Antiretroviral potential of human tripartite motif-5 and related proteins

Fengwen Zhang, Theodora Hatziioannou, David Perez-Caballero, David Derse, Paul D. Bieniasz

Abstract

TRIM5α is a potent inhibitor of infection by diverse retroviruses and is encoded by one of a large family of TRIM genes. We found that several TRIM motifs among a panel of selected human TRIM proteins (TRIM1, 5, 6, 18, 19, 21, 22, 34) could inhibit infection when artificially targeted to an incoming HIV-1 capsid. Conversely, when ectopically expressed as authentic full-length proteins, most lacked activity against a panel of retroviruses. The exceptions were TRIM1, TRIM5 and TRIM34 proteins. Weak but specific inhibition of HIV-2/SIVMAC and EIAV by TRIM34 was noted, and human TRIM5α modestly, but specifically, inhibited an HIV-1 strain carrying a mutation in the cyclophilin binding loop (G89V). Restriction activity observed in ectopic expression assays was sometimes not detectable in corresponding RNAi-based knockdown experiments. However, endogenous owl monkey TRIMCyp potently inhibited an SIVAGM strain. Overall, sporadic examples of intrinsic antiretroviral activity exist in this panel of TRIM proteins.

Keywords: HIV-1; SIV; MLV; TRIM5; TRIM genes; Retrovirus; Intrinsic immunity

Introduction

Mammalian evolution has resulted in at least three independent acquisitions of genes whose primary role appears to be an intrinsic defense against retroviral infection (Best et al., 1996; Bieniasz, 2004; Goff, 2004; Sheehy et al., 2002; Stremlau et al., 2004). One group of genes, exemplified by APOBEC3G, act primarily by deaminating cytidines in nascent retroviral DNA (Bishop et al., 2004; Harris et al., 2003; Lecossier et al., 2003; Liddament et al., 2004; Maneutz et al., 2003; Wiegand et al., 2004; Yu et al., 2004; Zhang et al., 2003; Zheng et al., 2004), while two others, Fv1 and tripartite motif-5 (TRIM5), target incoming retroviral capsids to block infection by as yet poorly defined mechanisms that may involve accelerated disassembly (Kozak and Chakraborti, 1996; Sebastian and Luban, 2005; Stremlau et al., 2004, 2006). The APOBEC3 locus appears to have been under evolutionary pressure during mammalian speciation (Sawyer et al., 2004). Indeed, while mice have only one APOBEC3 gene, humans have no less than seven, that have arisen as a result of gene duplications at the APOBEC3 locus, and exhibit varying levels of activity against numerous retroviruses (Bishop et al., 2004; Doehle et al., 2005; Liddament et al., 2004; Wiegand et al., 2004; Yu et al., 2004; Zheng et al., 2004). Conversely, Fv1 has a rather different evolutionary history and is apparently a remnant of an ancient endogenous retrovirus (Best et al., 1996). It exists as a single gene, is unique to the mouse, and specifically inhibits murine leukemia viruses (MLV) (Baumann et al., 2004; Hatziioannou et al., 2004a). Indeed, divergence in Fv1 and MLV capsid sequences is a major determinant of the sensitivity of various mouse strains to infection by various MLV strains (Bock et al., 2000; Kozak and Chakraborti, 1996; Lassaux et al., 2005; Qi et al., 1998; Stevens et al., 2004).

While Fv1 does not appear to target any retrovirus other than MLV that has been tested thus far (Baumann et al., 2004; Hatziioannou et al., 2004a), a similar type of intrinsic defense encoded by the mammalian (TRIM5) gene has far broader antiretroviral potential. Indeed, the TRIM5α protein is capable of targeting a variety of widely divergent retroviral capsids (Hatziioannou et al., 2004a; Keckesova et al., 2004; Perron et al., 2004; Saenz et al., 2005; Yap et al., 2004). The extent to
which TRIM5 occurs in various mammalian lineages has not yet been fully documented, but all primates that have been examined have a TRIM5 gene, while mice appear to lack a close relative (Song et al., 2005b). Interestingly, TRIM5 is one member of a family of dozens of genes that share a similar architecture (Reymond et al., 2001). Indeed, TRIM5 itself exists as part of a small cluster of closely related TRIM genes on human chromosome 11 (Reymond et al., 2001).

Each TRIM gene encodes a protein with an amino terminal tripartite motif, comprised of a RING domain, one or two additional zinc binding or ‘B-box’ domains, and a coiled-coil domain. The TRIM domain can be linked to one of a number of C-terminal domains, and sometimes a single TRIM gene can encode several variant proteins with different C-terminal domains (Reymond et al., 2001). The TRIM5α protein contains a C-terminal SPRY domain, which is related to domains found in other proteins of diverse organization and function, as well as in several other members of the TRIM protein family. Functional dissection of the TRIM5α protein has revealed that (1) the amino terminal RING domain is important for potent inhibition of retrovirus infection but is not absolutely required, (2) the B-box domain is required for inhibition, (3) the central coiled-coil is necessary and sufficient for TRIM5 multimerization, and (4) the C-terminal SPRY domain likely binds to incoming capsids and is the principle determinant of specificity, i.e., it determines which retroviruses are inhibited by a given TRIM5α variant (Javanbakht et al., 2005; Nakayama et al., 2005; Perez-Caballero et al., 2005a,b; Stremlau et al., 2005, 2006; Yap et al., 2005; Sawyer et al., 2005). Remarkably, a retrotransposition event in Owl monkeys has resulted in the almost precise replacement of the C-terminal SPRY domain with a cyclophilin A (CypA) domain (Nisole et al., 2004; Sayah et al., 2004). The resulting protein, TRIMCyp, potently inhibits HIV-1 infection as a consequence of the apparently unique ability of the HIV-1 capsid to bind to CypA.

The existence of numerous mammalian TRIM genes encoding proteins with similarity to TRIM5 begs the question of whether they are capable of inhibiting retrovirus infection and, if not, what is special about the TRIM5 protein that enables it to do so. Other TRIM proteins have been implicated in defense against infection by retroviruses and other viruses, but none has yet been shown to exhibit the potency and specificity of TRIM5α (Chelbi-Alix et al., 1998; Maul and Everett, 1994; Turelli et al., 2001; Yap et al., 2004, 2005). In this study, we have begun to examine the antiretroviral potential of a collection of human TRIM proteins, focusing primarily on those most closely related to TRIM5α.

Results

To begin to address whether TRIM proteins might have a role in antiretroviral defense, we performed a variety of assays designed to uncover antiretroviral activities in human TRIM proteins. TRIM proteins were selected for study based on close sequence similarity to the known restriction factor, TRIM5α, occurrence in the same cluster of TRIM genes on chromosome 11 as TRIM5α (TRIM6, TRIM21, TRIM22 and TRIM34) or previously reported activity against N-MLV or other viruses (TRIM1, TRIM18, TRIM19). A variety of criteria were applied to determine (1) whether the TRIM motif of each TRIM protein had antiretroviral potential when targeted to an incoming retrovirus by known capsid binding protein; (2) whether siRNA-mediated depletion of, or expression of putative dominant-negative versions of each TRIM protein could enhance the infection sensitivity of human cells; (3) whether overexpression of the TRIM genes in nonhuman cells could inhibit retrovirus infection. We first examined wild-type or mutant MLV and HIV-1 strains that are known or suspected to be restricted in human cells. Subsequently, we examined a panel of very diverse retroviruses including representatives that have, or have not, colonized humans.

Examination of the anti-HIV-1 activity of various TRIM domains fused to CypA

First, we determined whether the TRIM motif (consisting of RING, B-box and coiled-coil domains) of each of the aforementioned TRIM proteins intrinsically possessed the ability to inhibit retrovirus infection when targeted to an incoming capsid. To accomplish this, we took advantage of the fact that a fusion protein that naturally occurs in owl monkeys, TRIMCyp, contains a C-terminal CypA domain in place of the usual SPRY domain (Nisole et al., 2004; Sayah et al., 2004). The presence of the CypA domain targets TRIMCyp specifically to HIV-1 capsids through the well-characterized CypA:CA interaction (Luban et al., 1993; Yoo et al., 1997). Therefore, each of the human TRIM domains was fused in frame to the intervening spacer peptide and CypA domain from the owl monkey TRIMCyp protein in place of the existing owl monkey TRIM5 domain. The details of the construction are outlined in Fig. 1A, and the configuration was designed to recapitulate as accurately as possible the configuration of the TRIM domain-CypA fusion found in the owl monkey TRIMCyp protein. In addition, we have previously shown that appending the C-terminus of owl monkey TRIMCyp with a fluorescent protein (CFP or GFP) does not measurably affect its ability to inhibit HIV-1 infection (Perez-Caballero et al., 2005b). Thus, each of the artificial TRIMCyp fusion proteins was also appended with GFP to facilitate determination of the localization and antiretroviral activity of the various TRIMCyp proteins.

Each of the TRIMCyp-GFP fusion proteins was expressed in MDTF cells using retroviral vectors, and as can be seen in Fig. 1B, there was some variation in expression level. To ameliorate the potentially confounding effects of expression level on restriction activity, we carried out subsequent studies using cells that were either untreated or treated with sodium butyrate. In each case, butyrate treatment increased the level of TRIMCyp-GFP expression (Fig. 1B). The degree to which the expression of each protein was increased upon sodium butyrate treatment varied but, in most cases, allowed the influence of expression level on restriction activity to be assessed.

The ability of each TRIMCyp-GFP fusion protein to inhibit HIV-1 infection was measured using pools of G418-
resistant MDTF cells obtained after transduction with LNCX2/TRIMCyp-GFP retroviral vectors. Cells were challenged with VSV-G pseudotyped HIV-1 vectors carrying a DsRed reporter gene or, as a control, otherwise identical vectors containing a chimeric Gag protein in which the CA domain was from SIVMAC239 (HIV(SIVCA)). The percentage of DsRed-positive cells in the GFP-positive cell population was determined. As can be seen in Fig. 1C, there was significant variation in the ability of the TRIMCyp fusion proteins to block HIV-1 infection, but none was as potent as the owl monkey TRIMCyp-GFP protein (designated ‘o5’ in Fig. 1). Surprisingly, even the TRIMCyp-GFP protein based on human TRIM5 was less potent than owl monkey TRIMCyp-GFP (Fig. 1C), although this may have been due to its rather low level of expression (Fig. 1B).

Among the other TRIMCyp-GFP proteins, those based on TRIM1, TRIM6, TRIM18, TRIM21 and TRIM5 inhibited HIV-1 infection by 4- to 12-fold while that based on TRIM34 had weak activity (~2.5-fold inhibition) and those based on TRIM19 and TRIM22 were almost inactive (<2-fold inhibition) (Fig. 1C). These results were only marginally affected when the expression level of the TRIMCyp-GFP proteins was increased by pretreating cells with sodium butyrate (Fig. 1D), suggesting that expression level was not a major determinant of restriction activity.

We also determined the subcellular localization of each TRIMCyp-GFP fusion protein, with or without induction of higher level expression by sodium butyrate. In some, but not all, cases, increasing expression level had quite substantial effects on protein localization (Fig. 1E). As we have
previously reported, the owl monkey TRIMCyp-GFP fusion protein exhibited both diffuse and punctate distributions with the latter being favored under conditions of high expression (Perez-Caballero et al., 2005b). Similarly, sodium butyrate treatment led to a change in the localization of the TRIM1 and TRIM22-based TRIMCyp-GFP proteins from a diffuse cytoplasmic distribution to obvious accumulations in aggregates, or so-called ‘cytoplasmic bodies’. Curiously, in the latter case, nuclear bodies were lost when expression levels were enhanced. While the reasons for this are unclear, we speculate that rapid formation of cytoplasmic aggregates under conditions of high-level expression inhibits nuclear import of TRIM22 and formation of nuclear bodies. With the exceptions of TRIM19 and TRIM21, the other TRIMCyp-GFP proteins formed cytoplasmic bodies and their localization was less affected by increased expression level, although we did note that the size and number of bodies were sometimes affected by increasing expression level. For both TRIM6 and TRIM34, the distribution of the Cyp-GFP proteins was clearly asymmetric within the cytoplasm. Indeed there was a tendency of small puncta to accumulate in a single perinuclear cluster, perhaps suggesting some influence of the cytoskeleton on their localization. Again, this characteristic localization was lost upon induction of overexpression with sodium butyrate. As expected, the fusion protein based on TRIM19 (also known as PML) formed nuclear bodies, although we also noted large cytoplasmic aggregates in many cells. The TRIM21-based TRIMCyp-GFP was unique in that its distribution remained entirely diffuse, even upon induction of high-level expression by sodium butyrate.

Notably, however, localization did not appear to be a determinant of restriction activity. TRIMCyp-GFP proteins that were diffuse or concentrated in cytoplasmic aggregates/bodies could inhibit HIV-1 infection. Thus, as we and others have previously concluded, cytoplasmic body formation is not required for restriction activity and may simply be an artifact of overexpression (Diaz-Griffero et al., in press; Perez-Caballero et al., 2005a,b; Song et al., 2005a).

Restriction activity of authentic TRIM proteins against MLV and HIV-1

Given that the TRIMCyp fusion proteins based on TRIM1, 5, 6, 18, 21 and 34 were able to restrict HIV-1 infection, we next tested whether the corresponding proteins that retained their authentic C-terminal domains could inhibit infection by retroviruses that are known or suspected to be restricted in human cells. Specifically, wild-type HIV-1 was tested along with two CA mutants, namely G89V and G94D. The G89V CA mutant lacks the ability to bind CypA (Saphire et al., 1999) and exhibits reduced titer, specifically in human cells. However, it is unclear whether this reduced infectivity is due to restriction by TRIM5α (Towers et al., 2003; Yap et al., 2004). HIV-1 (G94D) also exhibits reduced titer compared to WT HIV-1 in some human target cells (e.g., HeLa and H9) but not in others (e.g., HOS and Jurkat). Interestingly, the apparently restricted phenotype conferred by G94D requires CypA:CA interaction but is not mediated by human TRIM5α (Hatzioannou et al., 2005; Sokolskaja et al., 2004).

Thus, because HIV-1 (G89V) and HIV-1 (G94D) are apparently restricted in a possibly TRIM5α-independent manner in human cells, they were good candidates to test for restriction by the TRIM5α related proteins. N-MLV that is restricted in human cells by TRIM5α, and B-MLV, which is not, were included in these experiments as controls for specific and nonspecific effects. We adopted several strategies to determine whether candidate TRIM proteins were capable of or responsible for restriction of these HIV-1 mutants. We first used siRNA-induced depletion of each of the TRIM proteins that were able to restrict WT HIV-1 in the context of TRIMCyp-GFP (TRIM1, 5, 6, 18, 21, 34) to determine whether the endogenously expressed proteins were responsible for apparent restriction of HIV-1 mutants in human cells. Target cells consisted of TE671 cells, which restrict N-MLV and HIV-1 (G89V), and HeLa cells, which more weakly restrict N-MLV and HIV-1 (G89V), but exhibit reduced sensitivity to HIV-1 (G94D).

To verify that each TRIM-specific siRNA was indeed capable of depleting the TRIM proteins to which they were targeted, each siRNA, or a control siRNA, was cotransfected with plasmids expressing full-length TRIM proteins bearing an amino-terminal HA epitope tag in HeLa cells. As can be seen in Fig. 2A, all of the siRNAs profoundly reduced the expression of the epitope tagged TRIM proteins, to virtually undetectable levels. Next, we transfected TE671 or HeLa cells with each siRNA and subsequently inoculated them with N- and B-MLV, as well as wild-type and mutant HIV-1 vectors. As can be seen in Figs. 2B and C, depletion of TRIM5 cells enhanced N-MLV titers, by approximately 30-fold in TE671 cells and 5-fold in HeLa cells, such that they approached those of B-MLV. Conversely, TRIM protein depletion had no effect on B-MLV infection, which is not thought to be restricted in human cells. Notably, none of the siRNAs that inhibited TRIM protein expression had significant effects on infection of TE671 or HeLa cells by either wild-type HIV-1 or the G89V or G94D mutants (Figs. 2B and C). Similar results were obtained when multiple TRIM proteins were depleted using pools of TRIM-specific siRNAs (Supplementary Fig. 1). Thus, these data suggested the apparent restriction of G89V and G94D mutant HIV-1 in human cells was not due to the endogenous expression of the TRIM proteins targeted here.

As an alternative approach to determine whether endogenous TRIM proteins might be responsible for restriction in human cells, we used an approach in which truncated TRIM proteins lacking the C terminal SPRY domain are over expressed in target cells. We have previously shown that a truncated form of TRIM5 lacking the SPRY domain (6SPRY) can completely abolish N-MLV restriction in human cells, by sequestering the endogenous full-length TRIM5α heteromers via interactions mediated by the coiled-coil domain (Perez-Caballero et al., 2005a). This approach is most successful in HeLa cells.
stably transduced with retroviral vectors expressing truncated TRIM proteins.

As can be seen in Fig. 3A, each of the truncated human TRIM (δSPRY) proteins was expressed in human cells at the same or higher levels than the protein derived from human TRIM5. In each case, expression could be further enhanced by pretreatment of cells with sodium butyrate. As we have previously shown, overexpression of the TRIM5 (δSPRY) protein completely abolished N-MLV restriction and increased titers to the same level as that of B-MLV (Perez-Caballero et al., 2005a). However, as shown in Fig. 3B, both TRIM6 (δSPRY) and TRIM34 (δSPRY) exhibited the same activity and almost completely abolished N-MLV restriction, although TRIM6 (δSPRY) was marginally less active than TRIM34 (δSPRY) and TRIM5 (δSPRY). In fact, the truncated TRIM proteins were even more effective inhibitors of restriction than were siRNAs, with TRIM5 (δSPRY) or TRIM34 (δSPRY) expression resulting in complete restoration of N-MLV infectivity in HeLa cells (Fig. 3B and Supplementary Fig. 2). Conversely, expression of other TRIM (δSPRY) proteins had only slight effects on N-MLV titer, with increases of 2-fold or less as compared to unmodified HeLa cells.

While the ability of TRIM6 (δSPRY) and TRIM34 (δSPRY) expression to enhance N-MLV infection in human cells was initially surprising, given that N-MLV restriction therein is thought to be entirely due to TRIM5, it has previously been shown that TRIM6 can form heteromultimers with TRIM5 (Reymond et al., 2001). To test whether this was also true of TRIM34, we used yeast two hybrid assays. As can be seen in Table 1, TRIM5 fused to a GAL4 DNA binding domain efficiently formed homomultimers with TRIM5. Notably, however, TRIM5 also formed heteromultimers, albeit less efficiently, with both TRIM6 and TRIM34. Because of this finding and the fact that siRNA-mediated depletion of TRIM5, but not TRIM6 or

Fig. 2. Effects of TRIM protein depletion by RNAi on the sensitivity of human cells to infection by MLV and HIV-1. (A) Western blot analysis of HA-tagged TRIM protein expression following cotransfection of HeLa cells with LNCX-derived plasmids expressing the various HA-tagged TRIM proteins and either a specific siRNA (+) or a control siRNA directed against luciferase (−). (B, C) Sensitivity of TE671 cells (B) or HeLa cells (C) to infection by N-MLV, B-MLV, wild-type or mutant HIV-1 vectors, following transfection with siRNAs specific for the indicated TRIM proteins. Controls included cells that were transfected with an siRNA that targeted luciferase (L) or were not transfected (−). The percentage of infected (GFP-positive) cells is plotted.
TRIM34, relieves N-MLV restriction, we surmise that overexpression of truncated versions of TRIM6 and TRIM34 likely inhibits N-MLV restriction by sequestering endogenous TRIM5 into inactive heteromultimers.

We next tested whether HeLa cells that overexpressed the TRIM (δSPRY) proteins exhibited altered sensitivity to the wild-type or G89V or G94D mutant HIV-1 vectors. In general, there was little or no effect on infection by these two HIV-1 mutants (Fig. 3 B), with the exception that truncated forms of TRIM5 and TRIM34 modestly increased the titer of HIV-1 (G89V) when cells were treated with sodium butyrate to enhance expression of the truncated proteins (Supplementary Fig. 2). Thus, these data suggested that HIV-1 (G89V) may be weakly restricted in HeLa cells by endogenous TRIM5, TRIM34 or an unknown protein capable of forming heteromultimers with these two truncated TRIM proteins.

**Anti-MLV and HIV-1 activity of full-length TRIM proteins expressed in MDTF cells**

As a further test of the antiretroviral potential of the panel of TRIM proteins, each was stably expressed as a full-length, HA-tagged protein in MDTF cells using LNCX-derived retroviral vectors. As expected, there was some degree of variation in expression level among the TRIM proteins (Fig. 4A). Therefore, we tested restriction with and without sodium butyrate induction, as before, to examine effects of expression level in restriction activity. As can be seen in Fig. 4B, only TRIM5 significantly inhibited N-MLV infection in uninduced cells. Marginal inhibition was also apparent in cells expressing TRIM1, as has been previously reported (Yap et al., 2004). In general, manipulation of TRIM protein expression level using sodium butyrate had little effect on these results with the exception that N-MLV inhibition by TRIM1 became more dramatic upon treatment of target cells with sodium butyrate (Supplementary Fig. 3), which clearly enhanced TRIM1 expression (Fig. 4A).

Inoculation of the panel of TRIM protein-expressing cells with wild-type or G89V or G94D mutant HIV-1 vectors did not reveal any restriction activities among the intact TRIM proteins, with one exception. Notably, infection by HIV-1 (G89V) was modestly but specifically and significantly inhibited (3- to 4-fold) by human TRIM5α (Fig. 4B). This phenotype was predicted by our earlier studies which showed that G89V mutant but not wild-type HIV-1 capsids can saturate N-MLV

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**Table 1**

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<th>Yeast two-hybrid analysis of TRIM protein heteromultimerization</th>
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<td>GAL4</td>
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<td>VP16</td>
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<tr>
<td>VP16-TRIM5</td>
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<tr>
<td>VP16-TRIM6</td>
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<td>VP16-TRIM34</td>
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* Values represent β-galactosidase activity (given in OD590 units) in a pool of Y190 yeast transformants expressing the indicated GAL4 DNA binding domain and VP16 transcription activation domain TRIM fusion proteins.
restriction in human cells but has not been observed by others (Sokolskaja et al., 2006; Yap et al., 2004). However, we obtained this result numerous times in separately derived pools and clones of murine MDTF and feline CRFK cells (data not shown and see below). Nonetheless, the degree of HIV-1 (G89V) restriction by human TRIM5α is clearly more modest than that of N-MLV and suggests that recognition of incoming HIV-1 (G89V) capsids by human TRIM5α and is quite inefficient. Moreover, these data indicate that restriction activities that are not readily apparent at endogenous levels of TRIM protein expression can be revealed by exogenous overexpression of TRIM proteins.

**Antiretroviral potential of TRIM proteins against diverse retroviruses**

We next tested whether any of the aforementioned TRIM proteins could inhibit infection by retroviruses other than HIV-1 or MLV. Because several reporter viruses were available only as nearly full-length lentiviral genomes, with a GFP reporter gene placed in the Nef position, we slightly altered the format of the restriction assay, because murine MDTF cells do not support efficient reporter gene expression due to incompatibility of the primate lentivirus Tat and murine cyclinT1 proteins (Bieniasz et al., 1998; Garber et al., 1998). Specifically, we generated feline CRFK cells expressing each of the full-length, HA-tagged TRIM proteins by transduction with LNCX-derived retroviral vectors and selection of TRIM protein-expressing pools.

We inoculated the pools of TRIM protein-expressing CRFK cells with GFP-expressing reporter viruses based on the genomes of HIV-2ROD, SIVMAC, SIVAGMTan, SIVAGMSab as well as GFP-expressing EIAV, HTLV-I and prototypic foamy virus (PFV) based vectors. With the exception of PFV, which requires its own envelope to mediate assembly, the naturally occurring envelope proteins were removed and the reporter viruses/vectors were pseudotyped with VSV-G. As can be seen in Fig. 5, most human TRIM proteins did not inhibit infection by most retroviruses with the following exceptions: in particular, HIV-2ROD infection was inhibited approximately 3- to 5-fold by TRIM5α (‘5’ in Fig. 5) and marginally (<2-fold) by TRIM34 and owl monkey TRIMCyp (‘5’ and ‘o5’ in Fig. 5). Additionally, SIVMAC was inhibited 2-fold by TRIM5 and 3-fold by TRIM34. Conversely, SIVAGMSab and SIVAGMTan were unaffected by any human TRIM protein but SIVAGMTan was inhibited 8-fold by owl monkey TRIMCyp (‘o5’, Fig. 5). EIAV was inhibited 7- to 9-fold fold by TRIM5 and marginally (<2-fold) by TRIM34. Thus, these results suggested that TRIM34, which is one of the closest relatives of TRIM5, might also modestly inhibit retroviral infection of human cells in some instances and that TRIMCyp might also inhibit SIVAGMTan infection of owl monkey cells.

**Weak restriction of lentivirus infection by ectopically but not endogenously expressed TRIM34**

The aforementioned results were obtained using pooled G418-resistant cells transduced with LNCX-derived vectors.
expressing TRIM proteins. In some cases, restriction by TRIM proteins can be underestimated because a fraction of the cells in the G418-resistant pool fail to express the transduced TRIM protein and this contributes a background, unrestricted level of infectivity (Perez-Caballero et al., 2005a). This can make the discrimination of strong and weak restriction by TRIM proteins somewhat difficult. To circumvent this problem, we used a previously described strategy in which single cell clones expressing the TRIM proteins of interest were derived. This approach ensures that close to 100% of the target cells actually express the TRIM protein and, in cases where the TRIM protein is highly active, reduces the ‘background’ level of infection. This enables the degree of inhibition mediated by TRIM proteins to be more accurately assessed (Perez-Caballero et al., 2005a). In this instance, for comparative purposes, we derived single cell clones of CRFK cells expressing HA-tagged human TRIM5 and TRIM34. Western blot analysis showed that the clones expressed nearly identical levels of HA-TRIM5 or HA-TRIM34 (data not shown).

As can be seen in Table 2, clones of CRFK cells expressing human TRIM5 were only marginally less sensitive to wild-type HIV-1 and SIV\textsubscript{MAC} as compared to unmodified CRFK cells. Conversely, human TRIM5-expressing CRFK clones were 5-fold less sensitive to HIV-2\textsubscript{ROD} infection and 10-fold less sensitive to EIAV than unmodified CRFK cells. Relatively strong inhibition of EIAV infection by human TRIM5 and partial inhibition of HIV-2 and SIV\textsubscript{MAC} has been described previously (Hatzioannou et al., 2004a,b; Stremlau et al., 2004; Ylinen et al., 2005), and these findings are similar to previous reports. Incidentally, human TRIM5-expressing CRFK clones were also 5-fold less sensitive to HIV-1 (G89V) as compared to unmodified cells, consistent with our findings in MDTF cells (Fig. 4). Moreover, as can be seen in Table 2, CRFK clones expressing human TRIM34 also exhibited altered sensitivity to retrovirus infection.

Table 2

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<tr>
<th>Virus</th>
<th>Infection sensitivity of CRFK cell clones expressing(^a)</th>
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<tr>
<td></td>
<td>TRIM5</td>
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<tr>
<td>HIV-1 (WT)</td>
<td>0.71 ± 0.17</td>
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<tr>
<td>HIV-1(G89V)</td>
<td>0.22 ± 0.02</td>
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<tr>
<td>HIV-2\textsubscript{ROD}</td>
<td>0.18 ± 0.03</td>
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<tr>
<td>SIV\textsubscript{MAC}</td>
<td>0.51 ± 0.12</td>
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<tr>
<td>EIAV</td>
<td>0.08 ± 0.01</td>
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<tr>
<td>N-MLV</td>
<td>0.006 ± 0.001</td>
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\(^a\) Infection sensitivity is given relative to that of unmanipulated CRFK cells which was arbitrarily set at a value of one. The values are given as a mean ± standard deviation of 3 determinations of relative infection sensitivity of TRIM expressing CRFK cells using virus doses that resulted in infection of 5 to 30% of the unmodified CRFK cells.
Specifically, they were approximately 2-fold less sensitive to HIV-2ROD and EIAV and 4-fold less sensitive to SIVMAC. However TRIM34 expression did not affect or only marginally affected CRFK sensitivity to HIV-1 (WT), HIV-1 (G89V) or N-MLV. Thus, human TRIM34 is intrinsically capable of inhibiting infection by some retroviruses, albeit significantly less potently than TRIM5α.

Next, we used siRNA-mediated depletion to test whether TRIM5 and or TRIM34 endogenously expressed by human TE671 or HeLa cells could affect infection by HIV-2ROD, SIVMAC or EIAV. In fact, only TRIM5 depletion enhanced HIV-2ROD (4-fold) and EIAV (5-fold) infection in TE671 cells (Fig. 6A), as previously reported (Hatziioannou et al., 2004a,b; Ylinen et al., 2005). Depletion of TRIM5 had little effect on SIVMAC infection. Surprisingly, these effects were only evident in TE671 cells and not in HeLa cells (Fig. 6B). We presume that this is because HeLa cells express less TRIM5 than TE671 cells, or that TRIM5 is less active therein. This notion is consistent with the observation that N-MLV is clearly more strongly restricted in TE671 than in HeLa cells (Fig. 2). Notably, depletion of endogenous TRIM34 had no effect on HIV-2ROD, SIVMAC, or EIAV infection, either in TE671 or in HeLa cells (Figs. 6A, B). Previous reports have indicated that TRIM34 mRNA expression is inducible by interferon-α in HeLa cells (Orimo et al., 2000). Nonetheless, even when cells were treated with interferon-α, as described previously (Orimo et al., 2000), in neither HeLa nor TE671 cells did TRIM34 depletion affect infection by any of the aforementioned viruses (data not shown). We conclude from these results that the weak antiretroviral activity of TRIM34 against HIV-2, SIVMAC, or EIAV is likely only measurable under conditions of ectopic expression, which is probably higher than occurs in TE671 or HeLa cells.

Restriction of SIVAGMTan by endogenously expressed owl monkey TRIMCyp

We also tested whether SIVAGMTan infection was blocked by the levels of TRIMCyp that are found endogenously in owl monkey cells. We first tested whether SIVAGMTan was indeed restricted in owl monkey cells by inoculating OMK cells with a fixed dose of the SIVAGMTan reporter virus in the presence of increasing amounts of noninfectious SIVAGMTan or HIV-1 virus-like particles (VLPs) that lacked viral genomic RNA. As can be seen in Fig. 7A, the noninfectious VLPs enhanced SIVAGMTan reporter virus infection by up to 100-fold. Since it is known that HIV-1 VLPs can abolish resistance to HIV-1 infection by saturating owl monkey TRIMCyp (Nisole et al., 2004; Sayah et al., 2004; Towers et al., 2003), this result suggested that SIVAGMTan is restricted by the same factor. To confirm this notion, OMK cells were inoculated with the SIVAGMTan reporter virus in the presence (or absence) of CsA, which abolishes the activity of TRIMCyp by competitively inhibiting interaction between TRIMCyp and incoming capsids. As can be seen in Fig. 7B, CsA treatment of OMK cells increased their sensitivity to SIVAGMTan by almost 100-fold. Thus the results shown in Fig. 7 indicate that endogenous levels of TRIMCyp are capable of strongly inhibiting infection by SIVAGMTan.

Discussion

Previous studies have shown that TRIM5α is capable of inhibiting infection by a wide array of retroviruses, with large variations in antiretroviral potency depending on the particular retrovirus that is targeted and the species from which the TRIM5α protein is obtained (Hatziioannou et al., 2004a,b; Keckesova et al., 2004; Perron et al., 2004; Stremlau et al., 2004; Yap et al., 2004). In this study, we tested whether a selected group of additional human TRIM proteins could exhibit inhibitory activity against a range of retroviruses. Reasoning that TRIM proteins most closely related to TRIM5α were the most likely candidates for novel restriction activities, the TRIM proteins were selected on the basis of whether they were homologous to TRIM5α, were part of a cluster of TRIM genes on human chromosome 11, or have previously been shown to exhibit antiviral activity. We found that several of the TRIM proteins tested have the potential to prevent retroviral infection because they could exhibit reasonable degrees of inhibition when targeted to an incoming HIV-1 capsid (by fusion to CypA). While most authentic,
full-length TRIM proteins did not exhibit antiretroviral activity, presumably because they fail to recognize the retroviruses tested, we confirmed previous findings that human TRIM5 can exhibit broad antiretroviral activity, and that human TRIM1 can inhibit N-MLV (Hatzioannou et al., 2004a,b; Keckesova et al., 2004; Perron et al., 2004; Yap et al., 2004). In addition, we found that human TRIM5 can weakly but specifically inhibit an HIV-1 carrying a capsid mutation in the CypA binding loop (G89V), that human TRIM34 could exhibit weak antiretroviral activity against certain lentiviruses (HIV-2, SIV, and EIAV). Finally, we found that owl monkey TRIMCyp strongly restricts the AGM lentivirus SIVAGMTan.

Aside from the findings of novel restriction activities, the results presented herein do highlight some experimental difficulties in studying retrovirus restriction by TRIM proteins. In some cases, restriction was only evident when the TRIM proteins were ectopically expressed. For example, TRIM1 inhibition of N-MLV and TRIM34 inhibition of lentiviruses was observed when the TRIM proteins were expressed in MDTF or CRFK cells, but siRNA induced depletion of TRIM1 or TRIM34 in human HeLa or TE671 cells did not affect sensitivity to N-MLV or lentivirus infection. It is possible that TRIM34 is simply not expressed in the cell lines that were tested or is expressed at levels that are too low to recapitulate the modest inhibitory activity exhibited in the engineered TRIM1 or TRIM34-expressing CRFK cell lines. In addition, human cell lines clearly vary in level of TRIM5-attributable restriction that can be detected. For example, in TE671 cells, restriction of HIV-2, and particularly EIAV, can be convincingly demonstrated using siRNA based strategies, while in HeLa cells, TRIM5 knockdown had virtually no effect. Determining whether novel TRIM protein-based restriction activities have relevance in vivo will obviously, therefore, be complicated.

These limitations notwithstanding, we were able to show novel and specific antiretroviral activities among TRIM proteins. A surprising finding was that SIVAGMTan was very potently inhibited by owl monkey TRIMCyp. In this case, inhibition was clearly not due to TRIMCyp overexpression because SIVAGMTan was restricted in OMK cells in a CsA-reversible manner. This finding strongly suggests that the SIVAGMTan capsid has at least some affinity for CypA, which is unexpected. Indeed, previous work has shown that SIVAGM strains, albeit from different SIVAGM subspecies do not package CypA into virions (Braaten et al., 1996). The significance of this observation is difficult to determine, since lentiviruses appear to be absent from new world primates. However, the fact that a retrovirus other than HIV-1 is sensitive to owl monkey TRIMCyp restriction makes more plausible the notion that this unique restriction factor was evolutionarily selected in owl monkeys to confer resistance to some ancient retrovirus (or other virus) whose capsid exhibited CypA binding activity.

At this point, it is difficult to know the biological significance of weak antiretroviral activity that was observed in the context of some ectopically expressed human TRIM genes (e.g., TRIM1, TRIM34). However, it is possible that weakly restricting TRIM genes could influence and partly constrain the spectrum of sequences that are explored by retroviruses during natural infections. Alternatively, restriction activities that are weak or absent when entry is mediated by the VSV-G envelope protein used in these studies might be accentuated when incoming capsids are delivered to a particular location in the cell. Indeed, sensitivity to the restriction activity referred to as Lv2 is influenced by both envelope and capsid (Marchant et al., 2005; Reuter et al., 2005; Schmitz et al., 2004). Finally, it is reasonable to think that potent restriction activities were selected (by retroviral epidemics) from weak TRIM gene restriction activities that preexist in a given species. Indeed, TRIM5α likely arose as a result of duplication of genes at locus that also contains a weak restrictor, i.e., TRIM34. Sporadic occurrences of potent antiretroviral activity (e.g., TRIMCyp on SIVAGM Tan and HIV-1 and even rhesus TRIM5 on HIV-1) that are uncovered in the laboratory are probably the indirect and accidental consequence of distant evolutionary events. While these instances of strong restriction could recapitulate the characteristics of past virus–host interactions, it is also possible that reciprocal evolutionary pressure exerted on TRIM genes and retroviruses could be far more subtle and complex.
Materials and methods

Construction of a panel of human TRIM protein expression vectors

cDNAs encoding full-length versions of several members of TRIM protein family, including TRIM1, TRIM6, TRIM18, TRIM19, TRIM21, TRIM22, TRIM34, were amplified using PCR from human placenta cDNA (Clontech) using the primers corresponding to the 5′ and 3′ end of the coding sequences and appended with sequences encoding XhoI restriction site at 5′ end and Sall at 3′ end. The PCR products were subcloned into the retroviral expression vector pLNCX2 (Clontech) which was modified such that the expressed protein encoded an HA epitope tagged at its amino-terminus. Similar vectors, encoding HA-tagged, but truncated forms of each TRIM protein, that lacked the carboxy-terminal SPRY domain, were also constructed. In this case, the panel of full-length TRIM expression vectors described above were used as templates for PCR amplification using reverse primers targeted to the 3′ end of these corresponding coiled-coil domains (at amino acid residue 483 for TRIM1, residue 298 for TRIM5, residue 300 for TRIM6, residue 483 for TRIM18, residue 582 for TRIM19, residue 286 for TRIM21, residue 300 for TRIM22 and residue 300 for TRIM34) appended with a stop codon (TAG) and a Sall restriction site. The PCR products, after digestion with XhoI and NotI, were fused in-frame to a CypA-GFP cassette in pLNCX2/CypA-GFP, which was constructed as follows: A DNA fragment encompassing a 3′ fragment of Owl monkey TRIMCyp, comprising the CypA domain as well as the spacer peptide that naturally exists between the TRIM5 and CypA domains in Owl monkey TRIMCyp (Nisole et al., 2004; Sayah et al., 2004), was amplified using a 5′ primer that encoded a NotI site underlying three Ala residues in the spacer peptide and a 3′ CypA primer appended sequences derived from the 5′ end of GFP. This was linked to a GFP encoding fragment via PCR-mediated recombination so that the amplified CypA-GFP fusion insert included NotI and Sall restriction sites at 5′ and 3′ ends respectively. This DNA fragment was inserted into LNCX2 (Clontech) to generate pLNCX2/CypA-GFP. This plasmid was then used to generate the panel of TRIMCyp-GFP expression vectors, by inserting the various TRIM motifs lacking the SPRY domain described above.

Cell lines, viruses and vectors

Adherent cell lines from human (HeLa, TE671, 293T), mouse (Mus Dunni tail fibroblast, MDTF), cat (CRFK) and owl monkey (OMK) were grown in DMEM/10%FCS/antibiotics. To construct the panel of full-length TRIM expression vectors described above, were used as templates for PCR amplification using reverse primers targeted to the 3′ end of each coiled-coil domain, without appending a stop-codon, but instead appending sequences encoding a NotI restriction site. The PCR products, after digestion with XhoI and NotI, were fused in-frame to a CypA-GFP cassette in pLNCX2/CypA-GFP, which was constructed as follows: A DNA fragment encompassing a 3′ fragment of Owl monkey TRIMCyp, comprising the CypA domain as well as the spacer peptide that naturally exists between the TRIM5 and CypA domains in Owl monkey TRIMCyp (Nisole et al., 2004; Sayah et al., 2004), was amplified using a 5′ primer that encoded a NotI site underlying three Ala residues in the spacer peptide and a 3′ CypA primer appended sequences derived from the 5′ end of GFP. This was linked to a GFP encoding fragment via PCR-mediated recombination so that the amplified CypA-GFP fusion insert included NotI and Sall restriction sites at 5′ and 3′ ends respectively. This DNA fragment was inserted into LNCX2 (Clontech) to generate pLNCX2/CypA-GFP. This plasmid was then used to generate the panel of TRIMCyp-GFP expression vectors, by inserting the various TRIM motifs lacking the SPRY domain described above.

Control and TRIM protein-expressing cells were seeded in 24-well plates and inoculated with GFP reporter viruses (for MDTF, CRFK or Hela cells expressing full-length and truncated TRIM proteins) or DsRed reporter viruses (for MDTF cells expressing the Cyp-GFP-fused TRIM proteins) in the presence of 5 μg/ml polybrene. For some experiments, indicated in the text, cells were treated with 5 mM sodium butyrate 1 day prior to infection, to increase the expression of the transduced TRIMs proteins. Two days after inoculation with reporter viruses, FACS analysis was carried out using Guava EasyCyte instrument (Guava Technologies). For cells expressing the Cyp-GFP fused TRIM proteins that were challenged with DsRed reporter viruses, GFP-positive cells were gated and the percentage of DsRed+GFP+ as compared to the total GFP+ population was taken as the measure of sensitivity to infection.

RNAi

Human HeLa and TE671 cells were mock transfected or transfected with 60 pmol of each TRIM-specific RNA duplex (SMART pool Dharmacon) or a control firefly luciferase duplex using Oligofectamine (Invitrogen) according to manufacturers instructions and replated 24 h later. Forty-eight hours after transfection, cells were inoculated with GFP reporter viruses followed by FACS analysis 2 days later. In some experiments, where multiple siRNAs were mixed, 15 pmol of each TRIM-specific siRNA was used.

Western blot analysis

Cell suspensions were normalized for cell number and aliquots containing 5 × 10^3 cells were lysed directly in SDS sample buffer, separated on SDS-PAGE gels and transferred to
nitrocellulose membranes. Due to the very high expression level of full-length, truncated and CypA-GFP fused versions of TRIM19, extracts were diluted 5-fold such that only $1 \times 10^3$ cells were loaded per lane. The blots were probed with a mouse α-HA antibody (Covance Inc.) or α-GFP antibody (Roche) and a peroxidase conjugated secondary antibody and were developed with chemiluminescent detection reagents (Pierce).

Microscopy

MDTF cells expressing each of the TRIMCyp-GFP fusion proteins were fixed with paraformaldehyde, stained with DAPI and subjected to deconvolution microscopy using a Deltavision microscope (Applied Precision). Images were acquired and processed as previously described (Martin-Serrano et al., 2005).

Yeast two-hybrid assays

A bait plasmid expressing a GAL4-huTRIM5α fusion protein was generated by insertion of huTRIM5α coding sequences into pGBK7T (Clontech). Yeast Y190 cells were transformed with pGBK7T/huTRIM5α and pVP16/HA (Bogerd et al., 1993) derivatives expressing the various TRIM proteins. Interactions were measured using a β-galactosidase reporter assay, as previously described (Bogerd et al., 1993; Martin-Serrano et al., 2001).

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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.2006.05.035.

References


Braaten, D., Franke, E.K., Luban, J., 1996. Cyclophilin A is required for the replication of group M human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus SIV (CPZ)GAB but not group O HIV-1 or other primate immunodeficiency viruses. J. Virol. 70 (7), 4220–4227.


Song, B., Diaz-Griferio, F., Park do, H., Rogers, T., Stremlau, M., Sodroski, J., 2005a. TRIM5alpha association with cytoplasmic bodies is not required for antiretroviral activity. Virology 343 (2), 201–211.


