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Unique features of TRIM5α among closely related human TRIM family members

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Abstract

The tripartite motif (TRIM) protein, TRIM5α, restricts some retroviruses, including human immunodeficiency virus (HIV-1), from infecting the cells of particular species. TRIM proteins contain RING, B-box, coiled-coil and, in some cases, B30.2(SPRY) domains. We investigated the properties of human TRIM family members closely related to TRIM5. These TRIM proteins, like TRIM5α, assembled into homotrimers and co-localized in the cytoplasm with TRIM5α. TRIM5α turned over more rapidly than related TRIM proteins. TRIM5α, TRIM34 and TRIM6 associated with HIV-1 capsid–nucleocapsid complexes assembled *in vitro*; the TRIM5α and TRIM34 interactions with these complexes were dependent on their B30.2(SPRY) domains. Only TRIM5α potently restricted infection by the retroviruses studied; overexpression of TRIM34 resulted in modest inhibition of simian immunodeficiency virus (SIV_{mac}) infection. In contrast to the other *TRIM* genes examined, *TRIM5* exhibited evidence of positive selection. The unique features of TRIM5α among its TRIM relatives underscore its special status as an antiviral factor.

Keywords: Tripartite motif; Restriction factor; Retrovirus; Nonsynonymous/synonymous; Positive selection; Capsid binding; B30.2(SPRY) domain; RING; RBCC

Introduction

Retroviruses encounter dominant post-entry restrictions in the cells of particular species. Human immunodeficiency virus type 1 (HIV-1) infection is blocked in the cells of Old World monkeys, whereas simian immunodeficiency virus (SIV_{mac}) infection is blocked in most New World monkey cells (Himathongkham and Luciw, 1996; Hofmann et al., 1999; Shibata et al., 1995). These restrictions are mediated by a host protein, TRIM5α, that exhibits species-specific variation in primates (Besnier et al., 2002; Bieniasz, 2003; Hatziioannou et al., 2003; Stremlau et al., 2004; Song et al., 2005b, 2005c; Stoye, 2002). Human TRIM5α also accounts for the N-tropic murine leukemia virus (N-MLV)-blocking activity found in human cells (Hatziioannou et al., 2004b; Yap et al., 2004; Perron et al., 2004; Keckesova et al., 2004). The viral determinant of susceptibility to TRIM5α-mediated restriction is the capsid protein (Cowan et al., 2002; Hatziioannou et al., 2004a; Kootstra et al., 2003; Owens et al., 2003, 2004; Towers et al., 2000).

TRIM5α is a member of the large family of tripartite motif proteins (TRIM) (Reymond et al., 2001). Members of this family contain RING, B-box and coiled-coil domains and thus are also called RBCC proteins (Reymond et al., 2001). The C-terminal portions of these proteins are more variable due to differential splicing of the primary RNA transcript. The alpha isoforms of many cytoplasmic TRIM proteins, like TRIM5α, contain a B30.2 or SPRY domain. To date, more than 60 TRIM

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proteins have been identified in the human genome; homologues exist in other species as well. TRIM proteins arose with the metazoans and have expanded in number during vertebrate evolution (Reymond et al., 2001). Although many members of the TRIM protein family have been linked to diverse biological processes, such as transcriptional regulation, apoptosis, inflammation, cell polarity determination, and antiviral activities, none of them have been well characterized in terms of their precise molecular and cellular functions (Jensen et al., 2001; Saurin et al., 1996).

Besides TRIM5α, a few other TRIM proteins have been reported to exhibit antiviral activities. TRIM1 from African green monkey, human and owl monkey cells weakly restricts N-MLV, but not HIV-1, infection (Yap et al., 2004). Overexpression of TRIM19 confers resistance to vesicular stomatitis virus (VSV) (a rhabdovirus) and influenza A virus (an orthomyxovirus), but not encephalomyocarditis virus (a picornavirus) (Chelbi-Alix et al., 1998). Recently, TRIM19 has been implicated in cellular resistance to human cytomegalovirus and herpes simplex type 1 (Tavalai et al., 2006; Everett et al., 2006). TRIM32 specifically binds to the activation domain of Tat proteins from HIV-1, HIV-2 and EIAV with high affinity, suggesting a potential role in transcription regulation of the viral genome (Fridell et al., 1995). TRIM22 has been reported to attenuate transcription directed by the long terminal repeat of HIV-1 in transfected cells (Tissot and Mechti, 1995). Many TRIM proteins, including TRIM19, TRIM21, TRIM22, TRIM34, and TRIM5α itself, can be up-regulated by interferon, supporting their potential role as effectors in the anti-viral cellular response (Asaoka et al., 2005; Chelbi-Alix et al., 1995; Gongora et al., 2000; Orimo et al., 2000; Tissot and Mechti, 1995). Thus, it has been speculated that the TRIM proteins may represent a new family of antiviral molecules involved in innate immunity (Nisole et al., 2005).

In humans, the *TRIM5* gene is located in a paralogous cluster on chromosome 11 with *TRIM6*, *TRIM34* and *TRIM22*. Although *TRIM6* and *TRIM34* have orthologs in mammalian genomes sequenced to date, orthologs of *TRIM5* have been unambiguously identified only in primates. TRIM12 and TRIM30 in rodents and a cluster of ungulate TRIM proteins are phylogenetically related to TRIM5/6/34/22 (Si et al., 2006). One of these ungulate proteins, cow 505265, restricts the infection of several retroviruses (Si et al., 2006; Ylinen et al., 2006). *TRIM5* and *505265* exhibit evidence of positive selection, particularly in the exons that encode the B30.2 domain (Sawyer et al., 2005; Song et al., 2005b; Si et al., 2006). Thus, in at least two mammalian lineages, proteins from the same subfamily of TRIM proteins exhibit antiretroviral activity.

In this study, we investigate the properties and antiviral activities of six human TRIM family members, including those closely related to TRIM5α. Only TRIM5α exhibited potent antiretroviral activity, although TRIM34 weakly inhibited a simian immunodeficiency virus. TRIM5α was found to be unique in other biochemical and genetic properties as well.

Results

Sequence comparison of human TRIM family proteins

The TRIM proteins appeared with the metazoans, and increased in number along with the evolution of vertebrates (Reymond et al., 2001). TRIM proteins are found in the nucleus as well as the cytoplasm. Many cytoplasmic TRIM proteins have a carboxy-terminal B30.2(SPRY) domain. Fig. 1 shows the phylogenetic relationship of human TRIM proteins that have B30.2(SPRY) domains. Because the B30.2(SPRY) domains of some TRIM proteins have been subjected to strong positive selection (Sawyer et al., 2005; Si et al., 2006; Song et al., 2005b), additional analyses were performed after removing the B30.2(SPRY) domain, thus reducing the possibility of highly variable or convergent residues affecting the phylogenetic reconstruction (Supplementary Figure 1). The analyses of the complete TRIM proteins and the B30.2(SPRY)-deleted TRIM proteins yielded similar results. TRIM6, TRIM34, and TRIM22 formed a clade with TRIM5, consistent with these proteins being paralogs. TRIM21 is the next closest human relative. The amino acid sequence identity of this subset of TRIM proteins ranges from 40 to 57%. Although TRIM5, TRIM6, TRIM22 and TRIM34 are clearly monophyletic, more detailed phylogenetic relationships among these proteins cannot be confidently deduced from these data alone. *TRIM6*, *TRIM34*, *TRIM22* and *TRIM5* form a cluster on human chromosome 11p15. Additional clusters of *TRIM* paralogs are found on human chromosomes 5, 6 and 17; the *TRIM* cluster on chromosome 6 is near the major histocompatibility complex locus.

Colocalization of several TRIM proteins with TRIM5α

A previous study (Reymond et al., 2001) suggested that many TRIM proteins form discrete bodies or speckles in the cytoplasm or nucleus of overexpressing cells. TRIM5α proteins from several primate species exhibit diffuse cytoplasmic staining as well as being incorporated into cytoplasmic bodies (Song et al., 2005a). These cytoplasmic bodies have been shown to represent pre-aggresomal structures (Diaz-Griffero et al., 2006a). To determine whether other cytoplasmic TRIM proteins also localize in similar structures, selected TRIM proteins were coexpressed with a V5-tagged rhesus monkey TRIM5α in HeLa cells. In addition to the human TRIM proteins most closely related to TRIM5, we studied TRIM4 and TRIM27 as examples of more distant TRIM relatives. The FLAG-tagged human TRIM4, TRIM6, TRIM22, TRIM27 and TRIM34 all colocalized with rhesus monkey TRIM5α (Figs. 2A and B). In at least some of the cells, these TRIM proteins were located in the cytoplasm and formed cytoplasmic bodies. In some of the TRIM22-expressing cells and most of the TRIM27-expressing cells, these proteins as well as TRIM5α_{rh} were partly located in the nucleus. Apparently, the intracellular location of TRIM5α_{rh} can be influenced by the coexpression of other TRIM proteins. Similarly, human TRIM5α was shown to colocalize with

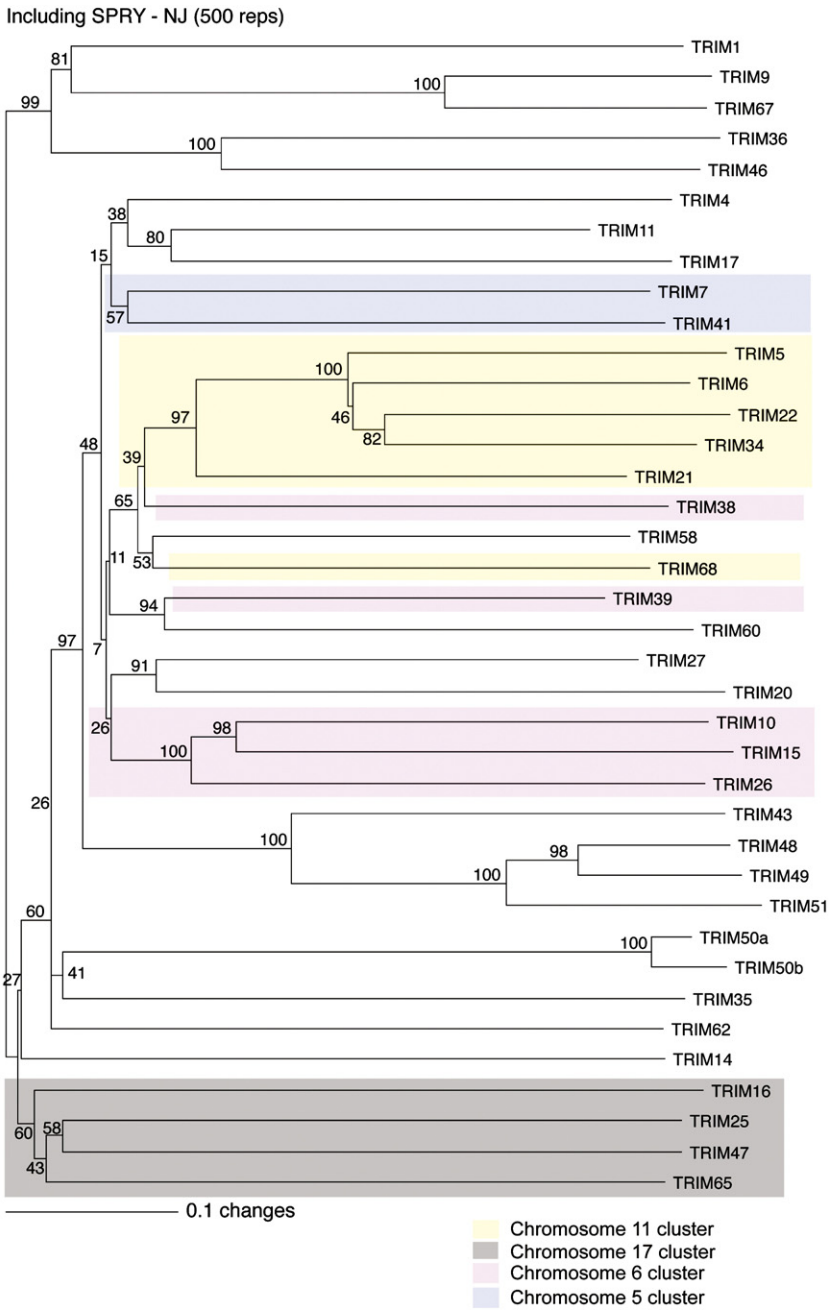

Fig. 1. Phylogeny of human TRIM family proteins containing a B30.2(SPRY) domain. A neighbor joining tree based on the amino acid sequences of 38 human TRIM proteins containing a B30.2(SPRY) domain is shown. Numbers indicate bootstrap proportions after 500 replications. The scale bar represents 0.1 changes per site. The highlighted TRIM proteins are encoded by human *TRIM* genes located in the indicated chromosomal clusters.

fusion proteins consisting of enhanced green fluorescent protein (GFP) and the human TRIM proteins listed above (data not shown).

TRIM proteins form trimers

It has been shown that wild-type TRIM5α proteins from different primate species form trimers (Mische et al., 2005). To investigate whether oligomerization is a common feature of cytoplasmic TRIM family members, cell lysates from HeLa cell lines stably expressing different TRIM proteins were incubated with increasing concentrations of the crosslinker glutaraldehyde and subsequently analyzed by Western blotting. All of the TRIM proteins were crosslinked into gel-stable complexes with molecular weights of approximately 150–180 kDa, consistent with the formation of trimers (Fig. 3). At high concentrations of the crosslinker, protein bands with even higher molecular weight (>250 kDa) were also seen for some TRIM proteins. These species may represent dimers of trimers. TRIM34 formed distinct high-molecular-weight complexes even in the absence of glutaraldehyde, indicating the unusual resistance of these forms to heat and

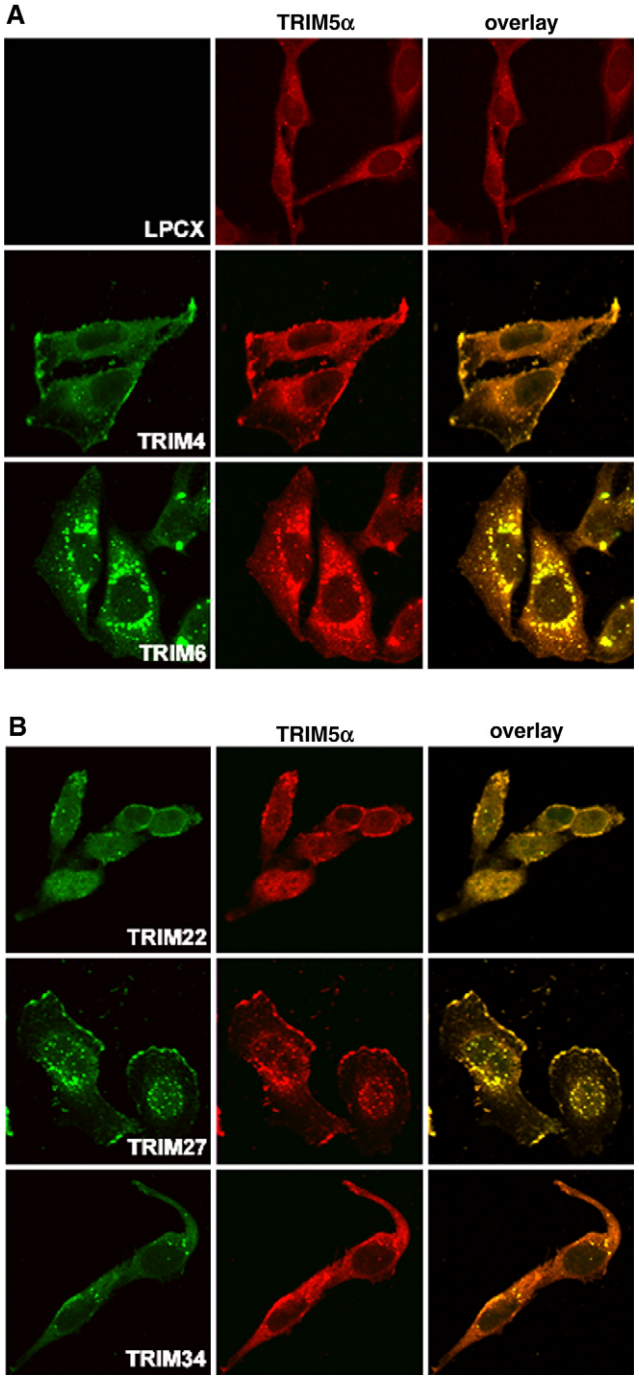

Fig. 2. Co-localization of rhesus monkey TRIM5α and other TRIM proteins. (A, B) HeLa cells stably expressing V5-tagged rhesus monkey TRIM5α and FLAG-tagged human TRIM proteins (or the empty LPCX vector) were fixed and permeabilized. Cells were stained with anti-V5 antibody conjugated to Cy3 (red) or an anti-FLAG antibody conjugated to FITC (green). Stained cells were examined using a confocal fluorescent microscope.

SDS. We conclude that cytoplasmic TRIM proteins are trimeric.

TRIM5α turns over faster than other related TRIM proteins

Both human and rhesus monkey TRIM5α proteins have short half-lives in cells stably expressing those proteins (Diaz-Griffero et al., 2006a). The RING and B-box 2 domains both contribute to this fast turnover rate. We examined the half-lives of other TRIM proteins to see whether rapid turnover is a shared feature.

HeLa cell lines stably expressing either C- or N-terminally tagged TRIM proteins were treated with cycloheximide and assayed for protein expression at different time points, as previously described (Diaz-Griffero et al., 2006a). As expected, TRIM5α almost completely disappeared within 2 h of treatment (Fig. 4). Of the other TRIM proteins, only TRIM4, which is not closely related to TRIM5 (26.8% identity), exhibited a rapid turnover, approximately two-fold slower than that of TRIM5α. The other TRIM proteins, including TRIM6, TRIM34 and TRIM22, which are closely related to TRIM5, exhibited half-lives of at least 5 h. TRIM21 and TRIM22 were very stable proteins. Of interest, replacement of the rhesus monkey TRIM5α RING domain with that of TRIM21 results in an increased half-life (Diaz-Griffero et al., 2006a). Apparently, features of the TRIM5α RING and B-box 2 domains are unusual in this subset of TRIM proteins, specifying rapid turnover.

Effect of TRIM proteins on the efficiency of retroviral infection

To examine the antiviral potential of the human TRIM proteins chosen for study, Cf2Th canine thymocytes expressing these proteins were infected with various recombinant retroviral vectors. A search of the dog genome failed to reveal a *TRIM5* ortholog (data not shown). Thus, canine cells are free of potential complications resulting from interactions of the TRIM proteins with endogenous TRIM5α. The TRIM proteins were tagged at the N-terminus with a FLAG epitope. Addition of the FLAG tag at the N- or C- terminus did not affect the ability, or lack thereof, of these TRIM proteins to restrict virus infection (data not shown). The rhesus monkey TRIM5α construct from a previous study was tagged at the C-terminus with an HA epitope derived from influenza virus (Stremlau et al., 2004). Some variation in the steady-state level of expression of these TRIM proteins was observed on Western blots (Fig. 5A); however, with the exception of TRIM27, all of the TRIM proteins were expressed as efficiently as $TRIM5\alpha_{hu}$. To assess antiretroviral activity, the Cf2Th stable cell lines were infected with different GFP-expressing recombinant retroviruses that were pseudotyped with the vesicular stomatitis virus (VSV) G glycoprotein. The retroviruses tested were human immunodeficiency virus (HIV-1), simian immunodeficiency virus of macaques (SIV_{mac}), simian immunodeficiency virus of African green monkeys (SIV_{agm}), N-tropic and B-tropic murine leukemia viruses (N-MLV and B-MLV, respectively), equine infectious anemia virus (EIAV), feline immunodeficiency virus (FIV), and bovine immunodeficiency virus (BIV). As expected, rhesus monkey TRIM5α potently restricted HIV-1, N-MLV, SIV_{agm}, FIV and EIAV infections, and exerted modest inhibitory activity against SIV_{mac} infection (Figs. 5B and C). Human TRIM5α potently blocked N-MLV infection and modestly inhibited HIV-1, SIV_{mac}, EIAV, FIV and BIV infections. Of the other TRIM proteins tested, only TRIM34 was associated with any antiviral activity, modestly inhibiting SIV_{mac} infection; very slight

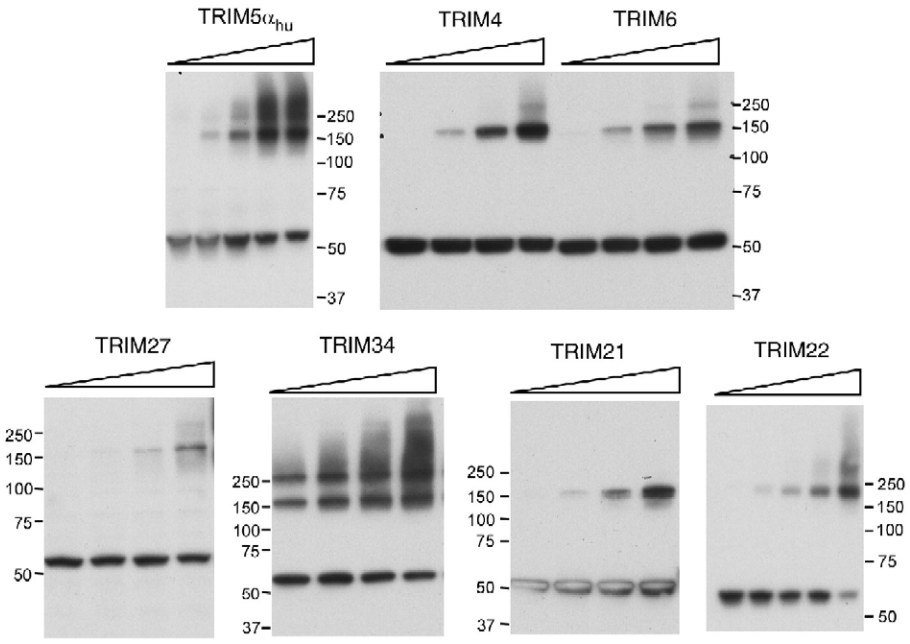

Fig. 3. Oligomerization of TRIM proteins. Lysates from HeLa cells stably expressing TRIM proteins were cross-linked with increasing concentrations of glutaraldehyde (0, 0.2, 0.4, 0.8 and 2.0 mM for TRIM5α$_{hu}$ and TRIM22, and 0, 0.05, 0.1 and 0.2 mM for the other TRIM proteins). The cross-linked products were resolved by SDS-PAGE and visualized by Western blotting with an anti-FLAG antibody. The position of the molecular weight markers (in kiloDaltons) is indicated on the figure.

decreases in FIV, EIAV and BIV infections were observed in TRIM34-expressing cells. Thus, the human and rhesus monkey TRIM5α proteins exhibit greater restricting activity against specific members of this retrovirus panel than the related human TRIM proteins.

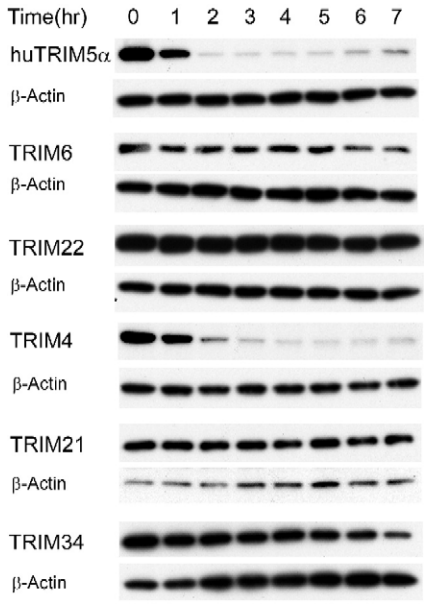

Fig. 4. Turnover of TRIM proteins. HeLa cells expressing different FLAG-tagged TRIM proteins were treated with cycloheximide for a period of 7 h. Cells were lysed at the indicated time points after the initiation of treatment. Cell lysates containing equal amounts of total proteins were analyzed by Western blotting with an anti-FLAG antibody or anti-actin antibody as controls. Both N- and C-terminally tagged proteins were examined, with comparable results; only one representative set of results is shown here.

Dominant-negative effects of some TRIM proteins on human and rhesus monkey TRIM5α

The antiviral activities of the TRIM proteins were also examined in HeLa cells. The steady-state expression levels of these TRIM proteins in HeLa cells were comparable to those in Cf2Th cells (data not shown). The potent restricting activity of rhesus monkey TRIM5α against HIV-1 and N-MLV, and that of human TRIM5α against N-MLV, were evident in the HeLa cells (Fig. 6). HIV-1 and N-MLV infections of TRIM34-expressing HeLa cells were slightly but reproducibly more efficient than those seen in control cells transduced with the empty LPCX vector. N-MLV infection was also slightly enhanced by expression of TRIM6 in the HeLa cells. TRIM6- and TRIM34-expressing cells exhibited increased susceptibility to infection by BNBB-MLV compared with control cells transduced with the empty LPCX vector (data not shown). The BNBB-MLV-GFP vector is identical to B-MLV-GFP except that residue 110 of the viral capsid has been changed from glutamic acid to arginine, the amino acid residue found at that position in the N-MLV capsid protein. This change in the capsid renders BNBB-MLV-GFP susceptible to restriction mediated by human TRIM5α (Towers et al., 2000; Perron et al., 2004). These results suggest that TRIM6 and TRIM34 may exert very mild dominant-negative effects on the endogenous human TRIM5α protein in the HeLa cells.

The effect of expression of the human TRIM proteins on the anti-HIV-1 activity of rhesus monkey TRIM5α was examined. HeLa cells were stably transduced with an empty LPCX vector or an LPCX vector expressing FLAG-tagged human TRIM4, 6, 22, 27 or 34. These cells were then transduced with a pLenti vector expressing V5-tagged TRIM5α$_{rh}$ or with the empty pLenti

vector. The expression of the TRIM proteins in these cell lines is shown in Fig. 7A. The cells were then challenged with recombinant HIV-1 expressing GFP (Fig. 7B). TRIM5α_{rh} inhibited HIV-1 infection in all of the cell lines except the cells coexpressing TRIM34. Apparently, TRIM34 can act as a dominant-negative inhibitor of TRIM5α_{rh}.

Association of other TRIM proteins with TRIM5α

The colocalization of these TRIM proteins with TRIM5α and the dominant-negative effects seen in the above assays suggested that some TRIM relatives might interact with TRIM5α. To address this possibility, we coexpressed TRIM5α and the TRIM proteins in cells and attempted to coprecipitate the proteins from cell lysates. HeLa cells stably expressing V5-tagged rhesus monkey TRIM5α from a pLenti vector were transduced with LPCX retroviral vectors encoding FLAG-tagged human TRIM proteins. The resulting cell lines expressed both TRIM5α_{rh}-V5 and FLAG-tagged TRIM$_{hu}$ proteins (Fig. 8A). Cell lysates were then prepared from these cell lines and used for immunoprecipitation with anti-V5 and anti-FLAG antibodies. Human TRIM5α was efficiently co-immunoprecipitated with rhesus monkey TRIM5α, and *vice versa* (Figs. 8B and C). This is consistent with yeast two-hybrid

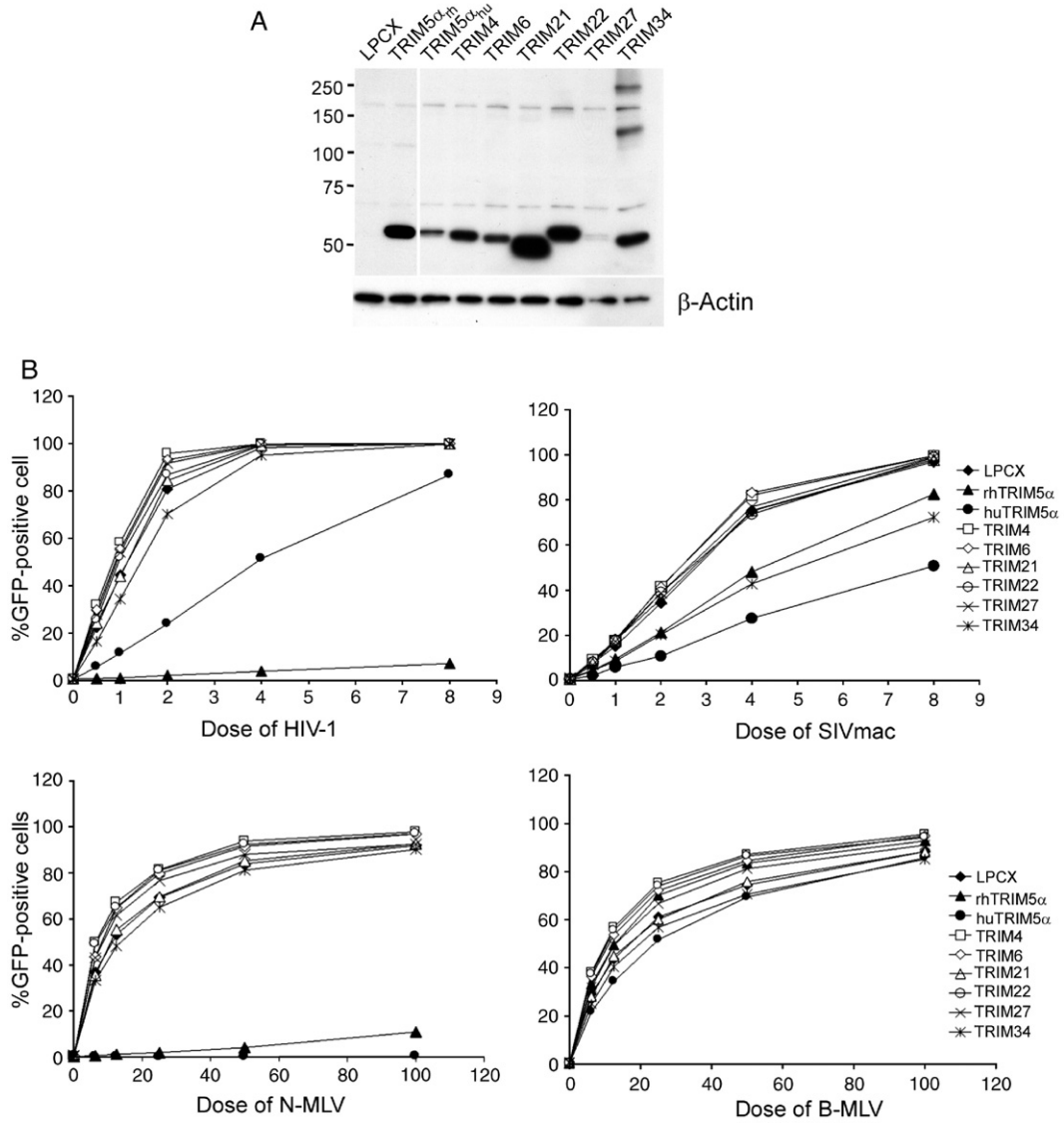

Fig. 5. Expression and antiretroviral activity of TRIM family proteins stably expressed in Cf2Th cells. (A) Expression of TRIM proteins stably expressed in Cf2Th cells was assessed by Western blotting with antibodies directed against epitope tags. TRIM5α_{rh} carries an HA tag at the C-terminus; TRIM5α_{hu} carries a FLAG tag at the C-terminus; all the other TRIM proteins are FLAG-tagged at the N-termini. As a control, the amount of β-actin in the cell lysates was also examined. (B) To assess the effects of the TRIM proteins on retroviral infection, Cf2Th cells expressing different TRIM proteins, or control Cf2Th cells transduced with the empty LPCX vector, were incubated with various amounts of recombinant HIV-1, SIV$_{mac}$, N-MLV or B-MLV viruses expressing GFP. Infected GFP-positive cells were counted by FACS. Ten dose units of HIV-1 or SIV$_{mac}$ correspond to ~3000 cpm reverse transcriptase units; 100 dose units of N-MLV or B-MLV correspond to ~400 cpm reverse transcriptase units. (C) Cf2Th cells expressing the different TRIM proteins, or control Cf2Th cells transduced with the empty pLPCX vector, were incubated with a dose of recombinant BIV, EIAV, FIV or SIV$_{agm}$ virus expressing GFP that was sufficient to infect 50–80% of the control Cf2Th cells. Infected GFP-positive cells were counted by FACS.

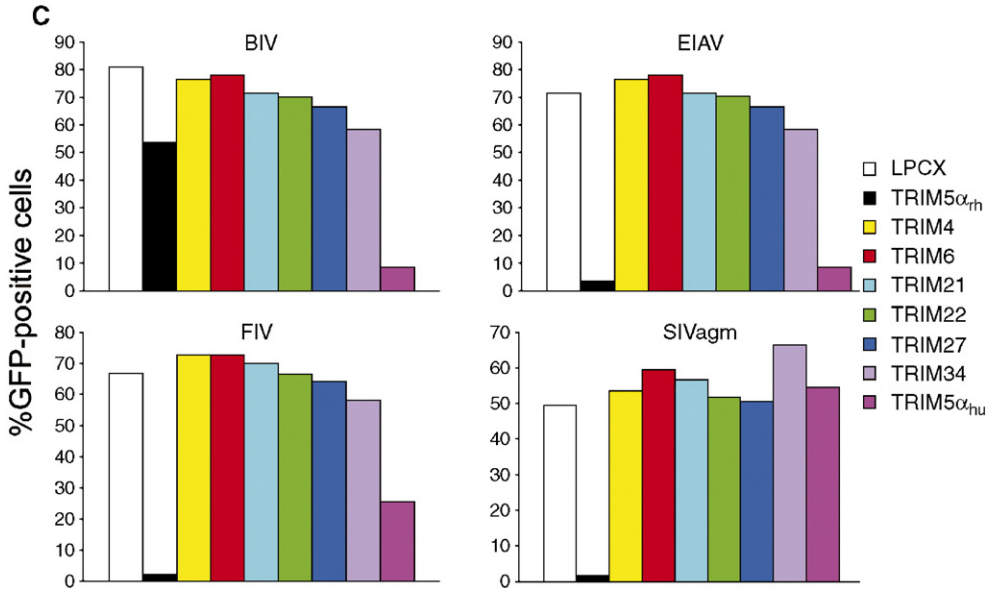

Fig. 5 (*continued*).

studies with human TRIM5α (Reymond et al., 2001), and previous co-immunoprecipitation experiments with rhesus monkey TRIM5α and its derivatives (Javanbakht et al., 2005). None of the heterologous TRIM proteins interacted efficiently with TRIM5α_{rh} under the conditions used (Figs. 8B and C). Only TRIM6 and TRIM34 were very weakly co-immunoprecipitated with rhesus monkey TRIM5α in both directions, which may help to explain their mild dominant-negative activities. The weakness of the heterologous interactions supports previous observations made in the yeast two-hybrid system that TRIM proteins preferentially form homo-oligomers (Reymond et al., 2001).

To examine whether TRIM5α_{rh} might associate with other TRIM proteins after synthesis and trimerization, cytosolic extracts were prepared from 293T cells transiently expressing V5-tagged TRIM5α_{rh} and from separate cells transiently

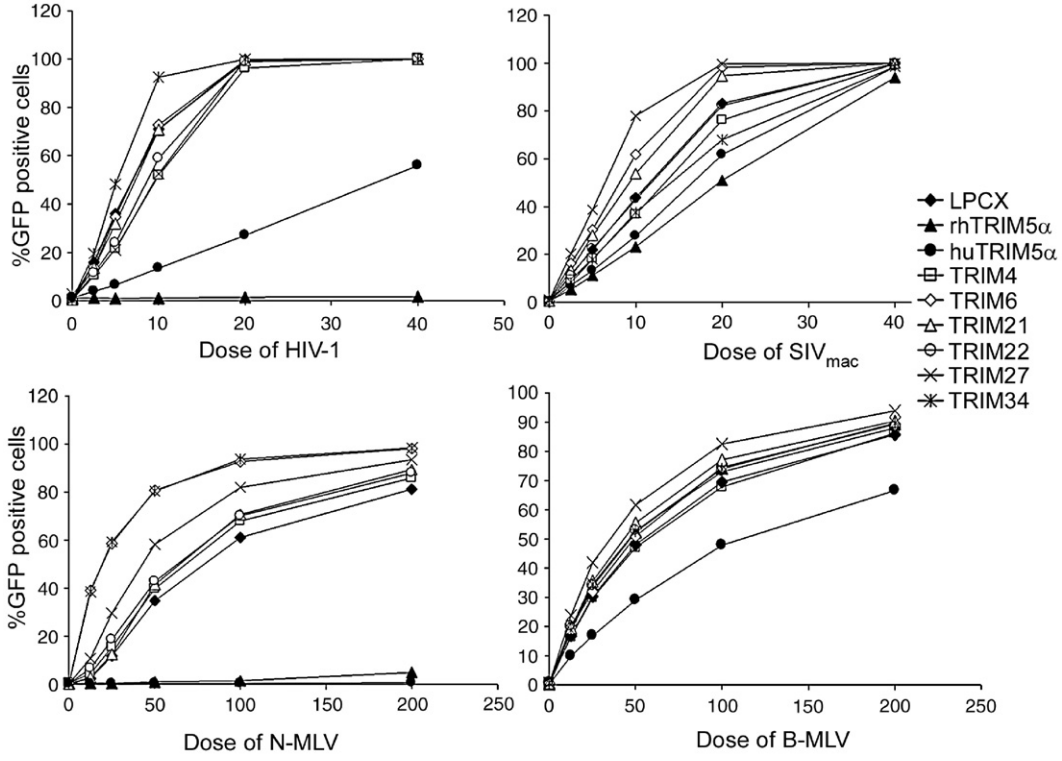

Fig. 6. Antiretroviral activity of TRIM family proteins stably expressed in HeLa cells. The effects of stable TRIM protein expression in HeLa cells on infection of the indicated recombinant viruses were assessed as described in the legend to Fig. 5. The control cells were transduced with the empty LPCX vector.

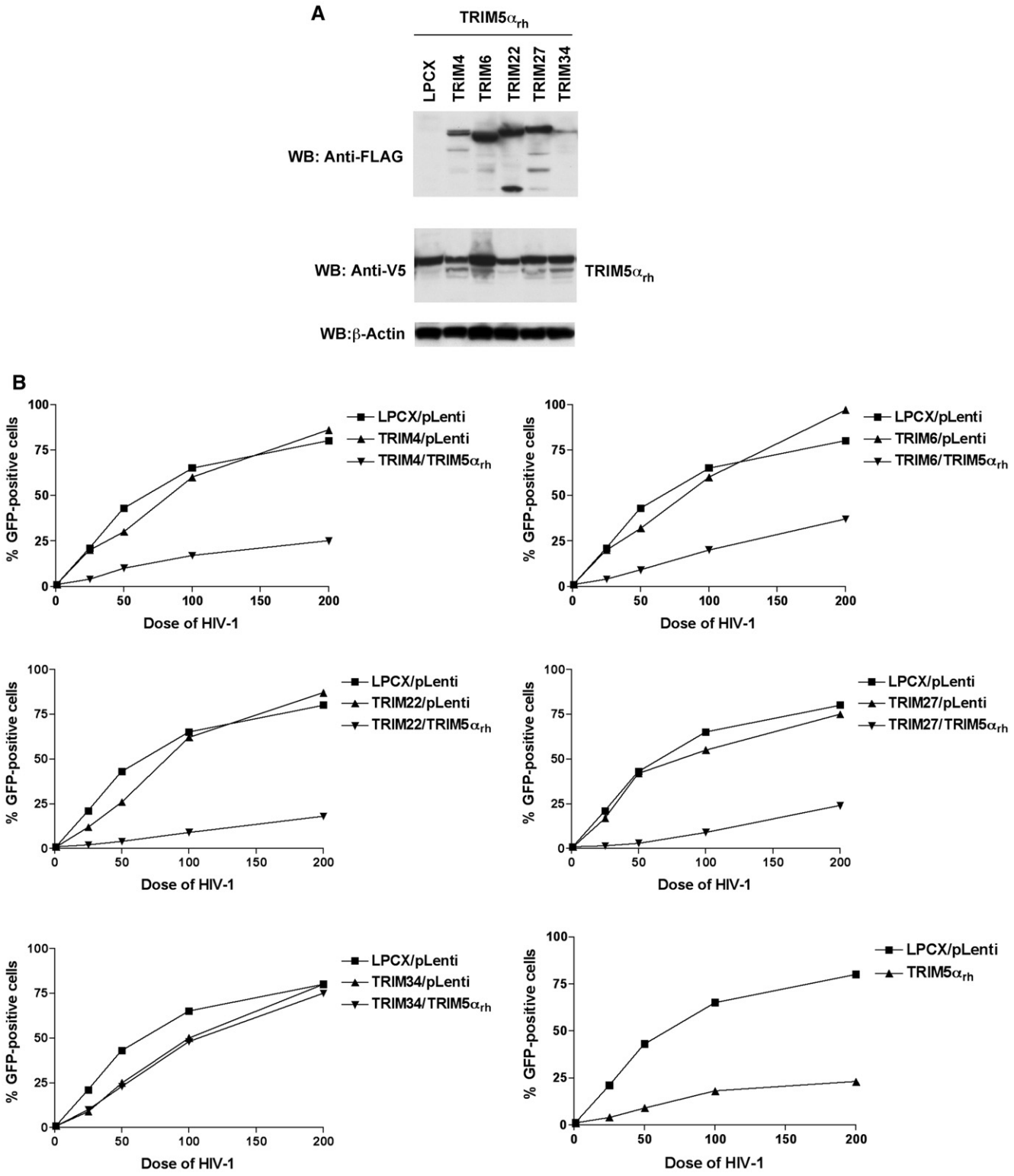

Fig. 7. Effect of expression of human TRIM proteins on the anti-HIV-1 activity of rhesus monkey TRIM5α. HeLa cells were stably transduced with an empty LPCX vector or an LPCX vector expressing the indicated FLAG-tagged human TRIM proteins. The cells were then transduced with a pLenti vector expressing V5-tagged TRIM5α_{rh} or with an empty pLenti vector. (A) Cell lysates were Western blotted (WB) with an anti-FLAG antibody (upper panel), an anti-V5 antibody (middle panel), or an antibody directed against β-actin (lower panel). (B) The HeLa cells were incubated with different doses of recombinant HIV-1 expressing GFP and pseudotyped with VSV G glycoprotein. Flow cytometry was used to determine the percentage of GFP-positive cells. The results shown are from a single experiment and are typical of those obtained in three independent experiments.

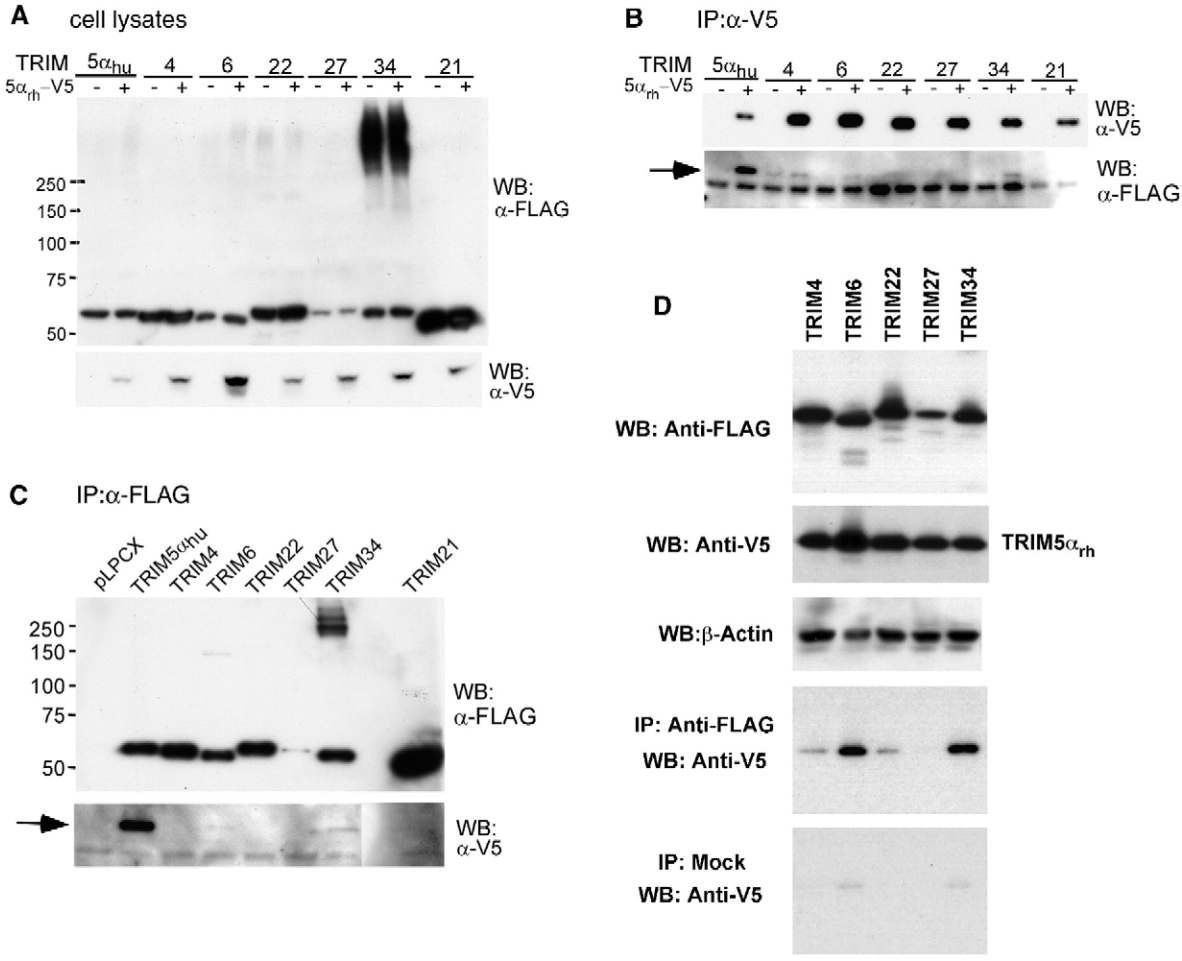

Fig. 8. Co-immunoprecipitation of human TRIM proteins and rhesus TRIM5α. HeLa cells stably expressing both a FLAG-tagged human TRIM protein from an LPCX vector and V5-tagged rhesus monkey TRIM5α from a pLenti vector were established. As a control, HeLa cells expressing the FLAG-tagged human TRIM proteins but carrying an empty pLenti vector were established in parallel. (A) To assess the level of expression of the TRIM proteins, cell lysates were analyzed by SDS-PAGE and Western blotted (WB) with anti-FLAG and anti-V5 antibodies. TRIM34 forms high-molecular-weight aggregates when boiled for 5 min. (B) Immunoprecipitation of the TRIM5α_{rh}-V5 protein with an anti-V5 antibody was used to detect coprecipitated proteins. The upper panel shows the rhesus monkey TRIM5α protein that was precipitated by the anti-V5 antibody; the lower panel shows the coprecipitated TRIM proteins that were detected by Western blotting (WB) with an anti-FLAG antibody (arrow). A faster-migrating non-specific band, which was detected in all the samples, conveniently serves as a loading control. (C) Cell lysates containing FLAG-tagged TRIM proteins were precipitated with an anti-FLAG antibody. Precipitates were Western blotted with an anti-V5 antibody to detect coprecipitated TRIM5α_{rh}-V5 protein. The upper panel shows the FLAG-tagged TRIM proteins that were precipitated; the lower panel shows the coprecipitated TRIM5α_{rh} protein. (D) Cytosolic extracts from 293T cells transiently expressing TRIM4, 6, 22, 27 or 34 (all tagged with a FLAG peptide) were mixed in a 1:1 ratio with cell extracts from 293T cells transiently expressing TRIM5α_{rh} (tagged with a V5 peptide). The mixtures were Western blotted (WB) with antibodies directed against FLAG, V5 and β-actin (upper three panels). The mixtures were also used for immunoprecipitation (IP) by an anti-FLAG antibody, or by control Protein A-Sepharose beads only (Mock). The precipitates were Western blotted with an anti-V5 antibody (bottom two panels). Similar results were obtained in three independent experiments.

expressing FLAG-tagged human TRIM4, TRIM6, TRIM22, TRIM27 or TRIM34. The cytosolic extracts were mixed in a 1:1 ratio and used for precipitation by the antibody recognizing the FLAG epitope on the human TRIM proteins. The precipitates were analyzed by Western blotting with an anti-V5 antibody to detect any coprecipitated TRIM5α_{rh}. Under these conditions, TRIM34 and TRIM6 associated with TRIM5α_{rh} more efficiently than did the other human TRIM proteins tested (Fig. 8D).

Ability of TRIM proteins to bind HIV-1 capsid preparations

The ability of TRIM5α proteins to bind the assembled retroviral capsid is thought to be necessary but not sufficient for the restriction of infection (Diaz-Griffero et al., 2006b; Stremlau et al., 2006). To examine the ability of the different TRIM proteins to associate with the HIV-1 capsid, we used an assay that measures the cosedimentation of epitope-tagged TRIM proteins with HIV-1 capsid–nucleocapsid (CA–NC) complexes that were assembled *in vitro* (Stremlau et al., 2006; Li et al., 2006b). Rhesus monkey TRIM5α associated with the HIV-1 CA–NC complexes that sedimented through 70% sucrose (Fig. 9A). The human TRIM6 and TRIM34 proteins also cosedimented with the HIV-1 CA–NC complexes, whereas the other TRIM proteins tested did not (Figs. 9A and B and data not shown). The TRIM5α_{rh}, TRIM6 and TRIM34 proteins did not pellet through the 70% sucrose cushion in the absence of added

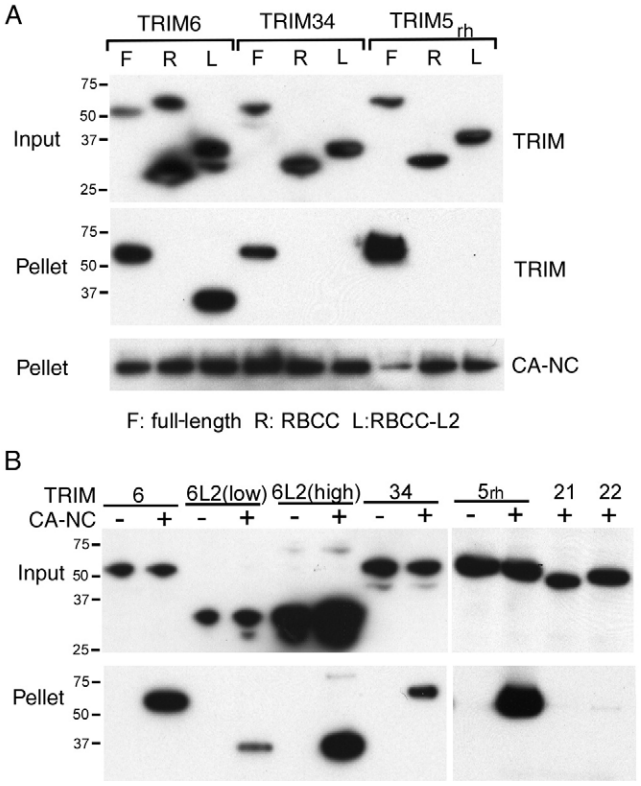

Fig. 9. Ability of TRIM proteins to bind HIV-1 capsid complexes. (A) Cell lysates of 293T cells transiently transfected with N-terminally FLAG-tagged TRIM variants were used in the HIV-1 CA–NC binding assay. The top and middle panels show the amounts of TRIM protein in the input and pellet, respectively; the bottom panel shows the amount of HIV-1 CA–NC protein that was pelleted through the 70% sucrose cushion. The variants of TRIM6, TRIM34 and TRIM5α_{rh} that were studied include the full-length proteins (F), the RBCC protein (R), and the RBCC-L2 proteins (L). The positions of the molecular weight markers are indicated on the left side of the gels. (B) TRIM proteins were examined for the ability to sediment through the 70% sucrose cushion in the absence and presence of the *in vitro* assembled HIV-1 capsid complexes. The upper and lower panels show the amount of TRIM protein in the input and pellet, respectively, assayed by Western blotting. Mutant TRIM6 RBCC-L2 (6L2) was tested at two different protein concentrations (low and high) for quantitative comparison. Two other TRIM family proteins (TRIM21 and TRIM22) exhibit no and very weak capsid-binding capabilities, respectively.

HIV-1 CA–NC complexes (Fig. 9B), indicating the specificity of the association.

The recognition of HIV-1 capsids by TRIM5α_{rh} is dependent upon the B30.2(SPRY) domain (Stremlau et al., 2006). To examine whether the association of TRIM6 and TRIM34 with the HIV-1 CA–NC complexes is similarly dependent on their B30.2(SPRY) domains, we created and tested the RBCC and RBCC-L2 mutants of these proteins in the HIV-1 capsid binding assay. The RBCC constructs include the RING, B-box 2 and coiled-coil domains of the proteins; the RBCC-L2 constructs contain, in addition, the L2 linker located between the coiled-coil and B30.2(SPRY) domains. As expected, the RBCC and RBCC-L2 variants of rhesus monkey TRIM5α did not detectably associate with the HIV-1 CA–NC complexes (lanes R and L, respectively, in Fig. 9A). The cosedimentation of TRIM34 with the HIV-1 CA–NC complexes was also dependent upon the integrity of the B30.2(SPRY) domain (Fig. 9A). By contrast, the RBCC-L2 variant of TRIM6, but not the RBCC variant, exhibited efficient association with the HIV-1 CA–NC complexes (Figs. 9A and B). Thus, the association of human TRIM34, but not human TRIM6, with the assembled HIV-1 capsid complexes is dependent upon the B30.2(SPRY) domain, as is the case for TRIM5α_{rh}.

Analysis of nonsynonymous/synonymous variation in different TRIM genes

Repeated exposure of a species to rapidly evolving viruses is expected to exert strong positive selective pressure on elements of the host immune system that directly interact with viral components (Haldane, 1949). Analysis of nonsynonymous/synonymous variation (K_a/K_s ratios) can provide insight into selection for or against change in the coding capacity of a gene (Li, 1993). *TRIM5* exhibits an unusual pattern of nonsynonymous/synonymous variation. The 5′ portion of *TRIM5* has a low K_a/K_s ratio, indicative of purifying selection. The 3′ end of *TRIM5*, which encodes the B30.2(SPRY) domain, exhibits a high K_a/K_s ratio indicative of positive selection (Liu et al., 2005; Sawyer et al., 2005; Song et al., 2005b). To investigate potential selection operating on related *TRIM* genes, we calculated the K_a/K_s ratios at various codon positions for pairwise comparison of *TRIM5*, *TRIM6*, *TRIM22* and *TRIM34* cDNAs from a hominoid (human), an Old World monkey (baboon), a New World monkey (marmoset) and a prosimian primate (lemur, only for TRIM6) (Fig. 10). The K_a/K_s ratios for *TRIM6* are well below 1 throughout the coding sequence, suggesting that purifying selection has operated on *TRIM6* to preserve the amino acid sequences. For *TRIM22* and *TRIM34*, the K_a/K_s ratios average around 1; apparently, these genes have undergone neutral evolution.

Discussion

In addition to the domain structure common to all TRIM proteins, TRIM5α shares several properties with the other TRIM proteins examined here. At least some of these TRIM proteins (TRIM6, TRIM34 and TRIM22) are encoded by *TRIM5* paralogs that, along with *TRIM5*, comprise a cluster of genes on human chromosome 11p15.4. All of the human TRIM proteins examined herein are trimeric. Trimerization, which involves TRIM5 sequences in the coiled coil and adjacent regions, is important for the antiretroviral activity of TRIM5α (Javanbakht et al., 2006). Trimerization occurs preferentially in an homologous fashion (Reymond et al., 2001); however, we did observe very weak heterologous association between rhesus monkey TRIM5α and either human TRIM6 or human TRIM34. This weak association might contribute to some of the mild dominant-negative effects observed for TRIM6 and TRIM34 with respect to TRIM5α antiretroviral activity. The RBCC domains of related TRIM proteins, TRIM6 and TRIM34, can functionally substitute for those of rhesus monkey TRIM5α (Li et al., 2006a). Heterologous RBCC domains have recently been reported to allow the HIV-1-restricting ability of TRIMCyp, an

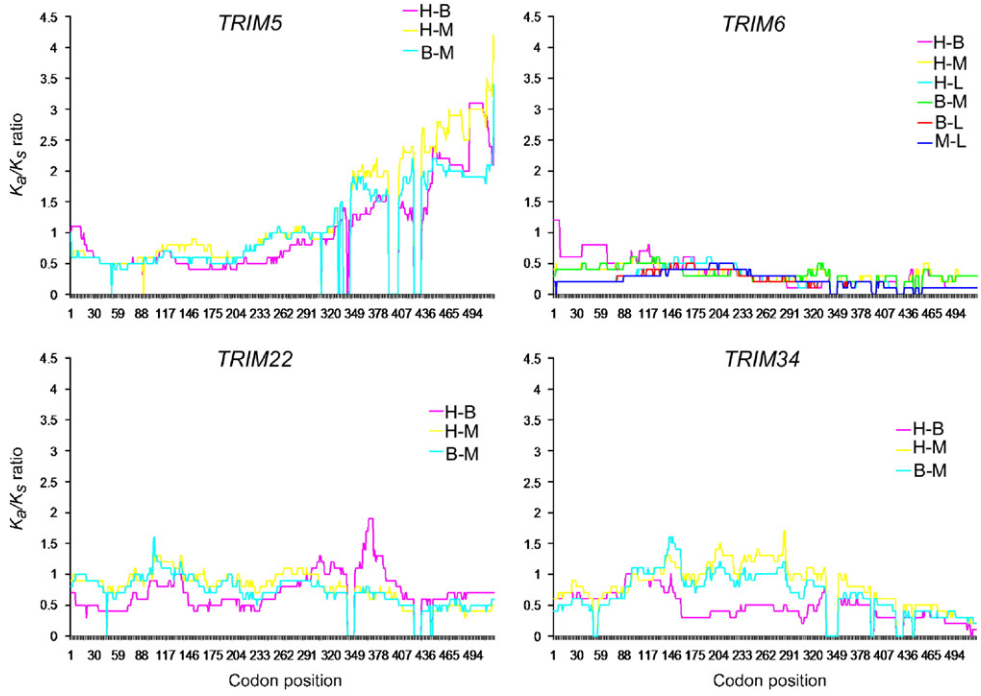

Fig. 10. Nonsynonymous/synonymous ratios (K_a/K_s) for pairwise comparisons of TRIM cDNAs. The plots show the K_a/K_s ratios at various codon positions for pairwise comparisons of human (H), baboon (B), marmoset (M) and lemur (L) *TRIM* cDNAs. The K_a/K_s ratios, calculated by a previously described method (Li, 1993), were estimated as rolling averages for a window of 150 codons.

owl monkey restriction factor consisting of the TRIM5 RBCC domains fused with cyclophilin A (Nisole et al., 2004; Sayah et al., 2004; Zhang et al., 2006).

Like TRIM5α, the TRIM relatives studied herein localize in the cytoplasm and, when overexpressed, form cytoplasmic bodies or speckles. Cytoplasmic localization is likely important for antiretroviral activity, as TRIM5α and TRIMCyp have been shown to exert their inhibitory effects on the retroviral capsid within the first hour following virus entry (Perez-Caballero et al., 2005; Yap et al., 2006; Diaz-Griffero et al., 2006b). The association of TRIM5α and TRIMCyp with cytoplasmic bodies is not necessary for their antiretroviral activity (Song et al., 2005a; Perez-Caballero et al., 2005), although other aspects of the tendency for self-aggregation that TRIM proteins exhibit may contribute in as-yet-unknown ways to the antiviral effect. At a minimum, the properties of trimerization and cytosolic location would be conducive to the evolution of host factors that interact with incoming retroviral capsids, which exhibit trimeric pseudosymmetry (Li et al., 2000).

TRIM5α exhibits several features that are unique among the primate TRIM proteins examined. Most importantly, only TRIM5α exhibited potent inhibitory activity against the retroviruses tested. Weak activity against some retroviruses, such as SIV_{mac}, was observed for human TRIM34, consistent with a recent report (Zhang et al., 2006). The biological relevance of retroviral restriction by TRIM34 is uncertain. TRIM34 exhibits a high propensity for self-aggregation and may, when overexpressed, be prone to associate with other moieties in a less specific fashion. TRIM34, and TRIM6 as well, were able to associate efficiently with the HIV-1 capsid; however, only the association of TRIM34 with the HIV-1 capsid appeared to require its B30.2(SPRY) domain. The mechanistic basis, specificity and biological relevance of the TRIM6 and TRIM34 association with the HIV-1 capsid will require additional investigation. Capsid association could contribute to the weak dominant-negative activities detected for these two TRIM proteins. The ability of a TRIM6 and TRIM34 ancestor to associate with viral capsids might have provided an evolutionary starting point for the specific binding of TRIM5-like paralogs to retroviral capsids.

Analysis of the nonsynonymous/synonymous changes in this group of *TRIM* genes revealed that the high K_a/K_s ratio that characterizes the B30.2(SPRY) domain-coding portion of *TRIM5* is not evident in the other *TRIM* genes examined. Host components of the innate or adaptive antiviral immune response that directly interact with evolutionarily labile viral targets would be expected to exhibit such evidence of positive selection (Haldane, 1949). The *TRIM* genes most closely related to TRIM5 had K_a/K_s ratios less than or equal to 1, indicative of purifying or neutral selection, respectively. This observation suggests that, if TRIM6, TRIM34 and/or TRIM22 are involved in antiviral defenses, they probably do not directly recognize a changeable viral component. A study of the evolution of the APOBEC family of cytidine deaminases also found that certain members of the family known to possess antiretroviral activity were under strong positive selection, whereas other members of the family were under purifying selection (Sawyer et al., 2004). Thus, although certain structural or biochemical properties of the APOBEC and TRIM protein family are conducive to the evolution of antiviral factors, only some family members may be selected

to assume those roles in innate intracellular immunity against viruses.

At least in the context of the overexpression systems studied herein, TRIM5α turned over more rapidly than any of the closely related TRIM proteins. Only the distantly related TRIM4 protein exhibited a half-life that approached the short half-life of TRIM5α. The TRIM5α RING and B-box 2 domains both contribute to the rapid turnover of this protein (Diaz-Griffero et al., 2006a). Studies of a chimeric protein in which the TRIM5α_{rh} RING domain is replaced by that of the more stable TRIM21 indicated that rapid turnover is not necessary for antiviral function (Diaz-Griffero et al., 2006a). However, rapid turnover of TRIM5α may minimize any detrimental effects of this protein on cells when viruses are not present, and also allow rapid upregulation of TRIM5α expression when virus infection is detected.

The *TRIM5* relatives likely arose by duplication events involving ancestral *TRIM* genes. Gene duplication lessens the selective constraints on one copy of the gene, thus allowing the evolution of new functions while the other copy continues to perform the essential original function (Hancock, 2005). The signs of stringent purifying selection in *TRIM6* suggest the probable functional importance of its protein product. Overall, *TRIM22* and *TRIM34* appear to be under more relaxed selective constraints. Understanding the natural cellular function of these TRIM proteins would be of interest in its own right and might provide additional insight into TRIM5α function.

Materials and methods

Phylogenetic analyses

The amino acid sequences of human TRIM proteins that contain a B30.2(SPRY) domain were extracted from the Ensembl database (http://www.ensembl.org/). Amino acid alignments were constructed using the online MAFFT v5.8 server (http://timpani.genome.ad.jp/%7Emafft/server/) with the E-INS-i iterative refinement method (Katoh et al., 2005). Phylogenetic analysis was performed using PAUP v4.0b10 (Swofford, 2002). A neighbor joining bootstrap analysis was performed on the amino acid alignment using the BioNJ setting (Gascuel, 1997) and 500 replications.

Because the B30.2(SPRY) domain may be a hotspot for positive selection (Sawyer et al., 2005; Si et al., 2006; Song et al., 2005b), additional analyses similar to those described above were performed after removing the B30.2(SPRY) domain (as defined in Song et al., 2005b). The B30.2(SPRY)-deleted TRIM analysis was performed to reduce the possibility of highly variable or convergent residues affecting the phylogenetic reconstruction.

Nonsynonymous/synonymous variation

The nonsynonymous/synonymous (K_a/K_s) ratios at various codon positions for pairwise comparisons of TRIM cDNAs were calculated by the method of Li (Li, 1993). The K_a/K_s ratio was estimated as a rolling average for a window of 150 codons, with the center of the window being moved codon-by-codon to produce a plot showing local variation in the degree of sequence conservation.

Creation of cells stably expressing TRIM family proteins

The cDNAs encoding human TRIM4β (NM_033091), TRIM6 isoform 2 (NM_058166), TRIM21 (NM_003141), TRIM22 (NM_006074), TRIM27 (NM_006510) and TRIM34 isoform 1 (NM_021616) were obtained either from a human kidney cDNA library by PCR (Polymerase Chain Reaction) or purchased from Open Biosystems. A sequence encoding the FLAG epitope tag (DYKDDDDK) was inserted at the 5′ end of the cDNAs, which were subsequently cloned into the pLPCX retroviral vectors using the *Cla*I and *Eco*RI/*Xho*I sites. Recombinant viruses were produced in 293FT cells (Invitrogen) by cotransfecting the pLPCX plasmids expressing the different TRIM proteins with the pVPack-GP and pVPack-VSV-G packaging plasmids (Stratagene). The pVPack-VSV-G plasmid encodes the vesicular stomatitis virus (VSV) G envelope glycoprotein, which allows efficient entry into a wide range of vertebrate cells (Yee et al., 1994). The resulting virus particles were used to transduce 2×10^5 Cf2Th cells or HeLa cells in six-well plates. Cf2Th cells are canine thymocytes, and HeLa are human epithelial cells. Cf2Th and HeLa cells were then selected in 5 μg/ml and 1 μg/ml puromycin (Sigma), respectively.

To create cell lines coexpressing two TRIM proteins, blasticidin-resistant HeLa cells stably expressing rhesus TRIM5α-V5 from a pLenti vector were first made using the Viral Power system (Invitrogen). These cells were then transduced as described above with the pLPCX retroviral vectors encoding different FLAG-tagged TRIM proteins and selected in puromycin.

Immunoblotting

Cf2Th or HeLa cells stably expressing the transduced proteins were lysed with phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH_2PO_4, 138 mM NaCl, 8 mM Na_2PO_4) containing 1% NP40 and protease inhibitor cocktail (Roche). The lysates were resolved by SDS-PAGE and Western blotted with horseradish peroxidase-conjugated anti-FLAG M2 antibody (Sigma) and anti-β-actin antibody (Sigma).

Infection with viruses expressing GFP

Recombinant HIV-1, SIV_{mac}, N-MLV, B-MLV, SIV_{agm}, FIV, BIV and EIAV viruses expressing green fluorescent protein (GFP) were made as previously described (Perron et al., 2004; Stremlau et al., 2004; Si et al., 2006). For infection, 3×10^4 cells were seeded in 24-well plates and incubated with the viruses for 60 h. Cells were then washed with PBS, fixed with 3.7% formaldehyde and subjected to fluorescence-activated cell sorting (FACS) analysis with a FACScan (Becton Dickinson).

Crosslinking of TRIM proteins

Cell lysates prepared in 1% NP40/PBS/protease inhibitor cocktail were incubated with varying concentrations (final concentration: 0, 0.2, 0.4, 0.8 and 2.0 mM) of glutaraldehyde (Sigma) at room temperature for 5 min, followed by adding excess glycine to quench the reaction. The crosslinked lysates were then subjected to SDS-PAGE and Western blotted with HRP-conjugated anti-FLAG antibody (Sigma).

Subcellular localization using immunofluorescence confocal microscopy

HeLa cells stably coexpressing human TRIM4, 6, 22, 27 or 34 and rhesus monkey TRIM5α were grown overnight on 12-mm-diameter coverslips and fixed in 3.9% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS; Cellgro) for 30 min. Cells were washed in PBS and permeabilized with 0.05% saponin for 30 min. Samples were blocked with 10% donkey serum (Dako, Carpinteria, CA) for 30 min, and incubated for 1 h with antibodies. The anti-V5-Cy3 antibody (Sigma) was used to stain V5-tagged TRIM5α_{rh} and anti-FLAG-FITC antibody (Sigma) was used to stain the FLAG-tagged human TRIM proteins (TRIM4, 6, 22, 27 and 34). Subsequently, samples were mounted for fluorescence microscopy by using the ProLong Antifade Kit (Invitrogen). Images were obtained with a BioRad Radiance 2000 laser scanning confocal microscope with Nikon 60× N.A.1.4 optics.

To study localization of GFP-TRIM fusion proteins and human TRIM5α, HeLa cells stably expressing HA-tagged human TRIM5α in 12-well plates were transfected with 1 μg of GFP-TRIM fusion constructs for 24 h using Gene Porter 2 (GTS, San Diego), and then subcultured on 8-well chamber slides. Twenty-four hours later, the cells were fixed, permeabilized, and incubated with rat anti-HA 3F10 antibody (1:200, Roche) followed by secondary anti-rat IgG conjugated with TRITC (1:200, Santa Cruz). The processed cells were analyzed using a confocal microscope (Nikon Eclipse E800) with laser (Bio-Rad MRC 1024), and the images were obtained using Bio-Rad Lasersharp 2000 software.

Co-immunoprecipitation

HeLa cells stably expressing both pLenti vector/rhesus TRIM5α-V5 and pLPCX vector/human FLAG-TRIM proteins from nearly confluent 100-mm plates were lysed in 1 ml of lysis buffer (1% NP40/PBS/protease inhibitor cocktail). The lysates were cleared of insoluble materials and aggregates by centrifugation at 100,000×*g* for 30 min. To minimize non-specific interaction with Protein A-Sepharose beads (Pharmacia), 0.5 ml of supernatant were first incubated with 20 μl beads (packed volume) for 4 h at 4 °C. The precleared lysates were then mixed with 20 μl fresh beads and 1 μl of either anti-V5 (Invitrogen) or anti-FLAG (Sigma) antibodies (~1.3 μg and 4.3 μg, respectively) overnight at 4 °C. The immunoprecipitates were washed three times with buffer I (300 mM NaCl, 50 mM Tris–HCl, 1% NP40) at 4 °C for 10 min each on a rocker and once with buffer II (150 mM NaCl, 10 mM Tris–HCl) for 10 min at 4 °C. The beads were then treated with 2× SDS sample buffer (125 mM Tris–HCl, 3% SDS, 16.7% glycerol, 3% β-mercaptoethanol, 0.01% bromophenol blue) and boiled for 5 min to release the precipitated proteins. Supernatants were analyzed by SDS-PAGE and Western blotting with HRP-conjugated anti-V5 antibody (1:5000, Invitrogen) or anti-FLAG antibody (1:500, Sigma).

To examine the ability of mature TRIM proteins to associate with TRIM5α, TRIM proteins (TRIM4, 6, 22, 27 and 34) tagged with a C-terminal FLAG peptide were transiently expressed in 293T cells by using Lipofectamine 2000 (Invitrogen). In a separate plate of 293T cells, TRIM5α_{rh} tagged with a C-terminal V5 peptide was transiently expressed. Cytosolic extracts were prepared by using a Dounce homogenizer (10 strokes) in buffer containing 100 mM KC1, 20 mM Tris–HCl pH 7.5, 5% glycerol and 0.5 mM DTT. Samples were centrifuged at 3000 rpm to remove nuclei and cell debris. Each of the TRIM protein-containing extracts was mixed with TRIM5α_{rh}-containing extracts in a 1:1 ratio. Mixtures were immunoprecipitated using an anti-FLAG monoclonal antibody (Sigma) at 4 °C for 3 h. Immunoprecipitates were washed 10 times using lysis buffer. Mixtures and precipitates were blotted using anti-FLAG, anti-V5 (Invitrogen) and anti-β-actin antibodies (Sigma).

The turn-over rate of TRIM proteins

HeLa cells expressing different TRIM proteins were seeded in 6-well plates 1 day prior to the experiment. When cells achieved 40–60% confluency, cycloheximide at 100 μg/ml (Sigma) was added to block protein synthesis. Treated cells were lysed at different time points and cell lysates containing equal amount of total proteins were subjected to SDS-PAGE and Western blotting analysis.

HIV-1 capsid-binding assay

Purification of recombinant HIV-1 capsid–nucleocapsid (CA–NC) protein from *Escherichia coli* was carried out as previously described (Ganser et al., 1999). High-molecular-weight HIV-1 capsid complexes were assembled using 300 μM CA–NC protein and 60 μM $(TG)_{50}$ DNA oligonucleotide in a volume of 100 μl of 50 mM Tris–HCl (pH 8.0) and 500 mM NaCl. The reaction was allowed to proceed overnight at 4 °C as previously described (Ganser et al., 1999). The assembled CA–NC complexes were stored at 4 °C until needed.

For a source of TRIM5 protein, 293T cells seeded in a six-well dish were transfected with amounts of pLPCX plasmids to produce roughly equivalent levels of TRIM protein expression, according to the manufacturer's protocol (Effectene, Qiagen). Forty-eight hours later, the cells were harvested in phosphate-buffered saline containing 5 mM EDTA and resuspended in 270 μl of hypotonic lysis buffer (10 mM Tris, pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT). The cells were lysed by freeze–thawing and the cell debris was removed by centrifugation at 4 °C for 10 min at maximum speed (14,000×*g*) in an

Eppendorf microcentrifuge. Two hundred thirty microliters of the cleared cell lysate was combined with 5 µl of HIV-1 CA–NC complexes from the assembly reaction mixture, and the concentration of salt was adjusted to 150 mM by adding 10× PBS buffer. The mixture was incubated for 1 h at room temperature with gentle rocking. After incubation, 20 µl of the mixture was assayed for input protein amount; the remainder was layered onto a 3.5-ml 70% sucrose cushion (prepared in 1× phosphate-buffered saline and 0.5 mM DTT) and centrifuged at 110,000×*g* for 1 h at 4 °C in a Beckman SW55Ti rotor. The pellet was resuspended in 80 µl of 1× sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS-polyacrylamide gel electrophoresis and Western blotting for TRIM proteins and HIV-1 p24 capsid protein, as described (Stremlau et al., 2006; Li et al., 2006b).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2006.10.035.

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