Role of the TRIM5α RING Domain E3 Ubiquitin Ligase Activity in Capsid Disassembly, Reverse Transcription Blockade and Restriction of Simian Immunodeficiency Virus

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Abstract

The mammalian tripartite motif protein, TRIM5α, recognizes retroviral capsids entering the cytoplasm and blocks virus infection. Depending on the particular TRIM5α protein and retrovirus, complete disruption of the TRIM5α RING domain decreases virus-restricting activity to varying degrees. TRIM5α 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 TRIM5α (TRIM5α_{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 (SIVmac and HIV-1, respectively). TRIM5α_{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 TRIM5α 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 TRIM5α_{AGM}. By contrast, TRIM5α proteins attenuated in RING ubiquitin ligase function still accelerated human immunodeficiency virus (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 TRIM5α RING function. Thus, ubiquitylation mediated by the RING domain of TRIM5α_{AGM} is essential for blocking SIV_{mac} infection at the stage of capsid uncoating.
Introduction

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 (HIV-1) infection is blocked at this stage in the cells of Old World monkeys (25, 52, 71). The simian immunodeficiency virus of macaques (SIVmac) encounters similar early blocks in the cells of New World monkeys and African green monkeys (25). These early, post-entry blocks are mediated by TRIM5α, and interspecies variation in TRIM5α accounts for species-specific patterns of viral restriction (23, 32, 62, 73, 75, 88). Differences in susceptibility to TRIM5α are determined by the retroviral capsid protein (8, 58).

TRIM5α 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 TRIM5α for the retroviral capsid by mediating the dimerization and higher-order association, respectively, of TRIM5α 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 potently restricting TRIM5α 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 TRIM5α 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 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). TRIM5α possesses E3 ubiquitin ligase activity and can ubiquitylate itself (87). Deletion of the TRIM5α/TRIMCyp RING domain or the disruption of RING domain folding by alteration of the zinc-binding cysteine residues results in varying effects on retroviral inhibition, depending on the TRIM5 protein and the restricted virus (12, 29, 48, 60, 75). The SIVmac-restricting activity of African green monkey TRIM5α (TRIM5αAGM) is severely attenuated by alteration of RING domain cysteines (48). However, because RING domain changes can affect TRIM5α steady-state levels, intracellular localization, higher-order self-association or capsid binding (14, 29, 34, and 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 TRIM5αAGM RING domain or complete deletion of the rhesus macaque TRIM5α (TRIM5αrh) RING domain only partially attenuated HIV-1-inhibiting activity (29, 60). Moreover, TRIM5αAGM and TRIM5αrh 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 TRIM5α blocks N-tropic murine leukemia virus
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 TRIM5α-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 SIVmac restriction mediated
by the TRIM5α 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 SIVmac
restriction potency. Subsequent study of SIVmac restriction by a panel of RING mutants
implicated E3 ubiquitin ligase activity in TRIM5αAGM-mediated premature disassembly of the
SIVmac capsid and disruption of reverse transcription. TRIM5α RING-mediated ubiquitin ligase
function and B30.2(SPRY) domain-mediated capsid binding independently contributed to SIVmac
restriction. By contrast, the E3 ubiquitin ligase activity of the RING domain exerted little impact
on the effect of TRIM5αAGM on HIV-1 capsid disassembly, reverse transcription or infection.
Replacement of the helix 4-5 loop of the SIVmac capsid with the corresponding region from the
HIV-1 capsid diminished the requirement for TRIM5αAGM ubiquitin ligase activity in the
restriction process.
Results

Determinants of restriction potency in TRIM5α proteins from African green monkey subspecies.

The TRIM5α proteins, TRIM5α<sub>AGM(Tan)</sub> and TRIM5α<sub>AGM(Pyg)</sub>, from the respective tantalus and pygerythrus subspecies of African green monkeys, both restrict HIV-1 infection potently [10]. However, only TRIM5α<sub>AGM(Tan)</sub> efficiently inhibits SIV<sub>mac</sub> infection. There are eight amino acid differences between TRIM5α<sub>AGM(Tan)</sub> and TRIM5α<sub>AGM(Pyg)</sub>; five of these involve amino acid residues in the B30.2(SPRY) domain (Figure 1A). Chimeras between these TRIM5α<sub>AGM</sub> proteins were created and tested for SIV<sub>mac</sub>-restricting ability (Figure 1A-C). These studies revealed that the TRIM5α<sub>AGM(Tan)</sub> B30.2(SPRY) domain did not determine the potency of TRIM5α<sub>AGM(Tan)</sub> in restricting SIV<sub>mac</sub>. Therefore, a subset of the three amino acid residues in the RING, coiled coil and linker 2 regions that differ between TRIM5α<sub>AGM(Tan)</sub> and TRIM5α<sub>AGM(Pyg)</sub> must contribute to SIV<sub>mac</sub> restriction potency. Each of these residues was individually changed in both TRIM5α<sub>AGM</sub> proteins to the amino acid found in the other TRIM5α<sub>AGM</sub> protein. HeLa cells stably expressing these TRIM5α<sub>AGM</sub> variants were challenged with SIV<sub>mac</sub> and HIV-1 (Figures 1D and S1B). The results indicated that phenylalanine 34 in the RING domain is necessary and sufficient to account for the potent SIV<sub>mac</sub>-restricting ability of TRIM5α<sub>AGM(Tan)</sub>-Leucine 34 in TRIM5α<sub>AGM(Pyg)</sub>, which is less commonly found in TRIM5 or its close relatives (74), compromises the ability of this protein to restrict SIV<sub>mac</sub> infection. The parental TRIM5α<sub>AGM(Pyg)</sub> protein and all the mutants containing leucine 34 were efficiently expressed (Figure 1B) and potently blocked HIV-1 infection (Figures 1D and S1B). We conclude that the TRIM5α<sub>AGM(Pyg)</sub> protein fails to restrict SIV<sub>mac</sub> infection because of the identity of RING domain residue 34.
To assess whether leucine 34 influences the global conformation of the TRIM5α<sub>AGM</sub> protein, other known properties of TRIM5 proteins were examined. The parental TRIM5α<sub>AGM</sub> proteins and residue 34 mutants were similar with respect to dimerization efficiency and binding to HIV-1 capsid-nucleocapsid (CA-NC) complexes (Figure 1E and 1F). TRIM5α<sub>AGM(Pyg)</sub> exhibited a slower turnover rate than TRIM5α<sub>AGM(Tan)</sub>, but leucine 34 in the RING domain only contributed slightly to this difference (Figure 1G). The ability to dimerize, bind HIV-1 capsids and restrict HIV-1 infection supports the structural and functional integrity of these TRIM5α<sub>AGM</sub> variants.

Replacement of TRIM5α<sub>AGM</sub> RING domain loops with those from other TRIM5α 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; Loop2 has more divergent sequences than Loop1 (Figure 2A) (74). To investigate the possible functional implications of these differences, Loop 1 and Loop 2 sequences of TRIM5α<sub>AGM(Tan)</sub> were replaced by those of TRIM5α proteins from other primates. Replacement of the TRIM5α<sub>AGM(Tan)</sub> Loop 1 with those of TRIM5α from humans, rhesus macaques or squirrel monkeys resulted in severe decreases in SIV<sub>mac</sub> restriction, despite efficient
expression of all three mutant proteins (Figure 2B and Figure S1A). These mutant proteins all
potently restricted HIV-1 infection (Figure S1C). In contrast to the phenotypes observed for
Loop1-substituted TRIM5αAGM(Tan) proteins, TRIM5αAGM(Tan) with the human TRIM5 Loop 2
inhibited SIVmac infection as efficiently as the parental TRIM5αAGM(Tan) protein (Figure 2C).
Apparent restriction of SIVmac infection by TRIM5αAGM(Tan) is sensitive to changes in Loop 1
of the RING domain.

Alteration of the TRIM5αAGM(Tan) RING domain Loop 1. The Loop 1 regions of human and
AGM TRIM5α RING domains differ in sequence only at residues 24 and 29 (Figure 2A), both
of which are surface-exposed in the TRIM5 RING structure (1). Each of these residues in
TRIM5αAGM(Tan) was altered to multiple other amino acids. Cells expressing these mutant
proteins were challenged with SIVmac. The SIVmac-restricting ability of TRIM5αAGM(Tan)
tolerated conservative changes in glutamic acid 24, but was completely eliminated by less
conservative substitutions at this position (Figure 2D). None of the TRIM5αAGM(Tan) proteins
with changes in proline 29 restricted SIVmac infection (Figure 2E). The sensitivity of
TRIM5αAGM 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 SIVmac-
restricting ability of TRIM5αAGM(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 to TRIM5αAGM(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, 85, 93, 84), 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 TRIM5αAGM(Tan) proteins, as well as TRIM5αAGM(Pyg), were
challenged with SIVmac and HIV-1. The TRIM5αAGM(Tan) L19A and E20A proteins exhibited
minimal anti-SIVmac activity (Figure 2F and Table 1). The TRIM5αAGM(Tan) L26A mutant was
nearly as effective as the wild-type TRIM5αAGM(Tan) protein in restricting SIVmac infection
(Figure 2F). However, all three Loop 1 TRIM5αAGM(Tan) mutants efficiently restricted HIV-1
infection (Figure S1C). Apparently, specific residues in Loop 1 of the TRIM5αAGM(Tan) RING
domain are more critical for restriction of SIVmac than HIV-1.

E3 ubiquitin ligase activity of TRIM5 variants. Purified rhesus monkey TRIM5α 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 activity of TRIM5α variants.
In this assay, the transiently expressed HA-tagged TRIM5α 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 TRIM5αAGM(Tan) were
observed; concomitant incorporation of the myc-ubiquitin into proteins is consistent with
TRIM5αAGM(Tan) poly-ubiquitylation (Figure S2A). To examine the possibility that other
coprecipitated cellular E3 ligase activities might mediate this ubiquitylation activity, we washed
the immunoprecipitated TRIM5αAGM(Tan) with buffers containing increasing concentrations (from
0-1 M) of salt, and then performed the in vivo ubiquitylation assay (Figure S2B). The lack of apparent effect of salt washing on polyubiquitylation activity is consistent with the observed E3 ligase activity being mediated by TRIM5α<sub>AGM(Tan)</sub> itself. This interpretation is strengthened by our observation that changes in the TRIM5α<sub>AGM(Tan)</sub> RING domain dramatically influenced the degree of observed self-ubiquitylation (see below). Of the different E2 enzymes tested in the in vivo ubiquitylation assay, only UbcH5 functioned as an E2 ubiquitin-conjugating enzyme in support of both TRIM5α<sub>ab</sub> and TRIM5α<sub>AGM(Tan)</sub> polyubiquitylation (Figure S2C and data not shown). No TRIM5α 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, TRIM5α<sub>ab</sub> polyubiquitylation occurred regardless of the position of the available lysine on the ubiquitin molecule (Figure S2D). In addition, TRIM5α<sub>ab</sub> was found to have at least 5 sites of auto-ubiquitylation (Figure S2E).

The E3 ubiquitin ligase activities of the TRIM5α variants studied herein were compared in the above in vitro assay. Of note, wild-type TRIM5α<sub>AGM(Tan)</sub> functioned as an efficient E3 ubiquitin ligase, whereas the wild-type TRIM5α<sub>AGM(Pyg)</sub> exhibited slower kinetics and a lower efficiency of polyubiquitylation (Figure 3A). As expected from the predicted E2-interacting residues of TRIM5, L19A and E20A changes both greatly reduced the E3 ligase activity of TRIM5α<sub>AGM(Tan)</sub>, but the E24Q and L26A mutants exhibited E3 ubiquitin ligase activity comparable to that of wild-type TRIM5α<sub>AGM(Tan)</sub> (Figure 3, B and C). Substantially less self-ubiquitylation was observed for the TRIM5α<sub>AGM(Tan)</sub> P29F and F34L mutants (Figure 3C and data not shown). For the TRIM5α<sub>AGM(Tan)</sub> variants above, a strong correlation between in vivo
E3 ubiquitin ligase activity and SIV<sub>mac</sub> restriction potency was observed (Figure 3D; Spearman rank correlation coefficient $r_s = 0.9893$, two-tailed $P < 0.0001$).

**Capsid-binding ability of TRIM5<sub>α</sub>AGM RING mutants.** To determine if any of the TRIM5<sub>α</sub>AGM RING changes affected recognition of the SIV<sub>mac</sub> capsid, we assembled SIV<sub>mac</sub> capsid-nucleocapsid (CA-NC) complexes in vitro (See electron micrograph in Figure S3), and measured the binding of the TRIM5<sub>α</sub> proteins to the complexes. TRIM5<sub>α</sub>AGM(Tan) bound SIV<sub>mac</sub> CA-NC complexes with an efficiency comparable to that of rhesus macaque TRIM5<sub>α</sub> (TRIM5<sub>α</sub>rh) (Table 1). Unexpectedly, the binding of the TRIM5<sub>α</sub>AGM(Pyg) protein to SIV<sub>mac</sub> CA-NC complexes was significantly better than that of the TRIM5<sub>α</sub>AGM(Tan) protein (Figure 4 and Table 1). The binding of the TRIM5<sub>α</sub>AGM(Tan) F34L, P29F, L19A, and E20A mutants to the CA-NC complexes was comparable to that of the wild-type TRIM5<sub>α</sub>AGM(Tan) protein. The TRIM5<sub>α</sub>AGM(Pyg) L34F mutant bound CA-NC complexes comparably to the wild-type TRIM5<sub>α</sub>AGM(Pyg) protein. Thus, these changes in the TRIM5<sub>α</sub>AGM RING domain, which exert dramatic effects on both restriction of SIV<sub>mac</sub> infection and E3 ligase activity, do not detectably affect the interaction of the mutant TRIM5<sub>α</sub>AGM proteins with the SIV<sub>mac</sub> capsid.

**Contribution of RING and capsid-binding functions to TRIM5<sub>α</sub> restriction.** To assess the relative contribution of RING-mediated ubiquitin ligase activity and capsid binding to TRIM5<sub>α</sub> restriction, we sought a system in which each of these components could be individually modulated. Because rhesus macaque TRIM5<sub>α</sub> (TRIM5<sub>α</sub>rh) is a weak inhibitor of SIV<sub>mac</sub> infection, we used TRIM5<sub>α</sub>rh as a backbone to modify ubiquitin ligase activity (by RING substitution) and SIV<sub>mac</sub> capsid-binding ability (by B30.2(SPRY) substitution). Thus, segments
of the TRIM5α<sub>rh</sub> RING domain and/or the B30.2(SPRY) domain were replaced by the equivalent regions from TRIM5α<sub>AGM(Tan)</sub> or TRIM5α<sub>AGM(Pyg)</sub> (Figure 5A). In the Rh-Rt and Rh-Rp chimeras, the RING domain of TRIM5α<sub>rh</sub> is replaced by the RING domain of TRIM5α<sub>AGM(Tan)</sub> and TRIM5α<sub>AGM(Pyg)</sub>, 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<sub>rh</sub> RING domain with the equivalent loop from TRIM5α<sub>AGM(Tan)</sub>. To modulate capsid binding, some of the RING domain chimeras were further modified by the replacement of the TRIM5α<sub>rh</sub> B30.2(SPRY) domain v1 variable region with that of TRIM5α<sub>AGM(Tan)</sub> or TRIM5α<sub>AGM(Pyg)</sub> (See Rh-RtVp, Rh-RtVt, Rh-RpVp and Rh-RpVt in Figure 5A).

The chimeric proteins and the parental TRIM5α proteins were stably expressed in canine Cf2Th cells, which were challenged with SIV<sub>mac</sub>. The Rh-Rt protein with the TRIM5α<sub>AGM(Tan)</sub> RING domain inhibited SIV<sub>mac</sub> infection better than either wild-type TRIM5α<sub>rh</sub> or the Rh-Rp protein with the TRIM5α<sub>AGM(Pyg)</sub> RING domain (Figure 5B, left panel and Table 1). However, all three TRIM5α variants restricted HIV-1 infection equivalently (Figure 5B, right panel). These results imply that the TRIM5α<sub>rh</sub> and TRIM5α<sub>AGM(Pyg)</sub> RING domains are not as functionally active as the TRIM5α<sub>AGM(Tan)</sub> RING domain, and that TRIM5α RING function is more important for restriction of SIV<sub>mac</sub> than HIV-1. Replacement of the TRIM5α<sub>rh</sub> RING domain Loop 1 sequence with that of TRIM5α<sub>AGM(Tan)</sub> was sufficient to achieve enhanced restriction of SIV<sub>mac</sub> infection (See Rh-loop 1(Tan) in Table 1). As only amino acid residue 29 differs between the Loop 1 sequences of TRIM5α<sub>rh</sub> and TRIM5α<sub>AGM(Tan)</sub>, we conclude that the gain in SIV<sub>mac</sub>-restricting activity results from the replacement of histidine 29 in TRIM5α<sub>rh</sub> with
proline. By contrast, the Rh-loop 2(Tan) protein, in which Loop 2 of TRIM5αrh was replaced by that of TRIM5αAGM(Tan), did not efficiently inhibit SIVmac infection (Table 1). The E3 ubiquitin ligase activity of the TRIM5αrh-TRIM5αAGM 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 (Figure 3C), supporting the correlation between E3 ligase activity and SIVmac restriction. Also consistent with this correlation is the observation that the potently restricting Rh-loop 1(Tan) chimera, with only the TRIM5αAGM(Tan) RING Loop 1 region, exhibited more ubiquitin ligase activity than the poorly restricting protein Rh-loop 2(Tan) protein (Figure 3C). No significant difference in the turnover rates of the TRIM5αrh, Rh-Rp and Rh-Rt proteins was observed (Figure S4). These results support a role for the E3 ubiquitin ligase activity of the TRIM5α RING domain in restricting SIVmac infection.

The Rh-RtVp, Rh-RtVt, Rh-RpVp and Rh-RpVt chimeras contain RING domains and B30.2(SPRY) domain v1 regions from TRIM5αAGM(Tan) or TRIM5αAGM(Pyg) (Figure 5A). All of these chimeric proteins inhibited SIVmac infection more efficiently than TRIM5αAGM(Pyg) and TRIM5αrh (Figure 5C and Table 1). Several independent experiments resulted in the same order of anti-SIVmac 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 SIVmac restricting ability. The Rh-RtVp and Rh-RtVt chimeras with the TRIM5αAGM(Tan) RING domain inhibited SIVmac infection more efficiently than the matched proteins (Rh-RpVp and Rh-RpVt, respectively) with the RING domain of TRIM5αAGM(Pyg). Thus, we tested the hypothesis that the greater SIVmac restriction potency of the chimeric proteins with the TRIM5αAGM(Tan) RING domain was associated with higher E3 ubiquitin ligase activity. The Rh-RtVp and the Rh-RtVt
chimeras containing the TRIM5α<sub>AGM(Tan)</sub> RING domain exhibited dramatically more self-
ubiquitylation in the <i>in vitro</i> assay than the Rh-RpVp and Rh-RpVt chimeras with the
TRIM5α<sub>AGM(Pyg)</sub> RING domain (Figure 5D). TRIM5α E3 ubiquitin ligase activity was not
apparently influenced by the sequence of the B30.2(SPRY) v1 region (compare Rh-RtVp with
Rh-RtVt, and also Rh-RpVp with Rh-RpVt in Figure 5D). These results are consistent with our
hypothesis that superior E3 ubiquitin ligase activity contributes to the potency of SIV<sub>mac</sub>
restriction by the chimeric TRIM5α proteins with the RING domain of TRIM5α<sub>AGM(Tan)</sub>.

The B30.2(SPRY) domain v1 variable region is longer in TRIM5α<sub>AGM</sub> than in TRIM5α<sub>rh</sub>
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 TRIM5α<sub>AGM(Tan)</sub> or TRIM5α<sub>AGM(Pyg)</sub>
substitutes for that of TRIM5α<sub>rh</sub>. The v1 sequences of TRIM5α<sub>AGM(Tan)</sub> differ by five amino acid
residues from those of TRIM5α<sub>AGM(Pyg)</sub> (Figure 1A). The Rh-RtVp and Rh-RpVp chimeras with
the B30.2(SPRY) v1 region from TRIM5α<sub>AGM(Pyg)</sub> inhibited SIV<sub>mac</sub> infection more efficiently
than the matched proteins (Rh-RtVt and Rh-RpVt, respectively) with the TRIM5α<sub>AGM(Tan)</sub>
B30.2(SPRY) v1 region (Figure 5C). We tested the hypothesis that differences in the efficiency
of capsid binding determined the observed differences in SIV<sub>mac</sub> restriction potency by
measuring the binding of these TRIM5α variants to SIV<sub>mac</sub> CA-NC complexes. As described
above, the binding of the TRIM5α<sub>AGM(Pyg)</sub> protein to SIV<sub>mac</sub> capsid complexes was significantly
better than that of the TRIM5α<sub>AGM(Tan)</sub> protein (Figure 5E and Table 1). Both chimeric proteins
(Rh-RtVp and Rh-RpVp) with the B30.2(SPRY) v1 region from TRIM5α<sub>AGM(Pyg)</sub> bound SIV<sub>mac</sub>
capsid complexes more efficiently than the chimeric proteins (Rh-RtVt and Rh-RpVt) with the
TRIM5α<sub>AGM(Tan)</sub> v1 region (Figure 5E and Table 1). The SIV<sub>mac</sub> capsid-binding abilities of the
Rh-RtVp and Rh-RpVp chimeras were almost equivalent to that of TRIM5α<sub>AGM(Pyg)</sub>-

Differences in the RING domains of the TRIM5α chimeras had no apparent effect on the efficiency of binding to the SIV<sub>mac</sub> CA-NC complexes. Thus, the v1 variable region of the B30.2(SPRY) domain is the major determinant of differences in SIV<sub>mac</sub> capsid-binding ability among these TRIM5α 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<sub>mac</sub> restriction. Thus, the Rh-RtVt chimera, which has potent E3 ubiquitin ligase activity but poor capsid-binding ability, achieved a similar level of SIV<sub>mac</sub> restriction as the Rh-RpVp chimera, which has poor E3 ubiquitin ligase activity but potent capsid-binding ability (Figure 5C and Table 1).

SIV<sub>mac</sub> reverse transcription in TRIM5α-expressing cells. The TRIM5α variants studied herein exhibited defined differences in RING-mediated E3 ubiquitin ligase activity and, in some cases, in capsid-binding affinity as well. This afforded an opportunity to examine the impact of changes in TRIM5α ubiquitylation capacity and capsid binding on the reverse transcription process and the fate of the retroviral capsid in infected cells. The level of late SIV<sub>mac</sub> reverse transcripts following the infection of cells expressing TRIM5α<sub>AGM(Pyg)</sub> was comparable to that measured in cells transduced with the empty LPCX vector control (Figure 6A). By contrast, the levels of SIV<sub>mac</sub> late reverse transcripts were dramatically reduced in target cells expressing TRIM5α<sub>AGM(Tan)</sub> or the functionally active TRIM5α<sub>AGM(Tan) E24Q</sub> and L26A mutants (Figure 6A and Table 1). Efficient SIV<sub>mac</sub> reverse transcription was observed in cells expressing the
TRIM5α<sub>AGM(Tan)</sub> L19A, E20A and P29F mutants, which are defective in E3 ligase activity and SIV<sub>mac</sub> restriction (Figures 2 and 3 and Table 1). Thus, for this group of TRIM5α<sub>AGM</sub> variants, both RING-mediated ubiquitylation and inhibition of viral reverse transcription correlated with SIV<sub>mac</sub> restriction potency.

The levels of SIV<sub>mac</sub> late reverse transcripts were also examined in target cells expressing the TRIM5α<sub>α</sub>-TRIM5α<sub>AGM</sub> chimeric proteins (Figure 6A and Table 1). The levels of SIV<sub>mac</sub> cDNA were lowest in the target cells expressing the chimeric proteins (Rh-RtVp and Rh-RtVt) containing the TRIM5α<sub>AGM(Tan)</sub> RING domain, which specifies robust E3 ubiquitin ligase activity. SIV<sub>mac</sub> 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<sub>mac</sub> infection were nearly identical in target cells expressing the Rh-RtVt and Rh-RpVp proteins (Figure 5C), which contain counterbalancing RING and B30.2(SPRY) domain functions, the levels of SIV<sub>mac</sub> 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<sub>mac</sub> reverse transcripts (Figure 6B and data not shown), indicating that the TRIM5α-mediated blocks occur prior to or at the earliest stages of reverse transcription. For the entire panel of TRIM5α variants, inhibition of viral reverse transcription strongly correlated with the E3 ubiquitin ligase efficiency of the RING domain (Figure 6C) (Spearman rank correlation coefficient <i>r_s</i> = 0.9510, two-tailed <i>P</i> < 0.0001).

Effects of proteasome inhibition on TRIM5α-mediated restriction and disruption of reverse transcription. A previous study suggested that approximately 10 per cent of the TRIM5α<sub>AGM</sub>
restriction of SIV<sub>mac</sub> 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<sub>mac</sub> infection mediated by the TRIM5<sub>α</sub>Rh-TRIM5<sub>α</sub>AGM chimeras. Late SIV<sub>mac</sub> reverse transcripts in target cells expressing the Rh-RtVp protein increased after treatment of the target cells with the proteasome inhibitors MG132 or ALLN (Figure 6D). The proteasome inhibitor-mediated increase in Rh-RtVp late reverse transcripts was evident by one hour after infection (Figure 6E). Treatment with proteasome inhibitors minimally affected the levels of SIV<sub>mac</sub> late reverse transcripts in target cells expressing the Rh-RpVp protein or control cells transduced with the empty LPCX vector (Figure 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<sub>mac</sub> reverse transcripts in the Rh-RpVp-expressing or LPCX-transduced cells (Figure 6F). Thus, proteasome inhibition specifically diminishes the ability of a TRIM5<sub>α</sub> protein with potent RING-associated E3 ubiquitin ligase activity to mediate decreases in SIV<sub>mac</sub> cDNA following infection.

The effects of proteasome inhibition on the restriction of SIV<sub>mac</sub> 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<sub>mac</sub> infection, perhaps due to the effects of the treatment on cell viability (Figure 6G). MG132 treatment increased the efficiency with which SIV<sub>mac</sub> infected cells expressing the Rh-RtVp protein, but did not affect SIV<sub>mac</sub> infection of the Rh-RpVp-expressing cells. Thus, proteasome inhibition partially relieves SIV<sub>mac</sub> restriction by a TRIM5<sub>α</sub> protein with potent RING-associated
E3 ubiquitin ligase activity, but not by a matched TRIM5α protein with poor RING E3 ubiquitin ligase function.

**Fate of the SIV<sub>mac</sub> capsid in cells expressing the TRIM5α variants.** To investigate the impact of TRIM5 RING function on capsid disassembly, the amount of particulate SIV<sub>mac</sub> capsids in the cytoplasm of SIV<sub>mac</sub>-challenged cells expressing the TRIM5α variants was measured (77). Minimal amounts of SIV<sub>mac</sub> capsid proteins were detected in the cytosol of cells exposed to a recombinant SIV<sub>mac</sub> without envelope glycoproteins (Figure 7A). The amount of particulate SIV<sub>mac</sub> capsids detected in the cytosol of cells expressing a TRIM5α protein from spider monkeys (a New World monkey) was less than that detected in cells expressing TRIM5α<sub>AGM(Tan)</sub>, which in turn was less than that seen in TRIM5α<sub>rh</sub>-expressing cells (Figure 7A). This order corresponds to the level of SIV<sub>mac</sub> infection observed in the cells expressing the TRIM5α proteins from these three monkey species (17 and Table 1). The level of cytosolic particulate SIV<sub>mac</sub> capsids was lower in infected cells expressing TRIM5α<sub>AGM(Tan)</sub> compared with that in TRIM5α<sub>AGM(Pyg)</sub>-expressing cells (Figure 7B). The level of cytosolic particulate SIV<sub>mac</sub> capsids was lower in cells expressing the restricting TRIM5α<sub>AGM(Tan)</sub> L26A mutant than in cells expressing the poorly restricting L19A mutant (Figure 7B).

The levels of cytosolic particulate SIV<sub>mac</sub> capsids in infected cells expressing the TRIM5α<sub>rh</sub>-TRIM5α<sub>AGM</sub> chimeric proteins were also examined. A high level of particulate SIV<sub>mac</sub> capsids was observed in the cells expressing the Rh-RpVp and Rh-RpVt proteins (Figure 7C). Lower levels of particulate SIV<sub>mac</sub> capsids, comparable to those seen in TRIM5α<sub>AGM(Tan)</sub> expressing cells, were observed in cells expressing the Rh-RtVt and Rh-RtVp chimeras (Figure
The observed levels of particulate cytoplasmic SIV\textsubscript{mac} capsids inversely correlated with the E3 ubiquitin ligase activities of the TRIM5\textalpha\ variants expressed in the target cells (Figure 7E) (Spearman rank correlation coefficient $r_S=0.7467$ and $P=0.0013$). A strong correlation was observed between the amounts of particulate SIV\textsubscript{mac} capsids in the cytosol and the levels of late SIV\textsubscript{mac} reverse transcripts (Figure 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\textalpha\ protein were accompanied by an increase in capsid protein in the supernatant. These data support the contribution of TRIM5\textalpha\ RING E3 ubiquitin ligase function to the premature disassembly of the SIV\textsubscript{mac} capsid in infected cells. Moreover, TRIM5\textalpha\-mediated decreases in the amounts of particulate SIV\textsubscript{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\textsubscript{mac} capsid was examined. MG132 treatment resulted in little change in the levels of particulate SIV\textsubscript{mac} capsids in the LPCX control cells and in the Rh-RpVp-expressing cells (Figure 7D). By 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 Figure 6D. Thus, proteasome inhibition specifically diminishes the ability of a TRIM5\textalpha\ protein with potent RING-associated E3 ubiquitin ligase activity to accelerate the disassembly of SIV\textsubscript{mac} capsids and to disrupt reverse transcription in infected cells.
Influence of the capsid helix-4/5 loop on susceptibility to TRIM5α variants. Changes in the helix-4/5 loop (the “cyclophilin-binding loop”) of the HIV-1 capsid can influence sensitivity to TRIM5α-mediated restriction (4, 24, 33, 35-36, 47, 49, 59, 72, 78, 80, 92, 93). To investigate whether the helix-4/5 loop might modulate viral sensitivity to TRIM5α RING changes, we made and tested SIVmac(HIV 4/5), which is identical to SIVmac except that the helix-4/5 loop of its capsid has been replaced by that of HIV-1. SIVmac(HIV 4/5) infection was less efficient than that of SIVmac, and was potently inhibited in cells expressing the Rh-RtVp and Rh-RpVp proteins (Figure 8A). The ratios of particulate:soluble capsid proteins in the cytosol and the levels of viral late reverse transcripts were decreased for SIVmac, HIV-1, and SIVmac(HIV 4/5) in cells expressing the Rh-RtVp protein, compared with those in control cells transduced with the empty LPCX vector (Figure 8, B and C). In cells expressing the Rh-RpVp protein, with diminished RING E3 ubiquitin ligase function, the levels of SIVmac particulate capsids and late reverse transcripts were similar to those observed in the LPCX-transduced control cells (Figure 8B, left panel and Figure 8C). By contrast, the levels of SIVmac(HIV 4/5) late reverse transcripts were reduced in the Rh-RpVp-expressing cells compared with those in the LPCX control cells (Figure 8C). Moreover, the ratio of particulate:soluble capsid proteins following SIVmac(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 (Figure 8B, left panel). 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 SIVmac(HIV 4/5) reverse transcripts in cells expressing the Rh-RtVp and Rh-RpVp proteins (Figure S5). Lower amounts of HIV-1 particulate capsids were observed in the target cells expressing TRIM5αAGM(Tan) and all of the TRIM5αrh/ TRIM5αAGM chimeras, relative to those seen in the LPCX-transduced control cells (Figure 8B, right panel). 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 TRIM5α protein. Apparently, these effects are independent of the binding of cyclophilin A to the helix-4/5 loop.
Deletion of the TRIM5α 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 TRIM5α<sub>AGM</sub> is important for the restriction of SIV<sub>mac</sub> infection. The relative anti-SIV<sub>mac</sub> inhibitory activity of TRIM5α<sub>AGM</sub> variants that differed in RING domain sequences strongly correlated with the E3 ubiquitin ligase activity measured in vitro. The TRIM5α<sub>AGM</sub> RING variants were shown to dimerize, bind HIV-1 and SIV<sub>mac</sub> capsid complexes, and restrict HIV-1 infection, supporting their structural integrity. Thus, the RING-mediated E3 ubiquitin ligase activity represents an “effector function” for TRIM5α<sub>AGM</sub>-mediated SIV<sub>mac</sub> 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<sub>mac</sub> restriction can be achieved by combining robust RING with weak B30.2(SPRY) functions, or weak RING with robust B30.2(SPRY) functions, respectively.

The RING-associated E3 ubiquitin ligase activity of TRIM5α inversely correlated with the levels of the SIV<sub>mac</sub> particulate capsids in the cytosol of the infected cells and with SIV<sub>mac</sub> cDNA synthesis. Moreover, the levels of particulate cytosolic capsids and viral cDNAs correlated. As the capsid is the direct binding target of TRIM5α (31, 38, 77), our data suggest a model in which the ubiquitin ligase activity of TRIM5α leads to accelerated disassembly of cytosolic SIV<sub>mac</sub> capsids. As has been seen for unstable HIV-1 capsid mutants (17, 40, 42),...
premature uncoating of the SIVmac capsids by TRIM5α directly or indirectly leads to disruption of reverse transcription.

Our results indicate the importance of the E3 ubiquitin ligase activity of TRIM5αAGM to SIVmac restriction. What might be the functionally important target of TRIM5α-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 TRIM5α to be the ligand of the B30.2(SPRY) domain, i.e., the retroviral capsid. However, no ubiquitylation of the SIVmac capsid protein in either particulate or soluble forms was observed in the fate-of-capsid assay. Similarly, fate-of-capsid studies conducted with N-tropic murine leukemia virus (N-MLV) restricted by human TRIM5α also allowed visualization of the solubilized capsid proteins that resulted from TRIM5α 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 TRIM5α auto-ubiquitylation contributes to retroviral restriction. It has been reported that infection of cells with a retrovirus susceptible to TRIM5α restriction leads to a decrease in the level of TRIM5α protein in the cell (66). The aggregation of TRIM5α proteins on the capsid surface could favor ubiquitylation and proteasome-mediated turnover of a fraction of the TRIM5α 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 TRIM5α auto-ubiquitylation in SIVmac restriction. Finally, host cell factors could be ubiquitylated by TRIM5α. The identification of such host cell proteins and dissection of their role in early post-entry steps in SIVmac infection are worthy goals of future efforts.

Proteasome inhibition resulted in increases in the levels of SIVmac particulate cytosolic capsids and reverse transcripts in infected cells expressing particular restricting TRIM5α proteins. These effects were observed for a TRIM5α protein, Rh-RtVp, with potent RING E3 ubiquitin ligase function and were not seen in cells expressing the matched Rh-RpVp TRIM5α protein with diminished RING activity. The phenotypic similarity of proteasome inhibition and mutagenic inactivation of the TRIM5α RING E3 ubiquitin ligase supports the involvement of ubiquitin in pre-reverse transcription blocks to SIVmac infection. Previous studies suggested that, although proteasome activity is not required for TRIM5α 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 TRIM5α-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 TRIM5α protein (Figures 6F and 7D) (10, 12, 16, 70, 82). Moreover, proteasome inhibition causes the redistribution of TRIM5α into large cytoplasmic aggregates (12, 14), potentially introducing non-physiologic artefacts 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 TRIM5α-mediated pre-reverse transcription blocks of SIV<sub>mac</sub> 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<sub>mac</sub> inhibition than to HIV-1 inhibition by TRIM5α<sub>AGM</sub>. In a recent report (48), Maegawa and colleagues also observed that the phenotypes of TRIM5α 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<sub>mac</sub>-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 [89-91], quantitative or qualitative differences between the subunit interactions in the HIV-1 and SIV<sub>mac</sub> capsids may alter the requirements for the E3 ubiquitin ligase function of TRIM5α.

Two TRIM5α variants, Rh-RpVp and Rh-RpVt, with poor RING domain function restricted SIV<sub>mac</sub> infection moderately, even though corresponding decreases in the levels of viral reverse transcripts or particulate capsids were not detected. Apparently, SIVmac restriction by these two TRIM5α proteins occurs after reverse transcription and without detectable increases in the rate of capsid disassembly. Rh-RpVp blocked SIV<sub>mac</sub> 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 TRIM5α mutants should allow further exploration of the mechanism and relevance of these blocks.
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 TRIM5α variants, recombinant retrovirus vectors were produced, using the pLPCX vector plasmid and a packaging system (pVPack-GP and pVPack-VSV-G) (Stratagene), as described (75). The retroviral vectors were used to transduce either HeLa or Cf2Th cells, followed by selection in either 1 μg/ml or 5 μg/ml puromycin, respectively. Once the cell lines were generated, the level of TRIM5α expressed stably in the cells was determined by Western blotting with an antibody (Roche) directed against the hemagglutinin (HA) epitope tag on the TRIM5α proteins (see below).

The TRIM5 genes from the tantalus and pygerythus subspecies of African green monkeys were originally cloned by polymerase chain reaction (PCR) amplification from COS-1 and Vero cells, respectively, as previously reported (73). All TRIM5 genes were cloned by using the EcoR I and Cla I sites in pLPCX; the encoded TRIM5α proteins have an HA-tag at their C-terminus. All mutations were introduced by using overlap-PCR and were confirmed by sequencing at the DFCI Molecular Biology Core Facility. The chimerae between TRIM5αAGM(Tan) and TRIM5αAGM(Pyg), or between TRIM5αAGM(Tan), TRIM5αAGM(Pyg) and...
TRIM5α<sub>th</sub> were made swapping the corresponding gene fragments generated by combinations of EcoRI, BstXI, SphI, BamHI, and ClaI.

SIV<sub>mac</sub>(HIV 4/5) contains an SIV<sub>mac</sub> capsid with the helix 4/5 loop replaced by that of HIV-1. To generate the SIV<sub>mac</sub>(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<sub>mac</sub>-ΔNefΔEnv-GFP plasmid.

Single-round infection assay

Recombinant green fluorescent protein (GFP)-expressing viruses (SIV<sub>mac</sub>-GFP, HIV-1-GFP, and SIV<sub>mac</sub>(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 (25, 73, 75). For the single-round infection assay, virus stocks were serially diluted by 2-fold and used to infect 24-well plates seeded with 2 x 10<sup>4</sup> cells the day before. After 48-60 hours of incubation at 37°C, each well was washed with 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 level of SIV<sub>mac</sub> infection in cells expressing different TRIM5α variants was 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 TRIM5α variant was divided by the percentage of GFP-positive cells observed in the control LPCX-transduced cells to derive the relative SIV\textsubscript{mac} infection level.

**TRIM5α oligomerization and half-life**

TRIM5α-expressing Cf2Th cell lines were seeded in 6-well plates a day in advance. Cells were lysed with MPER lysis buffer (Pierce) containing 1x protease inhibitor cocktail (Roche). After centrifugation at 15,000 x g for 10 minutes 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 crosslinking TRIM5α, the same amount of protein from each lysate was mixed with increasing amounts (0, 0.5, and 2 mM) of ethylene glycol bis [sulfosuccinimidylsuccinate] (sulfo-EGS; Pierce) and incubated at room temperature for 30 minutes. The reactions were stopped with 1M Tris-HCl, pH 8.0. The crosslinked TRIM5α proteins were analyzed by Western blotting with an anti-HA antibody.

To measure the half-life of TRIM5α variants, TRIM5α-expressing Cf2Th cells were incubated in medium containing cycloheximide at a final concentration of 100 μg/ml. Cells were harvested at regular intervals, washed with PBS, and kept on ice until the final batch of cells was harvested. 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 \( \text{TRIM5} \alpha \) protein.

**TRIM5\( \alpha \) binding to HIV-1 and SIV\( \text{mac} \) capsids**

The HIV-1 and SIV\( \text{mac} \) capsid-nucleocapsid (CA-NC) fusion proteins were purified from IPTG-induced *Escherichia coli* as previously described (20). The assay measuring the binding of TRIM5\( \alpha \) variants to the assembled HIV-1 CA-NC complexes was performed as previously described (77). To assemble SIV\( \text{mac} \) CA-NC complexes, 0.6 mM purified protein was mixed with 16 \( \mu \)M (TG)\( _{50} \) DNA oligonucleotide in 50 mM Tris-HCl, pH 7.0 and 1 M NaCl and incubated at 37\(^\circ\)C for more than 1 day. The resulting SIV\( \text{mac} \) CA-NC complexes were negatively stained and examined under the electron microscope; these studies confirmed that the diameter and length of the cylindrical CA-NC complexes are similar to those of previously reported HIV-1 CA-NC complexes (20) (Figure S3).

Approximately 2 \( \times \) 10\(^6 \) HEK293T cells were transfected to express the different TRIM5\( \alpha \) proteins transiently and then harvested 24 hours 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\(^\circ\)C to remove large cell debris, the supernatant was spun at 110,000 \( \times \) g for 1 hour at 4\(^\circ\)C in a Beckman ultracentrifuge. After this pre-clearing spin, the retrieved supernatant was brought up to a final volume of 200 \( \mu \)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\textsubscript{mac} CA-NC complexes was added and incubated at 30°C for 1 hour. After incubation, 20 μl of the mixture was saved for further analysis (“Input”). The rest of the mixture was layered onto a 60% (w/v) sucrose cushion (prepared in 1x PBS) and centrifuged at 110,000 x g for 1 hour 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 TRIM5α proteins with an anti-HA antibody, and stained with Coomassie Brilliant Blue to visualize the SIV\textsubscript{mac} CA-NC proteins. Control experiments indicated that, in the absence of added SIVmac CA-NC complexes, no TRIM5α proteins were detected in the pellets (data not shown).

\textit{In vitro E3 ubiquitin ligase activity}

The E3 ubiquitin ligase activity of a TRIM5α variant was assessed by measuring self-ubiquitylation \textit{in vitro}. Either transfected HEK293T cells transiently expressing TRIM5α or Cf2Th cells stably expressing TRIM5α 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% NP40, 0.5% sodium deoxycholate, 0.1% SDS and 1x protease inhibitor cocktail) or NP40 buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5mM EDTA, 1% NP40, and 1x protease inhibitor cocktail). After removing cell debris by brief centrifugation, 5 μg of anti-HA antibody (Sigma) was added to the soluble fraction and incubated at 4°C for 1 hour or overnight. Magnetic protein G-beads (Invitrogen), pre-equilibrated with the lysis buffer, were used to precipitate the complex of TRIM5α 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 DTT), and resuspended in 10 μl HEPES buffer. The protein G-bead suspension was mixed with 0.44 μg E1, 0.34 μg UbcH5a,
and 20 nM myc-Ubiquitin (final concentration) either in the presence or absence of Energy regeneration mixture (1x Energy regeneration buffer and 1 mM ATP final concentration) in a reaction volume of 20 µl. 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 Figure S2C. 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 TRIM5α proteins were detected using an anti-HA antibody. Control experiments demonstrated that the ratio of polyubiquitylated TRIM5α 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 TRIM5α protein of interest. In two or three independent experiments, the amounts of unmodified and polyubiquitylated TRIM5α proteins were measured using a densitometer. After subtracting the background polyubiquitylated TRIM5α signal observed in the absence of the added Energy regeneration mixture, the ratio of poly-ubiquitylated:unmodified TRIM5α protein for each TRIM5α variant was normalized to that observed for wild-type TRIM5α<sub>AGM(Tan)</sub>. The relative E3 ubiquitin ligase activity is expressed as a percentage of the activity of wild-type TRIM5α<sub>AGM(Tan)</sub>, which is set at 100%.

**Measurement of viral reverse transcription products**
One day prior to infection, 5 x 10^5 CI2Th 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 quantified by an *in vitro* reverse transcriptase (RT) assay described previously (75), and 5 x 10^4 cpn of RT activity was used for infection. The virus stocks were all pre-treated with 60 units of turbo-DNase I (Ambion) at 37°C for 2 hours prior to incubation with cells. As a negative control, cells were treated with 25 μM azidothymidine (AZT) for 1 hour 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 hours; for measurement of the late reverse transcripts, a 10-12-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 PCR reactions, which were conducted in a final volume of 50 μl under the following conditions: 95°C for 3 minutes followed by 32 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 68°C for 30 seconds. The reaction products were analyzed on a 1.5% agarose gel. The primer pairs are as follows: for minus-strand strong-stop cDNA, forward 5′-AGTCGCTCTGCGGAGAGGCTG-3′, reverse 5′-TGCTAGGGATTTTCCTGCTTCGGTTT-3; for late reverse transcripts, forward 5′-CACTAGGATTTCTGCTTCGGTTT-3, 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 TRIM5α variants were seeded in T75 flasks. On the following day, the flask was incubated at 4°C for 15 minutes, and the cell medium was replaced by 6 ml cold cell culture medium containing 5 x 10^6 cpm RT 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 minutes, the cell-virus mixture was shifted to 37°C. After incubation at 37°C for 6-10 hours, the cells were washed three times with cold PBS and then treated with pronase (7 mg/ml in DMEM, Roche) for 5 minutes on ice. The cells were harvested, washed three times with cold PBS, resuspended in 250 μl hypolysis buffer (10 mM Tris-Cl, pH 8.0, 10 mM KCl2, 1 mM EDTA), and lysed by motorized pestle (Sigma) for 1 minute. After centrifugation at 1,500 x g for 3 minutes, the supernatant was transferred to a new pre-chilled tube and mixed with 250 μl cold hypolysis buffer. Fifty μl 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 minutes, and then loaded onto a 50% (w/v) sucrose cushion in PBS. The particulate viral capsids were pelleted by centrifugation at 30,000 rpm for 2 hours in a Beckman SW41 rotor at 4°C. From the top of each tube, 100 μl of supernatant was saved for analysis and the pellet was resuspended in 60 μl 1x 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 TRIM5α E3 ubiquitin ligase activity, the level of late reverse transcription products, the amount of pelletable capsid, and the level of SIV<sub>mac</sub> 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<sub>S</sub>) and the P value.

The TRIM5α variants included in each analysis were:

1) For relative level of SIV<sub>mac</sub> infection versus E3 ubiquitin ligase activity:

2) For late reverse transcripts versus E3 ubiquitin ligase activity:

3) For pelletable capsid versus late reverse transcripts, and for E3 ubiquitin ligase activity versus pelletable capsids:
Acknowledgments

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References


43. **Li, X., B. Song, S-H. Xiang, and J. Sodroski.** 2007. Functional interplay between the B-box 2 and the B30.2(SPRY) domains of TRIM5alpha. Virology **366**:234-244.
44. **Li, X., and J. Sodroski.** 2008. The TRIM5alpha B-box 2 domain promotes cooperative binding to the retroviral capsid by mediating higher-order self-association. J. Virol. 82:11495-11502.


Figure Legends

Figure 1. A single RING domain residue determines the potency of SIV\textsubscript{mac} restriction by TRIM5\textsubscript{AGM} variants. (A) The domains of TRIM5\textsubscript{a} are depicted in the upper figure, 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\textsubscript{a\textsubscript{AGM(Tan)}} and TRIM5\textsubscript{a\textsubscript{AGM(Pyg)}} are shown in single-letter code. The B30.2(SPRY) region chimeras are depicted. B = B-box 2 domain. (B) The steady-state expression levels of the TRIM5\textsubscript{a} 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 \(\beta\)-actin. (C,D) HeLa cells expressing the TRIM5\textsubscript{a\textsubscript{AGM}} variants or transduced with the empty LPCX vector were incubated with the indicated volume of recombinant VSV G-pseudotyped SIV\textsubscript{mac}-GFP or HIV-1-GFP virus stocks. After 72 hours, GFP-positive cells were analyzed by FACS. The results shown are representative of those obtained in three independent experiments. (E) The TRIM5\textsubscript{a} proteins in the lysates of stably expressing Cf\textsubscript{2Th} cells were incubated with sulfo-EGS at the indicated concentrations. The crosslinked TRIM5\textsubscript{a} 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\textsubscript{a} proteins are indicated. (F) The ability of the TRIM5\textsubscript{a}
variants to bind HIV-1 capsid complexes was tested by incubating the “Input” amounts of TRIM5α 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 TRIM5α protein. The CA-NC protein was detected by Coomassie Brilliant Blue staining of the gel. (G) The half-life of the indicated TRIM5α variants was investigated by incubating Cf2Th cells expressing the TRIM5α 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.

Figure 2. Specific sequences in Loop 1 of the TRIM5αAGM(Tan) RING domain are critical for SIVmac restriction. (A) The sequences of the RING domains of TRIM5α proteins from African green monkeys (tantalus subspecies), humans, rhesus monkeys and squirrel monkeys are aligned. The amino acid residue numbers are based on those of TRIM5αAGM(Tan). Areas where sequence differences exist are highlighted in the grey boxes. The dots above the sequence indicate cysteine and histidine residues involved in zinc coordination. The location of Loops 1 and 2 is shown. (B-F) HeLa (B-E) or Cf2Th (F) cells, either transduced with the empty LPCX vector or expressing TRIM5αAGM(Pyg) or the indicated TRIM5αAGM(Tan) variants, were incubated with different amounts of
recombinant VSV G-pseudotyped SIV\textsubscript{mac}-GFP virus stocks. Different preparations of the SIV\textsubscript{mac}-GFP virus stock were used in the experiments. After 72 hours, GFP-positive cells were analyzed by FACS. The results shown are representative of those obtained in three independent experiments.

**Figure 3.** Restriction of SIV\textsubscript{mac} infection by TRIM5\textsubscript{α} RING variants correlates with E3 ubiquitin ligase activity. (A) The ability of HA-tagged TRIM5\textsubscript{α}\textsubscript{AGM(Tan)} and TRIM5\textsubscript{α}\textsubscript{AGM(Pyg)} proteins precipitated from the lysates of stably expressing cells to mediate auto-ubiquitylation *in vitro* is shown. After the indicated times of incubation at 37°C, the reaction products were subjected to Western blotting with an anti-HA epitope tag. Poly-ubiquitylated forms of TRIM5\textsubscript{α} having more than 5 conjugated ubiquitins are indicated. The numbers beneath each lane represent the percent conversion of TRIM5\textsubscript{α} protein to polyubiquitylated forms. (B, C) The assay for *in vitro* auto-ubiquitylation of wild-type (wt) TRIM5\textsubscript{α}\textsubscript{AGM(Tan)}h the indicated TRIM5\textsubscript{α}\textsubscript{AGM(Tan)} mutants, and wild-type TRIM5\textsubscript{α}\textsubscript{rh} (Rh) or TRIM5\textsubscript{α}\textsubscript{rh} with the indicated RING substitutions was performed for two-hours at 37°C in the absence (-) or presence (+) of the energy regeneration system (ERS). The numbers beneath each lane represent the percent conversion of TRIM5\textsubscript{α} protein to polyubiquitylated forms. (D) The relationship between the *in vitro* E3 ubiquitin ligase activity of TRIM5\textsubscript{α} variants and the relative level of SIV\textsubscript{mac} infection.
observed in cells expressing those TRIM5α proteins is shown. The Spearman rank correlation coefficient ($r_s$) and $P$ value are shown.

**Figure 4.** **RING domain changes in TRIM5α$_{AGM}$ affecting SIV$_{mac}$ restriction do not alter capsid binding.** The ability of the TRIM5α variants to bind SIV$_{mac}$ capsid complexes was tested by incubating the “Input” amounts of TRIM5α 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 TRIM5α protein. The gel was stained with Coomassie Brilliant Blue to detect the CA-NC protein.

**Figure 5.** **RING domain-mediated ubiquitin ligase activity and B30.2(SPRY)-mediated capsid binding independently determine restriction potency.**

(A) The wild-type TRIM5α$_{rh}$ and chimeric proteins in which the TRIM5α$_{rh}$ RING domain and, in some cases, the B30.2(SPRY) v1 region were replaced by the corresponding regions of TRIM5α$_{AGM(Tan)}$ or TRIM5α$_{AGM(Pyg)}$ are depicted. (B, C) Cf2Th cells expressing the indicated TRIM5α$_{AGM}$ 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 hours, GFP-positive cells were analyzed by FACS. The results shown are representative of those obtained in three independent experiments. (D) The in vitro auto-ubiquitylation of the TRIM5α$_{AGM(Tan)}$, TRIM5α$_{AGM(Pyg)}$ and the TRIM5α$_{rh}$
chimeric proteins, after a two-hour incubation at 37°C in the absence (-) or presence (+) of the energy regeneration system (ERS), is shown. (E) The ability of the TRIM5α variants to bind SIV<sub>mac</sub> capsid complexes was tested by incubating the “Input” amounts of TRIM5α proteins with SIV<sub>mac</sub> CA-NC complexes assembled <i>in vitro</i>. After centrifugation through a 60% sucrose cushion, the pellet was analyzed by Western blotting for the bound TRIM5α protein. The gel was stained with Coomassie Brilliant Blue to detect the CA-NC protein.

**Figure 6.** TRIM5α RING-mediated ubiquitylation is essential for disruption of SIV<sub>mac</sub> reverse transcription. Cf2Th cells transduced with the empty LPCX vector or expressing the TRIM5α<sub>AGM(Tan)</sub>, TRIM5α<sub>AGM(Pyg)</sub> and TRIM5α<sub>rh-TRIM5α<sub>AGM</sub></sub> chimeras were incubated with 7.5 x 10<sup>5</sup> RT-cpm units of SIV<sub>mac</sub>-GFP virus, 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 pre-treated with AZT as a control. (C) A correlation is shown between the E3 ubiquitin ligase activity of TRIM5α proteins and the level of late reverse transcripts detected in cells expressing the TRIM5α proteins following incubation with the SIV<sub>mac</sub>-GFP virus. The Spearman rank correlation coefficient (r<sub>S</sub>) and P value are shown. (D) Cf2Th cells expressing the Rh-RpVp or Rh-RtVp TRIM5α proteins or transduced with the empty LPCX
vector were pre-treated for one hour with the proteasome inhibitors, MG132 (3 and 10 μM) or ALLN (2.5 and 5 μM), or with the DMSO solvent as a control. The SIV<sub>mac</sub>-GFP virus was added to the cells, and the virus-cell mixture was incubated at 37°C for 2 hours in the presence of the proteasome inhibitors. The medium was then replaced with fresh medium without proteasome inhibitors and the cells were cultured at 37°C for three hours 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 TRIM5α proteins were treated with proteasome inhibitors or DMSO solvent and exposed to the SIV<sub>mac</sub>-GFP virus, as in (D). At the indicated times, cells were harvested and used for DNA extraction and analysis of minus-strand strong-stop DNA. The bottom panel is a longer exposure of the middle panel. (F) Semi-quantitative PCR was performed with two-fold dilutions of the total DNA extracted from the LPCX-transduced control cells and the Rh-RpVp-expressing cells exposed to SIV<sub>mac</sub>-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 TRIM5α proteins or transduced with the empty LPCX vector were pre-treated with either DMSO (solid symbols) or MG132 (open symbols) for one hour. The cells were then incubated with SIV<sub>mac</sub>-GFP virus in the presence of 5 μg/ml polybrene for 2 hours in the continued presence of DMSO or MG132. The cells were then washed and returned to culture for 48 hours until FACS analysis was performed.
Figure 7. TRIM5α RING-mediated ubiquitylation is important for the accelerated disassembly of SIV\textsubscript{mac} capsids. (A-D) The fate of the SIV\textsubscript{mac} capsid was examined in Cf2Th cells expressing the indicated TRIM5α variants or transduced with the empty LPCX vector. The cells were incubated for 6-8 hours with SIV\textsubscript{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. In (D), the target cells were treated with MG132 (+) or DMSO (-) prior to and during addition of the SIV\textsubscript{mac}-GFP virus, as described in the legend to Figure 6. The experiments were performed 3-4 times, and typical results derived from a single experiment are shown. (E) The correlation between the E3 ubiquitin ligase activity of TRIM5α variants and the amount of pelletable cytoplasmic capsids in the TRIM5α-expressing cells 6-8 hours after incubation with the SIV\textsubscript{mac}-GFP virus is shown. The Spearman rank correlation coefficient (r\textsubscript{s}) and P value are shown. (F) The correlation between the amount of pelletable capsids in the cytoplasm of TRIM5α-expressing cells 6-8 hours after incubation with the SIV\textsubscript{mac}-GFP virus and the level of late reverse transcripts in the target cells is shown. The Spearman rank correlation coefficient and P value are shown.
Figure 8. Capsid helix-4/5 loop determines the requirement for RING ubiquitin ligase function in early restriction. Cf2Th cells stably expressing the indicated TRIM5α variants or transduced with the empty LPCX vector were incubated with VSV G-pseudotyped GFP-expressing viruses (SIVmac, SIVmac(HIV 4/5) and HIV-1). SIVmac(HIV 4/5) is SIVmac containing a chimeric SIVmac 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 the SIVmac-GFP and SIVmac(HIV 4/5)-GFP viruses were cultured for 48 hours 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 the Cf2Th cells 6-8 hours after incubation with the indicated viruses. (C) The Cf2Th cells exposed to the SIVmac-GFP, SIVmac(HIV 4/5)-GFP and HIV-1-GFP viruses were lysed at 3 hours after virus incubation and the single-strand strong-stop DNA was PCR-amplified from 100 ng of the total DNA.
Table 1. Phenotypes of TRIM5α variants.

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<th>Late SIV&lt;sub&gt;mac&lt;/sub&gt; Reverse Transcripts&lt;sup&gt;c&lt;/sup&gt;</th>
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<sup>a</sup>The relative SIV<sub>mac</sub> infection level in Cf2Th cells expressing the given TRIM5α variant was determined, as described in Materials and Methods. The level of SIV<sub>mac</sub> restriction represents the complement of the relative infection level (+++, more than 90% of wild-type TRIM5α<sub>AGM(Tan)</sub> level; ++, 50-80%; +, 25-50%; -, 0-25%; -/, not detected).

<sup>b</sup>The ubiquitin ligase activity of each TRIM5α variant was determined in the in vitro auto-ubiquitylation assay described in the Materials and Methods. The E3 ubiquitin ligase activity of each TRIM5α variant relative to wild-type TRIM5α<sub>AGM(Tan)</sub> is designated: ++++, 80-100% of wild-type TRIM5α<sub>AGM(Tan)</sub> E3 ligase activity; ++, 40-80%; +, less than 40%.

<sup>c</sup>Late SIV<sub>mac</sub> reverse transcripts were measured as described in Materials and Methods. The level relative to that observed following SIV<sub>mac</sub> infection of cells expressing TRIM5α<sub>AGM(Pyg)</sub> is reported: ++++, greater than 120% of the level in LPCX-expressing cells; ++++, 80-100%; ++, 40-80%; +, 10-40%; - not detected. ND = not determined.

<sup>d</sup>The binding of the TRIM5α variant to SIVmac CA-NC complexes was measured as described in Materials and Methods. The binding relative to that of the wild-type TRIM5α<sub>AGM(Pyg)</sub> is reported: ++++, 90-100% of the binding observed for the TRIM5α<sub>AGM(Pyg)</sub> protein; ++++, 60-80%; ++, 30-60%; +, less than 25%. ND = not determined.

<sup>e</sup>The amount of particulate SIV<sub>mac</sub> capsid in the cytosol of cells expressing the TRIM5α 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; ++++, 80-100%; ++, 50-70%; +, 30-50%; -, less than 30%; - , not detected. ND = not determined.
Viral dose, μl
% GFP   cells
SIVmac-GFP

RING
Coiled Coil
B30.2(SPRY)

Loop1
Loop2

Tan
Human
Rhesus monkey
Squirrel monkey

CPICLELLTQPLSLDCGHSFCQACLTANHKSMLDK-GERSSCPVC
CPICLELLTQPLSLDCGHSFCQACITANHKSMLKQ-GERSSCPVC
CPICLELLTQPLSLDCGHSFCQACITANHKSMLKLS-GERSCPLC

15 59

24 2919

181x400

221x400

130x430

127x493

178x569

111x496

115x462

109x429

105x396

195x333

148x269

177x281

127x311

111x374

109x429

105x396

195x333

148x269

177x281

127x311

111x374

109x429

105x396
Relative level of SIVmac infection (%)

rs=0.9893, P < 0.0001

E3 Ub ligase activity (% of TRIM5α activity)
A

SIVmac-GFP

SIVmac(HIV 4/5)-GFP

% GFP+ cells

Viral dose, µl

B

SIVmac

SIVmac(HIV 4/5)

HIV-1

C

SIVmac

SIVmac(HIV 4/5)

HIV-1