments were performed 3 or 4 times, and typical results derived from a single experiment are shown. (E) Correlation between the E3 ubiquitin ligase activity of TRIM5 α variants and the amount of pelletable cytoplasmic capsids in the TRIM5 α -expressing cells 6 to 8 h after incubation with SIV_{mac}-GFP. The r_s and P value are shown. (F) Correlation between the amount of pelletable capsids in the cytoplasm of TRIM5 α -expressing cells 6 to 8 h after incubation with the SIV_{mac}-GFP virus and the level of late reverse transcripts in the target cells. The Spearman rank correlation coefficient and P value are shown.

pressing cells, were observed in cells expressing the Rh-RtVp and Rh-RpVp chimeras (Fig. 7C). The observed levels of particulate cytoplasmic SIV_{mac} capsids inversely correlated with the E3 ubiquitin ligase activities of the TRIM5 α variants expressed in the target cells (Fig. 7E) ($r_s = 0.7467$ and $P = 0.0013$). A strong correlation was observed between the amounts of particulate SIV_{mac} capsids in the cytosol and the levels of late SIV_{mac} reverse transcripts (Fig. 7F) ($r_s = 0.8289$ and $P = 0.0003$). In experiments where the levels of capsid proteins in the input sample were well matched, decreases in pelletable capsid in cells expressing a restricting TRIM5 α protein were accompanied by an increase in capsid protein in the supernatant. These data support the contribution of TRIM5 α RING E3 ubiquitin ligase function to the premature disassembly of the SIV_{mac} capsid in infected cells. Moreover, TRIM5 α -mediated decreases in the amounts of particulate SIV_{mac} capsids are strongly associated with blocks in reverse transcription.

The effect of proteasome inhibition by MG132 treatment of

the Rh-RtVp- and Rh-RpVp-expressing cells on the fate of the SIV_{mac} capsid was examined. MG132 treatment resulted in little change in the levels of particulate SIV_{mac} capsids in the LPCX control cells and in the Rh-RpVp-expressing cells (Fig. 7D). In contrast, the low level of particulate capsids in Rh-RtVp-expressing cells was restored to much higher levels by MG132 treatment. These effects of MG132 treatment on the level of particulate cytosolic capsids mirrored the MG132 effects on reverse transcription shown in Fig. 6D. Thus, proteasome inhibition specifically diminishes the ability of a TRIM5 α protein with potent RING-associated E3 ubiquitin ligase activity to accelerate the disassembly of SIV_{mac} capsids and to disrupt reverse transcription in infected cells.

Influence of the capsid helix-4/5 loop on susceptibility to TRIM5 α variants. Changes in the helix-4/5 loop (the “cyclophilin-binding loop”) of the HIV-1 capsid can influence sensitivity to TRIM5 α -mediated restriction (4, 24, 33, 35, 36, 47, 49, 59, 72, 78, 80, 92, 93). To investigate whether the helix-4/5 loop

FIG. 8. Capsid helix-4/5 loop determines the requirement for RING ubiquitin ligase function in early restriction. Cf2Th cells stably expressing the indicated TRIM5 α variants or transduced with the empty LPCX vector were incubated with VSV G-pseudotyped GFP-expressing viruses [SIV_{mac}, SIV_{mac}(HIV 4/5), and HIV-1]. SIV_{mac}(HIV 4/5) is SIV_{mac} containing a chimeric SIV_{mac} capsid with the loop between helices 4 and 5 replaced by the corresponding loop of the HIV-1 capsid. (A) The Cf2Th cells exposed to SIV_{mac}-GFP and SIV_{mac}(HIV 4/5)-GFP were cultured for 48 h and then analyzed by FACS for GFP expression. The data from a single experiment, which was repeated with similar results, are shown. (B) The fate of the viral capsid was studied in the cytosol of Cf2Th cells 6 to 8 h after incubation with the indicated viruses. (C) Cf2Th cells exposed to SIV_{mac}-GFP, SIV_{mac}(HIV 4/5)-GFP, and HIV-1-GFP were lysed 3 h after virus incubation, and the single-strand strong-stop DNA was PCR amplified from 100 ng of the total DNA.

might modulate viral sensitivity to TRIM5 α RING changes, we made and tested SIV_{mac}(HIV 4/5), which is identical to SIV_{mac} except that the helix-4/5 loop of its capsid has been replaced by that of HIV-1. SIV_{mac}(HIV 4/5) infection was less efficient than that of SIV_{mac} and was potentially inhibited in cells expressing the Rh-RtVp and Rh-RpVp proteins (Fig. 8A). The ratios of particulate:soluble capsid proteins in the cytosol and the levels of viral late reverse transcripts were decreased for SIV_{mac}, HIV-1, and SIV_{mac}(HIV 4/5) in cells expressing the Rh-RtVp protein compared with those in control cells transduced with the empty LPCX vector (Fig. 8B and C). In cells expressing the Rh-RpVp protein, with diminished RING E3 ubiquitin ligase function, the levels of SIV_{mac} particulate capsids and late reverse transcripts were similar to those observed in the LPCX-transduced control cells (Fig. 8B, left, and C). In contrast, the levels of SIV_{mac}(HIV 4/5) late reverse transcripts were reduced in the Rh-RpVp-expressing cells

compared with those in the LPCX control cells (Fig. 8C). Moreover, the ratio of particulate:soluble capsid proteins following SIV_{mac}(HIV 4/5) infection of the Rh-RpVp-expressing cells was approximately 5.3-fold lower than that seen after infection of the LPCX control cells (Fig. 8B, left). Treatment of the target cells with cyclosporine, which blocks cyclophilin A interaction with the HIV-1 helix-4/5 loop, did not change the level of SIV_{mac}(HIV 4/5) reverse transcripts in cells expressing the Rh-RtVp and Rh-RpVp proteins (see Fig. S5 in the supplemental material). Smaller amounts of HIV-1 particulate capsids were observed in the target cells expressing TRIM5 α _{AGM(Tan)} and all of the TRIM5 α _{rh}/TRIM5 α _{AGM} chimeras than in the LPCX-transduced control cells (Fig. 8B, right). Thus, the helix-4/5 loop of the capsid protein can influence the sensitivity of the early blocks in retroviral infection to altered RING function of the restricting TRIM5 α protein. Apparently, these effects

are independent of the binding of cyclophilin A to the helix-4/5 loop.

DISCUSSION

Deletion of the TRIM5 α RING domain or disruption of RING domain folding by alteration of the zinc-coordinating cysteine residues has been shown to result in varying effects on retroviral inhibition, depending on the TRIM5 protein and the restricted virus (12, 29, 48, 60, 75). In this study, by using a panel of RING mutants with intact zinc-binding residues, we show that the E3 ubiquitin ligase function of TRIM5 α _{AGM} is important for the restriction of SIV_{mac} infection. The relative anti-SIV_{mac} inhibitory activities of TRIM5 α variants that differed in RING domain sequences strongly correlated with the E3 ubiquitin ligase activity measured *in vitro*. The TRIM5 α _{AGM} RING variants were shown to dimerize, bind HIV-1 and SIV_{mac} capsid complexes, and restrict HIV-1 infection, supporting their structural integrity. Thus, the RING-mediated E3 ubiquitin ligase activity represents an "effector function" for TRIM5 α -mediated SIV_{mac} restriction, contributing additively with capsid binding to determine restriction potency. The Rh-RtVt and Rh-RpVp chimeric proteins provide an example of how equivalent levels of SIV_{mac} restriction can be achieved by combining robust RING and weak B30.2(SPRY) functions, or weak RING and robust B30.2(SPRY) functions, respectively.

The RING-associated E3 ubiquitin ligase activity of TRIM5 α inversely correlated with the levels of the SIV_{mac} particulate capsids in the cytosol of the infected cells and with SIV_{mac} cDNA synthesis. Moreover, the levels of particulate cytosolic capsids and viral cDNAs correlated. As the capsid is the direct binding target of TRIM5 α (31, 38, 77), our data suggest a model in which the ubiquitin ligase activity of TRIM5 α leads to accelerated disassembly of cytosolic SIV_{mac} capsids. As has been seen for unstable HIV-1 capsid mutants (17, 40, 42), premature uncoating of the SIV_{mac} capsids by TRIM5 α directly or indirectly leads to disruption of reverse transcription.

Our results indicate the importance of the E3 ubiquitin ligase activity of TRIM5 α _{AGM} to SIV_{mac} restriction. What might be the functionally important target of TRIM5 α -mediated ubiquitylation, and what are the consequences of ubiquitylation of the potential target? RING-mediated E3 ubiquitin ligases simultaneously bind both the E2 enzyme partner and substrate to transfer the ubiquitin moiety from E2 to substrate; a few TRIM family members are known to transfer ubiquitin to their ligands, which are directly recognized by the B30.2(SPRY) domains (19, 30, 39, 50, 59). Based on these examples, one might expect the ubiquitylation target of TRIM5 α to be the ligand of the B30.2(SPRY) domain, i.e., the retroviral capsid. However, no ubiquitylation of the SIV_{mac} capsid protein in either particulate or soluble form was observed in the fate-of-capsid assay. Similarly, fate-of-capsid studies conducted with N-MLV restricted by human TRIM5 α also allowed visualization of the solubilized capsid proteins that resulted from TRIM5 α action; no modified form of the capsid protein was detected (10, 64, 77). Thus, if the targeted capsid is ubiquitylated, only a very small fraction of the total protein population is modified. Another possibility is that

TRIM5 α autoubiquitylation contributes to retroviral restriction. It has been reported that infection of cells with a retrovirus susceptible to TRIM5 α restriction leads to a decrease in the level of TRIM5 α protein in the cell (66). The aggregation of TRIM5 α proteins on the capsid surface could favor ubiquitylation and proteasome-mediated turnover of a fraction of the TRIM5 α protein in the cell, potentially leading to faster uncoating of the bound capsid. However, considering that the ubiquitin chains are also able to induce conformational changes and recruit several cellular factors, depending on the type of Ub-Ub chain linkages, further work is required to substantiate a role for TRIM5 α autoubiquitylation in SIV_{mac} restriction. Finally, host cell factors could be ubiquitylated by TRIM5 α . The identification of such host cell proteins and dissection of their roles in early postentry steps in SIV_{mac} infection are worthy goals of future studies.

Proteasome inhibition resulted in increases in the levels of SIV_{mac} particulate cytosolic capsids and reverse transcripts in infected cells expressing particular restricting TRIM5 α proteins. These effects were observed for a TRIM5 α protein, Rh-RtVp, with potent RING E3 ubiquitin ligase function and were not seen in cells expressing the matched Rh-RpVp TRIM5 α protein with diminished RING activity. The phenotypic similarity of proteasome inhibition and mutagenic inactivation of the TRIM5 α RING E3 ubiquitin ligase supports the involvement of ubiquitin in pre-reverse transcription blocks to SIV_{mac} infection. Previous studies suggested that, although proteasome activity is not required for TRIM5 α antiviral activity *per se* (2, 48, 61, 66, 77), treatment with proteasome inhibitors resulted in higher levels of HIV-1 and murine leukemia virus reverse transcripts and particulate capsids in TRIM5 α -expressing target cells (2, 10, 12, 84). Interpretation of results with proteasome inhibitors, however, is complicated by the observation that the levels of retroviral cDNA and particulate capsids in the cytosol can be increased by proteasome inhibition even in cells not expressing a restricting TRIM5 α protein (Fig. 6F and 7D) (10, 12, 16, 70, 82). Moreover, proteasome inhibition causes the redistribution of TRIM5 α into large cytoplasmic aggregates (12, 14), potentially introducing nonphysiologic artifacts into the restriction mechanism. Finally, proteasome inhibitors not only block the proteasome-mediated degradation pathway, but can also deplete the pool of free ubiquitin in cells by impeding the recycling of ubiquitin from protein conjugates (9, 86). Thus, although our results clearly implicate ubiquitylation in TRIM5 α -mediated pre-reverse transcription blocks of SIV_{mac} infection, it is formally possible that ubiquitin ligation contributes to this restriction in a manner that is independent of proteasome-mediated degradation.

RING-mediated E3 ubiquitin ligase activity was apparently more important to SIV_{mac} inhibition than to HIV-1 inhibition by TRIM5 α _{AGM}. In a recent report (48), Maegawa and colleagues also observed that the phenotypes of TRIM5 α mutants with changes in RING domain cysteines depended on the restricted virus. Although further work will be needed to understand the basis for the SIV_{mac}-HIV-1 difference, we found that the helix-4/5 loop on the capsid influenced the requirement for RING-mediated E3 ubiquitin ligase activity. As changes in this loop have been shown to modulate HIV-1 capsid stability and the rate of capsid disassembly in infected cells (15, 89–91), quantitative or qualitative differences be-

tween the subunit interactions in the HIV-1 and SIV_{mac} capsids may alter the requirements for the E3 ubiquitin ligase function of TRIM5 α .

Two TRIM5 α variants, Rh-RpVp and Rh-RpVt, with poor RING domain function restricted SIV_{mac} infection moderately, even though corresponding decreases in the levels of viral reverse transcripts or particulate capsids were not detected. Apparently, SIV_{mac} restriction by these two TRIM5 α proteins occurs after reverse transcription and without detectable increases in the rate of capsid disassembly. Rh-RpVp blocked SIV_{mac} infection more potently than Rh-RpVt, indicating that the strength of this apparent post-reverse transcription block is influenced by capsid-binding affinity. TRIM protein variants have been reported to block retroviral infection after viral cDNA synthesis (90, 92). The availability of well-characterized TRIM5 α mutants should allow further exploration of the mechanism and relevance of these blocks.

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