Determinants for the Rhesus Monkey TRIM5α-mediated Block of the Late Phase of HIV-1 Replication*

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Rhesus monkey TRIM5α (TRIM5αrh) includes RING, B-box, coiled-coil, and B30.2(PRYSFPRY) domains and blocks HIV-1 infection by targeting HIV-1 core through a B30.2(PRYSFPRY) domain. Previously, we reported that TRIM5αrh also blocks HIV-1 production in a B30.2(PRYSFPRY)-independent manner. Efficient encapsidation of TRIM5αrh, but not human TRIM5α (TRIM5αhu), in HIV-1 virus-like particles suggests the interaction between Gag and TRIM5αrh during viral assembly. Here, we determined responsible regions for late restriction activity of TRIM5αrh. The RING disruption, but not the replacement with human TRIM21 RING, ablated the efficient encapsidation and the late restriction, suggesting that a RING structure was essential for the late restriction and efficient interaction with HIV-1 Gag. The prominent cytoplasmic body formation of TRIM5αrh, which depended on the coiled-coil domain and the ensuing linker 2 region, was not required for the encapsidation. Intriguingly, TRIM5αrh coiled-coil domain mutants (M133T and/or T146A) showed impaired late restriction activity, despite the efficient encapsidation and cytoplasmic body formation. Our results suggest that the TRIM5αrh-mediated late restriction involves at least two distinct activities as follows: (i) interaction with HIV-1 Gag polyprotein through the N-terminal, RING, and B-box 2 regions of a TRIM5αrh monomer, and (ii) an effector function(s) that depends upon the coiled-coil and linker 2 domains of TRIM5αrh. We speculate that the TRIM5αrh coiled-coil region recruits additional factor(s), such as other TRIM family proteins or a cellular protease, during the late restriction. RBCC domains of TRIM family proteins may play a role in sensing newly synthesized viral proteins as a part of innate immunity against viral infection.

TRIM5α is a member of the tripartite motif (TRIM) family of proteins and contains RING, B-box 2, and coiled-coil domains (1). Similar to other cytoplasmic TRIM proteins, TRIM5α contains a C-terminal B30.2(PRYSFPRY) domain that is thought to mediate binding to specific ligands (1). Rhesus monkey TRIM5α (TRIM5αrh) blocks an early step of HIV-1 infection, prior to significant reverse transcription (2, 3), by recognizing the incoming HIV-1 core structure with the B30.2(PRYSFPRY) domain and promoting its degradation or premature disassembly (4, 5). Sequences in the B30.2(PRYSFPRY) domain dictate the potency and specificity of the restriction of particular retroviruses (4, 6–8). Disruption of the B-box 2 domain of TRIM5αrh eliminates the ability of the protein to block HIV-1 infection, thus indicating its importance (6, 9). A recent study suggests that the B-box 2 mediates higher order self-association of TRIM5αrh oligomers to potentiate the restriction of retroviral infection (10). The coiled-coil domain of TRIM5α contributes to protein oligomerization and efficient capsid binding and post-entry restriction (10, 11). Disruption of the TRIM5αrh RING domain decreases, but does not eliminate, the restriction of HIV-1 infection (8, 12). Although polyubiquitination and rapid degradation of TRIM5α depend upon intact RING and B-box 2 domains (13), rapid turnover of TRIM5α is not required for its post-entry restriction activity, and proteasome inhibitors cannot prevent the post-entry restriction (9, 14). These observations suggest the block of HIV-1 entry occurs independently of the ubiquitin/proteasome system. More recent studies, however, have demonstrated that proteasome inhibitors can relieve the TRIM5α-dependent inhibition of reverse transcription without impairing the block of HIV-1 nuclear entry (2, 15). Moreover, TRIM5α is rapidly degraded in cells exposed to a restriction-sensitive retrovirus, suggesting a role of proteasomal degradation in the restriction (5). Similar to other TRIM proteins, TRIM5α self-associates to form cytoplasmic bodies (11, 16), which turn over rapidly by exchanging with free cytoplasmic TRIM5α as well as neighboring TRIM5α bodies (17).

In addition to this well characterized post-entry restriction activity, TRIM5αrh has another “late restriction” activity to affect the production phase of the HIV-1 life cycle (12, 18, 19). Richardson et al. (19) have demonstrated that cell-associated HIV-1 transmission in human cells is blocked only when both donor and recipient cells express TRIM5αrh. We and others (18, 20) have shown that co-expression of the C-terminal hemagglutinin (HA)-tagged TRIM5αrh with HIV-1 proviral plasmids reduces the yield of infectious virus up to 20–100-fold, although the late restriction activity of endogenous TRIM5αrh remains controversial (18, 20). High levels of TRIM5αrh showed potent antiviral activity on HIV-1 production through degradation of Gag polyproteins, whereas modest TRIM5αrh expression blocks HIV-1 production by reducing the virion infectivity as well as the yield of infectious virus (12, 16). When HIV-1 production surpasses the late restriction activity, HIV-1 virions or virus-like particles (VLPs) produced in the presence of TRIM5αrh protein incorporate high levels of intact and truncated forms of TRIM5αrh (12, 16).
Efficient encapsidation of TRIM5αrh is also evident in HIV-1 Gag-only particles (12), suggesting that TRIM5αrh interacts with HIV-1 Gag polyproteins before or during Gag assembly and that Gag maturation is not necessary for the Gag-TRIM5αrh interaction during the late restriction. HIV-1 protease appears to cleave TRIM5αrh to produce the truncated 20-kDa form of TRIM5αrh in the VLPs, because HIV-1 protease inhibitors block the formation of the 20-kDa form in the VLPs (12). However, this truncation of TRIM5αrh is not necessary for the late restriction, because TRIM5αrh RBCC-TRIM5αhu B30.2(PRYSPRY) chimeras exhibit efficient VLP incorporation and potent late restriction activities without showing remarkable truncation (12).

When compared with TRIM5αrh, human TRIM5α (TRIM5αhu) shows marginal antiviral activity on HIV-1 production (12, 16, 18). Little or no encapsidation of TRIM5αh in protein in HIV-1 virions or HIV-1 VLPs (12, 16) implies weaker interaction of HIV-1 Gag polyproteins with TRIM5αhu than TRIM5αrh. A series of TRIM5αrh-TRIM5αhu chimeric constructs reveal that the RBCC (RING, B-box 2, and coiled-coil) domain, but not the B30.2(PRYSPRY) domain, of TRIM5αrh determines both the late restriction activity as well as the encapsidation efficiency (12). A series of C-terminally HA-tagged TRIM5αrh mutants with deletions in the N- or C-terminal regions indicate the following: (i) the B30.2(PRYSPRY) domain is dispensable for the late restriction activity and efficient encapsidation of TRIM5αrh; (ii) the coiled-coil domain is essential for the late restriction but not for the efficient encapsidation, and (iii) the N-terminal region is essential for efficient TRIM5α encapsidation (16).

In this study, we further characterized the domains responsible for the TRIM5α-mediated late restriction using TRIM5αrh-TRIM5αhu chimeras and deletion/point mutants. We found that a RING structure and the TRIM5α rh RING motif mutants, TRIM5α + 21R and C15A/C18A, as well as their parental pLPCX-TRIM5αrh were kindly provided by Dr. Sodroski (4, 22). Codon-optimized HIV-1 Gag-Pol- and Gag-expression plasmids, pH-GP and pH-Gag, were described previously (12). Infectious molecular clone pNL4-3 (23) was used to produce HIV-1.

**Materials and Methods**

**Cells**—293T and GHOST(3)R3/X4/R5 (21) cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotics.

**Plasmids**—C-terminally HA-tagged TRIM5αhu- and TRIM5αrh-expressing plasmids, pHuT5α and pRhT5α, were described previously (12). The N-terminal deletion mutant D0 has the first 14 amino acids replaced with a single amino acid Met, whereas the C-terminal deletion mutants SP1 and CC1 have the deletions in the B30.2(PRYSPRY) region and the B30.2(PRYSPRY) and coiled-coil region, respectively (16). All the RBCC chimeric constructs as well as point mutants were generated by extension PCR, pLPCX-based TRIM5αrh RING motif mutants, TRIM5α + 21R and C15A/C18A, as well as their parental pLPCX-TRIM5αrh were kindly provided by Dr. Sodroski (4, 22). Codon-optimized HIV-1 Gag-Pol- and Gag-expression plasmids, pH-GP and pH-Gag, were described previously (12). Infectious molecular clone pNL4-3 (23) was used to produce HIV-1.

**Encapsulation Assay**—293T cells (10⁶ cells/well in a 6-well plate) were transfected with 1.0 μg of a TRIM5α expression plasmid together with 0.2 μg of pNL4-3 by 6 μl of FuGENE 6 (Roche Applied Science), according to the manufacturer’s instructions. Total DNA concentrations were adjusted to 1.2 μg with pcDNA3.1(+) (Invitrogen). Forty eight hours post-transfection, supernatants were harvested and filtered through 0.45-μm filters. Viral titers in the supernatants were determined in GHOST(3)R3/X4/R5 indicator cells. The concentrations of p24 in the supernatants were assayed with the p24 enzyme-linked immunosorbent assay kit (ZeptoMetrix). For immunoblotting analysis of TRIM5α and HIV-1 Gag proteins, the transfected cells were lysed with RIPA buffer and subjected to SDS-PAGE with a 4–15% gradient gel (Bio-Rad) and immunoblotting analysis. Following transfer to a polyvinylidene difluoride membrane, TRIM5α protein was detected by rat anti-HA monoclonal antibody, 3F10 (Roche Applied Science), whereas mouse anti-p24 monoclonal antibodies, AG3.0 (24) and 183-H12–5C (25), were used to detect HIV-1 precursor Gag and p24 capsid proteins. For treatment with proteasome inhibitors, 293T cells, which were transfected with a TRIM5α expression plasmid and pNL4-3, were treated with various concentrations of proteasome inhibitor, MG132 (Sigma) or MG115 (Sigma), for 16 h before cells were harvested for immunoblotting.

**Immunofluorescent Analysis**—293T cells (10⁶ cells/well in a 6-well plate) were transfected with 0.3 μg of pH-GP together with 0.9 μg of a TRIM5α-expressing plasmid with 6 μl of FuGENE 6. The amounts of plasmid DNA were adjusted to 1.2 μg with pcDNA3.1(+) for each transfection. Forty eight hours post-transfection, culture supernatants were harvested and passed through a 0.45-μm pore-sized filter. VLPs in 1 ml of supernatants were purified by ultracentrifugation through a 20% sucrose cushion. The pellets of VLPs were washed by phosphate-buffered saline and then lysed in 10 μl of RIPA buffer. Proteins were separated by 4–15% gradient gel SDS-PAGE and subjected to Western blot analysis with rat anti-HA monoclonal antibody (3F10, Roche Applied Science) or anti-p24 monoclonal antibody mixtures (AG3.0 and 183-H12–5C). Use of gradient gels was critical to visualize both the intact and truncated forms of TRIM5αrh.
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FIGURE 1. RING domain is required to block HIV-1 production. A, schematic representation of TRIM5α RING-mutants. Black and white bars indicate the sequences from TRIM5αrhr and human TRIM21, respectively. White asterisks show the position of point mutations. B, 293T cells were co-transfected with a TRIM5αrr mutant expression plasmid along with pNL4-3. Forty eight hours post-transfection, cell lysates and VLPs in the supernatants were purified through a 20% sucrose layer. C, 293T cells were co-transfected with a TRIM5αrr mutant expression plasmid along with codon-optimized Gag-Pol expression plasmid. Forty eight hours post-transfection, VLPs in the supernatant were purified through a 20% sucrose layer and subjected to Western blot analysis. F and T indicate full-length and truncated forms (−20 kDa) of TRIM5α protein. D, 293T cells were co-transfected with a TRIM5αrr mutant expression plasmid along with pNL4-3 and treated with increasing concentrations of MG115. The HIV-1 Gag proteins in the proteasome inhibitor-treated cells were detected by Western blot analysis (upper panel). We also tested the effect of another proteasome inhibitor, MG132 (lower panel).

localization of TRIM5α proteins was determined under a confocal microscope.

RESULTS

RING Domain Is Required to Block HIV-1 Production—Previously, we have shown that the deletion in the first 14 amino acid residues prior to the RING consensus sequence abrogates the efficient VLP encapsidation as well as the strong late restriction activity of TRIM5αrhr (16). Similarly, N-terminally FLAG-tagged TRIM5αrhr did not show strong late restriction activity (12). These observations suggest that the N-terminal region of TRIM5αrhr is required for interaction with HIV-1 Gag polyprotein or for proper TRIM5α conformation. To further understand the domains necessary for the late restriction, we first tested the influence of mutations in the TRIM5αrhr RING domain on the late restriction activity. Two RING domain mutants, TRIM5-21R and C15A/C18A, were used to assess the importance of the RING motif in TRIM5αrhr (Fig. 1). TRIM5-21R is a TRIM5αrhr with the RING domain replaced with the human TRIM21 RING domain (13, 26, 27), whereas C15A/C18A is a TRIM5αrhr with a disrupted RING structure (27). To examine the influence of these RING mutations on HIV-1 production, 293T cells were transfected with a TRIM5α expression plasmid and an HIV-1 infectious molecular clone pNL4-3. As a control, we used the parental construct, pLPCX-TRIM5αrhr, which expresses an HA-tagged wild-type TRIM5αrhr. The viral titers in the supernatants were then determined in GHOST indicator cells. The TRIM5-21R showed late restriction activity comparable with the wild-type TRIM5αrhr, whereas C15A/C18A showed impaired late restriction (Fig. 1A). This observation indicates that a RING domain is required for the late restriction. Because TRIM5-21R exhibits a longer half-life than TRIM5αrhr (27), it is conceivable that rapid degradation of TRIM5αrhr is not required for the late restriction.

Next, we examined whether these RING mutants can interact with the Gag protein by encapsidation into VLPs made with a codon-optimized HIV-1 Gag-Pol-expressing plasmid. Forty eight hours post-transfection, cell lysates and VLPs in the supernatants were purified through a 20% sucrose layer and subjected to Western blot analysis. F and T indicate full-length and truncated forms (−20 kDa) of TRIM5α protein. D, 293T cells were co-transfected with a TRIM5αrhr mutant expression plasmid along with pNL4-3 and treated with increasing concentrations of MG115. The HIV-1 Gag proteins in the proteasome inhibitor-treated cells were detected by Western blot analysis (upper panel). We also tested the effect of another proteasome inhibitor, MG132 (lower panel).
protein were observed in cell lysates, the 20-kDa form was predominant in the VLPs, ruling out the possible precipitation of TRIM5αrh-containing cell debris without VLP encapsidation. These data indicate that a RING domain is required for the efficient interaction with HIV-1 Gag polyproteins.

The block of HIV-1 entry appears to occur independently of the ubiquitin/proteasome system during the TRIM5αrh-mediated post-entry restriction (9, 14), although more recent studies have demonstrated that proteasome inhibitors can partially relieve the TRIM5α-dependent inhibition of reverse transcription without affecting the restriction of HIV-1 nuclear entry (2, 15). We examined the influence of proteasome inhibitors on the late restriction. 293T cells were co-transfected with pRhT5α and pNL4-3. One day after transfection, the supernatants were replaced with fresh growth medium containing a proteasome inhibitor, MG132 or MG115. After an overnight treatment, culture supernatants and producer cells were harvested. Because of the potent toxicity of MG132 and MG115, we did not determine the viral titers. To assess the effects of proteasome inhibitors, the HIV-1 Gag proteins in the producer cells were detected by immunoblotting (Fig. 1E). Although we observed the toxicity associated with higher concentrations of proteasome inhibitor treatment, MG132 or MG115 treatment did not affect the TRIM5αrh-mediated block of HIV-1 Gag production. Our results therefore suggest that the late restriction occurs independently of the ubiquitin/proteasome system.

**TRIM5αrh Coiled-coil Domain Is Required for the Late Restriction Activity**—Our previous studies using TRIM5αhu-TRIM5αrh chimeras have shown that the TRIM5αrh RBCC domain contains the determinant for the late restriction (12, 16). To further map the responsible element(s) in TRIM5αrh-mediated restriction on HIV-1 production, we generated five additional TRIM5α rh RBCC chimeras, RBCC1 to RBCC5, by extension PCR. After verification of the expression of these chimeras (Fig. 2B), we examined the antiviral activity of each chimeric TRIM5α protein as described above. RBCC1 to RBCC4 showed strong antiviral activity comparable with the wild type, whereas the chimera RBCC5, which has the RING and B-box 2 domains of TRIM5αrh and coiled-coil and B30.2(PRYSRPR) domains of TRIM5αhu, exhibited impaired antiviral activities (Fig. 2C). These data indicate that the coiled-coil domain of TRIM5αrh is required for the late restriction. We then examined whether these chimeras could interact with HIV-1 Gag protein by encapsidation assay. We found all chimeras were incorporated into VLPs (Fig. 2D), although RBCC2 and RBCC3 showed reduced levels of incorporation. These results suggested that the five chimeric TRIM5αs can interact with the Gag protein, and the linker region between the RING and B-box 2 domains, or the conformation of this region, affects the interaction. RBCC5 showed reduced antiviral activity yet maintained the incorporation capacity of the protein, indicating that the interaction of TRIM5α with HIV-1 Gag alone is insufficient for the late restriction activity. In addition, TRIM5α rh RBCC5 was incorporated into VLPs as an intact, full-length form but not in a truncated form, which confirmed our previous observation that HIV-1 protease efficiently cleaves TRIM5αrh in the B30.2(PRYSRPR) domain but not TRIM5αhu (12).

**Met-133 and Thr-146 in the Coiled-coil Domain of TRIM5αrh Are Critical for the Efficient Late Restriction**—There are seven amino acid differences between human and rhesus monkey TRIM5α in the Bcl-2/HindIII region, which contains the B-box 2 and partial coiled-coil domains. To determine the amino acid residues responsible for the late restriction and encapsidation, we replaced each of these seven amino acids with a corresponding TRIM5αhu amino acid residue (Fig. 2A). After confirming the expression of these TRIM5αrh mutants (Fig. 2E), we examined their antiviral activities against HIV-1. Five TRIM5αrh mutants showed antiviral activities comparable with the wild type, although two TRIM5αrh coiled-coil domain mutants, M133T and T146A, showed impaired late restriction activity (Fig. 2F). We also generated a coiled-coil mutant, TRIM5αrh-MT/TA, with the two critical amino acid substitutions M133T and T146A. The antiviral activity of this mutant was comparable with the antiviral activities of M133T and T146A (Fig. 2F). We then examined the encapsidation efficiencies of these mutants. When HIV-1 VLPs were made in the presence of TRIM5αrh mutants, all point mutants, including the TRIM5αrh-MT/TA, were efficiently incorporated into VLPs (Fig. 2G). These data indicate that the amino acid residues Met-133 and Thr-146 are essential for the late restriction activity and further confirmed that incorporation of TRIM5α protein does not necessary lead to the late restriction. It is likely that the TRIM5αrh coiled-coil domain is required for the step(s) after the interaction of TRIM5α with HIV-1 Gag to block HIV-1 production.

To better understand the TRIM5αrh mutant with M133T and T146A (MT/TA), we examined whether the mutant shows post-entry restriction against HIV-1 infection. Feline CrFK cells, which are highly permissive to HIV-1 vector infection, were transduced by retroviral vectors that encode HA-tagged wild-type and mutant TRIM5α proteins, MT/TA and SP1 (B30.2(PRYSRPR)-deleted). CrFK cells stably expressing the TRIM5αrh proteins were selected under the presence of G418 (1 mg/ml). Empty retroviral vector-transduced cells were used as a control. TRIM5αrh expression was verified by immunoblotting (Fig. 3A). When the cells were infected by a green fluorescent protein–carrying HIV-1 vector at a multiplicity of infection of 0.05, MT/TA- and wild-type TRIM5αrh-expressing cells showed notable resistance to HIV-1 vector infection (Fig. 3B), suggesting the intact post-entry restriction activity of TRIM5αrh-MT/TA mutant. We also tested the potential of multimerization of the MT/TA mutant by using glutaraldehyde as cross-linker. 293T cells expressing the coiled-coil mutant were lysed in 1% Nonidet P-40 phosphate-buffered saline solution, cross-linked with 2.0 mM glutaraldehyde at room temperature for 5 min, and subsequently quenched by adding 20 mM glycine. Cross-linked substrates were then subjected to immunoblotting analysis with anti-HA antibody. The MT/TA mutant demonstrated typical multimerized signals, comparable with wild-type TRIM5αrh (Fig. 3C), suggesting that the M133T or T146A mutations in TRIM5αrh coiled-coil domain do not affect the TRIM5αrh multimerization.

**TRIM5αhu Mutant with the B-box 2 and Coiled-coil Domain of TRIM5αrh Restricts HIV-1 Production**—In an attempt to make a TRIM5αhu with the late restriction activ-
ity, we replaced the essential amino acid residues/region for the late restriction with corresponding TRIM5α/H9251 rh sequences. We introduced Met-133 and Thr-146 into the TRIM5α/H9251 hu sequence to make TRIM5α/H9251 hu-TA/MT. We also generated TRIM5α/H9251 hu-BcHd by replacing the BclI-HindIII region with the corresponding TRIM5α/H9251 rh region (Fig. 4A).

After verifying the expression of the TRIM5α/H9251 hu mutants by immunoblotting (Fig. 4B), we examined the antiviral activities of these mutants. TRIM5α/H9251 hu-TA/MT did not strongly restrict HIV-1 production, whereas TRIM5α/H9251 hu-BcHd restricted HIV-1 production by up to 100-fold, comparable with the antiviral activity of wild-type TRIM5α/H9251 rh (Fig. 4C). We also examined the ability of these TRIM5α/hu mutants to interact with HIV-1 Gag by the encapsidation assay. As shown in Fig. 4D, TRIM5α/hu-BcHd, but not TRIM5α/hu-TA/MT, was efficiently incorporated in the VLPs. These data further confirm that the TRIM5α/hr B-box 2 and coiled-coil regions are critical for the late restriction and the efficient encapsidation of HIV-1.
TRIM5α. Introduction of the Met-133 and Thr-146 into TRIM5αhu did not affect the antiviral activity or encapsidation efficiency of TRIM5αhu. It is plausible that the weak antiviral activity of TRIM5αhu-TA/MT on HIV-1 production is primarily due to its insufficient interaction with HIV-1 Gag polyproteins.

**Ability to Form Prominent Cytoplasmic Bodies Can Be Separated from the Late Restriction Activity and the VLP Encapsulation**—TRIM5α self-associates to form cytoplasmic bodies (1, 3, 28). Because aggregation of TRIM5α may play a role in the TRIM5αrh-HIV-1 Gag interaction, we examined the possible correlation between the cytoplasmic body formation of the TRIM5α mutants and their VLP encapsidation and late restriction activities. 293T cells were transfected with a TRIM5α expression plasmid, and the localization of C-terminally HA-tagged TRIM5α proteins were detected by anti-HA antibody (Fig. 5, A and B).

![FIGURE 5.](image)

**FIGURE 5.** Post-entry restriction by the TRIM5αrh mutant with M133T and T146A. A, feline CrFK cells were retrovirally transduced to express TRIM5αrh mutants, MT/TA and SP1. HA-tagged TRIM5α proteins were detected by anti-HA antibody. An empty vector, multiple cloning site (MCS), was used as a control. B, G418-selected bulk populations of CrFK cells were infected by a green fluorescent protein-expressing HIV-1 vector. Green fluorescent protein-positive cell populations were analyzed by fluorescence-activated cell sorter. C, cell lysates from HA-tagged TRIM5αrh- and TRIM5αrh-MT/TA-expressing cells were cross-linked by glutaraldehyde and analyzed for multimerization by immunoblotting using anti-HA antibody.

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The late restriction activities of TRIM5α mutants were determined by comparing the effects of transfected TRIM5α mutants on HIV-1 production. 293T cells were co-transfected with a TRIM5α expression plasmid along with pNL4-3. Two days post-transfection, viral titers in the supernatant were analyzed in GHOST indicator cells. The ability of TRIM5α mutants to form cytoplasmic bodies was assessed by immunoblotting using anti-HA antibody. The results showed that the ability to form cytoplasmic bodies was not correlated with the late restriction activity of TRIM5α mutants. The TRIM5αrh-BcHd with the B-box 2 and partial coiled-coil domains of TRIM5αrh showed efficient cytoplasmic body formation, comparable with wild-type TRIM5αrh. These results indicate that the TRIM5αrh B-box 2 and coiled-coil domains contain the determinant for the prominent cytoplasmic body formation. This was also supported by our observations that TRIM5αrh-based mutants D0, D1, and SP1, which have deletions in the N-terminal region, RING, or C-terminal B30.2(PRYSPRY) domains, retained the ability to form prominent cytoplasmic bodies. In sharp con-
contrast, most cells transfected with TRIM5αh mutants CC1 and SP2, which have C-terminal deletions in the coiled-coil, ensuing linker 2 and B30.2(PRYSPRY) domains, showed diffused cytoplasmic HA signals, similar to TRIM5α/H9251hu-transfected cells. Importantly, no correlation was observed between the cytoplasmic body formation and the expression levels of TRIM5α/H9251rh proteins (Fig. 5C). We therefore concluded that the coiled-coil domain of TRIM5αrh and the linker 2 region of either TRIM5αrh or TRIM5αhu are required for the efficient cytoplasmic body formation in 293T cells. Our results confirmed the previous report that, unlike the coiled-coil regions of other related TRIM proteins, efficient TRIM5αα multimerization and cytoplasmic body formation require the coiled-coil and the linker 2 region (29).

In terms of the relationship between the cytoplasmic body formation and VLP incorporation, it is notable that TRIM5αrh mutants D0 and D1 formed large cytoplasmic bodies (Fig. 5) despite their poor encapsidation efficiency (16). Clearly, the prominent cytoplasmic body formation is independent from the efficient encapsidation of TRIM5αrh proteins. On the other hand, the determinants for the efficient cytoplasmic body formation and the late restriction activity both localized in the coiled-coil and linker 2 domains of TRIM5αrh (Fig. 5) (16). We therefore assessed if these two activities of TRIM5αrh could be separated. As shown in Fig. 5, TRIM5αrh-MT/TA, which has the two point mutations in the coiled-coil domain critical for the late restriction activity, could form cytoplasmic bodies comparable with the wild-type TRIM5αrh. These results indicate that the amino acid residues Met-133 and Thr-146 in the coiled-coil domain do not play a role in the cytoplasmic body formation, and the ability to form prominent cytoplasmic bodies can be separated from the late restriction activity of TRIM5αrh.

FIGURE 5. Ability to form prominent cytoplasmic bodies can be separated from the late restriction activity and the VLP encapsidation. 293T cells were transfected with a plasmid-encoding TRIM5α mutant. HA-tagged TRIM5α mutants were detected with a rat anti-HA antibody 3F10 and a fluorescein isothiocyanate-conjugated anti-rat IgG antibody. Nuclei were counter-stained with 4',6-diamidino-2-phenylindole. Subcellular localization of TRIM5α proteins was determined under confocal microscopy. A, TRIM5α protein localization was tested for prominent cytoplasmic body formation, which was shown as cytoplasmic bodies or diffuse, depending on each cell showing more than three discrete cytoplasmic bodies or diffuse cytoplasmic signals. B, typical subcellular localizations of the TRIM5α mutants are shown. C, 293T cells were transfected with a TRIM5α mutant plasmid, and the TRIM5α expression levels were analyzed by immunoblotting using anti-HA antibody. All constructs expressed similar levels of TRIM5α proteins.
DISCUSSION

TRIM5αrh functions as a RING finger-type E3 ubiquitin ligase, which is self-ubiquitinated by the E2 ubiquitin-conjugating enzyme UbcH5B (3, 30). Although TRIM5αrh and TRIM5αhu proteins are rapidly turned over, rapid degradation of TRIM5α appears unnecessary for its antiretroviral activity against incoming viruses (9, 14, 22). Disruption of TRIM5αrh RING domain impairs, but does not eliminate, the post-entry restriction of HIV-1 infection (8, 12), although proteasome inhibitors do not impair the potency of post-entry restriction (9, 14, 22). By using a RING domain-disrupted mutant and a TRIM21-TRIM5 chimera, we demonstrated that a RING motif, but not the rapid degradation of TRIM5α protein, was required for the late restriction. Proteasome inhibitors failed to impair the late restriction. Although it remains possible that the proteasome system plays some role in the late restriction, these observations indicate that the block of HIV-1 production is largely ubiquitin/proteasome system-independent. Because disruption of the RING motif also resulted in the loss of TRIM5α incorporation into HIV-1 VLPs, it is likely that the RING structure is required for the efficient interaction with HIV-1 Gag polyproteins during viral assembly and that the lack of strong interaction resulted in the loss of late restriction activity of the RING disruption mutant.

Previously, we have demonstrated that the deletion in the first 14 amino acid residues prior to the RING consensus sequence abrogates the efficient VLP encapsidation and the late restriction activity of TRIM5αrh (16). In contrast, TRIM5αrh mutant CC1 with deletion in the C-terminal half of the protein, including the coiled-coil and B30.2(PRYSPRY) domains, shows efficient encapsidation, indicating that the coiled-coil and B30.2(PRYSPRY) domains are not required for the VLP incorporation (16). In this study, we found that replacement of the partial B-box 2 and coiled-coil domains of TRIM5αhu to their rhesus counterparts led to increased encapsidation of the TRIM5αhu mutant (Fig. 4). In addition, two RBCC domain chimeras, RBCC2 and RBCC3, showed impaired VLP encapsidation, suggesting that the region between the RING domain and its ensuing linker 1 region also plays a role in the efficient encapsidation of TRIM5αrh (Fig. 1). Taken together, we concluded that the N terminus, RING, ensuing linker 1, and the B-box 2 domains of TRIM5αrh determine the encapsidation efficiency. It is conceivable that the proper conformation of TRIM5αrh protein is required for the interaction with HIV-1 Gag in the producer cells.

TRIM5α concentrates in discrete structures in the cytoplasm, designated cytoplasmic bodies (1, 14). Although cytoplasmic body formation may not be required for the post-entry restriction (6), the cytoplasmic bodies are not protein aggregates or inclusion bodies that represent dead-end static structures but rather play a role in TRIM5α function or regulation (17). Importantly, the multimerizing properties of TRIM5α contribute to the formation of cytoplasmic bodies (16). We hypothesized that the multimerization/aggregation of TRIM5αrh might play a key role in the late restriction activity, particularly the interaction with HIV-1 Gag, and we examined whether cytoplasmic body formation of TRIM5α mutants/
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we speculate that the TRIM5αrh-mediated late restriction involves at least two distinct phases as follows: (i) interaction of a TRIM5α monomer with HIV-1 Gag, which depends on the N-terminal, RING, and B-box 2 regions of TRIM5αrh, and (ii) an effector function(s) that depends upon the coiled-coil, especially amino acid residues Met-133 and Thr-146. It is plausible that multimerization of TRIM5αrh, which depends on its coiled-coil domain and linker 2 region, plays a role in the second phase or prior to the second phase of the late restriction.

Previously, we have shown that TRIM5αrh restricts HIV-1 production in a cell line-specific manner. For instance, TRIM5αrh restricts HIV-1 production in 293T or MT4 cells but not in HeLa cells, when TRIM5αrh is overexpressed (16). Given that the cell type specificity is due to the differential expression of a cofactor, it is possible that the TRIM5αrh-coiled-coil motif recruits a cofactor during the late restriction through the amino acid residues Met-133 and Thr-146. The possible candidates include other TRIM family protein(s), such as TRIM22 or TRIM21, or a cellular protease. TRIM22 was recently identified to affect HIV-1 production through interaction with HIV-1 Gag polyprotein, and like TRIM5αrh-mediated late restriction, disruption of the RING motif ablates the late restriction activity of TRIM22 (15). Moreover, the type I interferon-responsive TRIM5 and TRIM22 genes share the enhancer region, suggesting a similar transcriptional regulation of the two proteins (16, 20). TRIM21 may also play a role as a cofactor in the late restriction, as it was shown to ubiquitinate virus-infected cells and play a role in the innate immunity against viral infection.

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