Published in final edited form as:

Virus Res. 2013 May ; 173(2): 306–314. doi:10.1016/j.virusres.2013.01.013.

A novel aminoacid determinant of HIV-1 restriction in the TRIM5 α variable 1 region isolated in a random mutagenic screen

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Abstract

Human-derived antiretroviral transgenes are of great biomedical interest and are actively pursued. HIV-1 is efficiently inhibited at post-entry, pre-integration replication stages by point mutations in the variable region 1 (v1) of the human restriction factor TRIM5 α . Here we use a mutated megaprimer approach to create a mutant library of TRIM5 α _{Hu} v1 and to isolate a mutation at Gly330 (G330E) that inhibits transduction of an HIV-1 vector as efficiently as the previously described mutants at positions Arg332 and Arg335. As was the case for these other mutations, modification of the local v1 charge toward increased acidity was key to inhibiting HIV-1. G330E TRIM5 α _{Hu} also disrupted replication-competent HIV-1 propagation in a human T cell line. Interestingly, G330E did not enhance restriction of HIV-1 when combined with mutations at Arg332 or Arg335. Accordingly, the triple mutant G330E-R332G-R335G bound purified recombinant HIV-1 capsid tubes less efficiently than the double mutant R332G-R335G did. In a structural model of the TRIM5 α _{Hu} PRYSPRY domain, the addition of G330E to the double mutant R332G-R335G caused extensive changes to the capsid-binding surface, which may explain why the triple mutant was no more restrictive than the double mutant. The HIV-1 inhibitory potential of Gly330 mutants was not predicted by examination of natural TRIM5 α orthologs that are known to strongly inhibit HIV-1. This work underlines the potential of random mutagenesis to isolate novel variants of human proteins with antiviral properties.

Keywords

HIV-1; TRIM5 α ; Restriction; Random mutagenesis; Genetic screen

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1. Introduction

It has long been recognized that treatment with type I IFN (IFN- α especially) efficiently inhibits HIV-1 propagation in vitro, suggesting an important role for innate immunity in the control of this infection (Chakrabarti and Simon, 2010). Several mediators of the anti-HIV-1 activity of type I IFNs in human cells have been discovered in the last 15 years. These proteins, sometimes called “restriction factors”, can inhibit the replication of retroviruses at various stages. All the restriction factors isolated so far are dominant and some are antagonized by HIV-1 gene products (Goffinet et al., 2009; Mangeat et al., 2009; Marin et al., 2003; Mehle et al., 2004). Some restriction factors show a high level of specificity and typically target non-cognate retroviruses more efficiently, implying an important role in the prevention of inter-species transmission (Cullen, 2006; Evans et al., 2010; Mariani et al., 2003).

TRIM5 α was isolated as a retroviral restriction factor in 2004 (Stremlau et al., 2004) and acts within the post-entry, pre-integration window (Owens et al., 2003; Perez-Caballero et al., 2005b). The viral molecular target of TRIM5 α is the correctly matured N-terminal domain of capsid (CA) proteins forming the outer surface of the retroviral core (Cowan et al., 2002; Forshey et al., 2005; Ikeda et al., 2004; Owens et al., 2003; Shi and Aiken, 2006; Stremlau et al., 2006). A direct interaction between the two proteins, each present as high molecular weight multimers, occurs shortly after entry and is required for downstream inhibition of viral replication (Kar et al., 2008; Langelier et al., 2008; Perez-Caballero et al., 2005a; Sebastian and Luban, 2005; Stremlau et al., 2006). The mechanism of TRIM5 α -mediated restriction can be broken down to discrete events, some of them inter-dependent: (i) virus entrapment into TRIM5 α cytoplasmic bodies (Campbell et al., 2008), (ii) decreased stability of the virus core (Black and Aiken, 2010; Perron et al., 2007; Stremlau et al., 2006; Zhao et al., 2011) (iii) targeting to a proteasome-dependent degradation pathway (Anderson et al., 2006; Diaz-Griffero et al., 2006; Lienlaf et al., 2011; Rold and Aiken, 2008), and (iv) inhibition of nuclear import (Anderson et al., 2006; Berthouix et al., 2004; Wu et al., 2006). The linear structure of TRIM5 α reveals a partition between effector domains at the N-terminus and a target recognition domain called PRYSPRY (also known as SPRY or B30.2) found at the C-terminus. The PRYSPRY domain contains evolutionarily variable regions that determine binding to CA and restriction potency (Diaz-Griffero et al., 2008; Ohkura et al., 2006; Sawyer et al., 2005; Song et al., 2005).

The human ortholog of TRIM5 α moderately restricts HIV-1 (~2-fold), even when over-expressed (Pham et al., 2010; Stremlau et al., 2004; Uchil et al., 2008), but many natural isolates of HIV-1 are more sensitive to TRIM5 α_{Hu} (Battivelli et al., 2011). Thus, stable transduction of HIV-1-targeting TRIM5 α has been proposed as a way to decrease the permissiveness of human cells to HIV-1 infection in medical applications (Rossi et al., 2007). Rhesus macaque TRIM5 α (TRIM5 α_{Rh}) over-expressed exogenously is dominant over its human endogenous counterpart and efficiently restricts HIV-1, including upon transduction into hematopoietic progenitor cells (Anderson and Akkina, 2005). Human/rhesus chimeric TRIM5 α , in which regions of the PRYSPRY domain originate from the rhesus ortholog, have also been used (Kambal et al., 2011). Other human/rhesus mapping experiments led to the discovery that mutations at Arg332 in the variable 1 (v1) region of

PRYSPRY conferred HIV-1 restriction activity (Li et al., 2006; Yap et al., 2005), although not to the level of TRIM5 α_{Rh} (Li et al., 2006; Pham et al., 2010). Based on the dual hypothesis that (i) TRIM5 α_{Rh} and other Old world primate TRIM5 α might be too immunogenic for human gene transfer, (ii) novel discrete mutations in TRIM5 α could increase its affinity for HIV-1 CA, we previously introduced a random mutagenic screen to isolate point mutants of TRIM5 α_{Hu} targeting HIV-1 (Pham et al., 2010). Using this approach, we found that several mutations abrogating the positive charge at position 335 of the PRYSPRY domain restricted HIV-1 in human cell lines. Here we describe a second, different mutagenic screen that led to the identification of additional HIV-1-restrictive mutants.

2. Material and methods

2.1. Plasmid DNAs

pMIP-TRIM5 α_{Hu} and pMIP-TRIM5 α_{Rh} express C-terminal FLAG-tagged versions of the corresponding proteins and have been extensively described before (Berthouet et al., 2005a,b; Bérubé et al., 2007; Pham et al., 2010; Sebastian et al., 2006). pNL-GFP, pMD-G, p R8.9, pTRIPCMV-GFP and pCL-Eco have all been described elsewhere (Berthouet et al., 2003, 2004, 2005a,b; Naviaux et al., 1996; Zufferey et al., 1997). The proviral clone pNL4-3 (Adachi et al., 1986) was used for propagation experiments.

2.2. Cells and retroviral vectors production

Human rhabdomyosarcoma TE671 cells, human embryonic kidney 293T cells and feline renal CRFK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at 37°C. Human T lymphoblast Sup-T1 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics at 37°C. All cell culture reagents were from Hyclone (Thermo Scientific, Logan, UT, USA). VSV G-pseudotyped HIV-1NL4-3 HIV-1 and MLV-based vectors were produced through transient transfection of 293T cells and collected as previously described (Bérubé et al., 2007; Pham et al., 2010). To produce HIV-1TRIP-CMV-GFP, cells were co-transfected with pTRIPCMV-GFP, p R8.9 and pMD-G. To produce MIP or MIP-TRIM5 α vectors, cells were co-transfected with the relevant pMIP vector, pCL-Eco and pMD-G.

2.3. Library construction and screening

The following oligodeoxynucleotide (ODN) named “38-doping” was ordered from Sigma-Genosys: 5' -aga caa gtg agc tct CCg AAa CCa CAg ATa ATa TAt GGg GCa CGa GGg ACa AGa TAc CAg ACa TTt GTg AAt ttc aat tat tgt act. This ODN was designed to introduce random mutations in the coding region for TRIM5 α_{Hu} (NCBI accession # NM 033034) residues Pro323 to Asn341. The manufacturer was instructed to introduce random mutations at nucleotides shown in capital letters in the sequence above. At each of these positions, 95% of the ODNs were requested to have the correct residue, while 5% would have any of the 3 other ones. Mutations were confined to the first and second residues of each codon in order to minimize the occurrence of silent mutations. Because 38 residues were targeted for mutagenesis, and assuming a 5% chance of being mutated for each, the

expected ODNs synthesized should bear 1.9 mutations on average. However, we found that TRIM5 α _{Hu} generated by this procedure had 3.2 mutations on average.

Sewing PCR was used to transfer the mutant library to a retroviral vector expressing TRIM5 α _{Hu}. For this, TRIM5 α _{Hu} upstream of the mutated region was amplified with primers TRIM5fwd (5'-GTTCTCGAGATGGCTTCTGGAATCCTGGTTAAT) and TRIM5963–940 (GCTCACTTGTCTCTTATCTTCAGA). The rest of TRIM5 α _{Hu} was amplified using the 38-doping ODN and TRIM5rev (5' - TCCTGAATTCTTACTTATCGTCGTCATCCTTGTAATC). Each PCR reaction was done in 1× High Fidelity (HF) buffer, contained 170 ng of matrix DNA, 20 units/ml of high fidelity Phusion enzyme from New England Biolabs (Pickering, ON), 0.2 mM of dNTP, 0.2 μM of each primer and proceeded for 25 cycles (98°C, 10 s; 56°C, 30 s; 72°C, 45 s). PCR products were agarose gel-purified using the QIAquick gel extraction kit. About 1/10 of the purified product from each PCR were combined and used to perform a second PCR reaction with ODNs TRIM5fwd and TRIM5rev. DNA was amplified for 25 cycles (98°C, 20 s; 56°C, 20 s; 72°C, 1 min) using the Phusion enzyme. The PCR product was purified using the QIAGEN QIAquick PCR purification kit, then digested with PshA1 and EcoR1. Digested DNA was again gel-purified and then ligated overnight to pMIP-TRIM5 α _{Hu} cut with the same enzymes. The ligation product was divided in 6 aliquots and then entirely transformed in *Escherichia coli* DH5 α bacteria by electroporation, yielding a 21,000-clone library.

MLV-based vectors carrying the mutant library were produced by transfection of 293T cells in a 10-cm plate with 10 μg of mutated pMIP-TRIM5 α _{Hu}, 10 μg of pCL-Eco and 5 μg of pMD-G. 100,000 TE671 cells in 6-well plates were infected with various amounts of the MLV-TRIM5 α _{Hu}, in triplicate. Two days later, cells were treated with 2 μg/ml of puromycin for 2–3 days, at which point we visually examined the cultures and discarded the ones for which transduction efficiency was higher than 15%. The remaining cells were pooled. Puromycin-resistant cells were subsequently challenged with a preparation of the GFP-expressing HIV-1 vector HIV-1TRIP-CMV-GFP at a high multiplicity of infection, exactly as before (Pham et al., 2010). GFP-negative cells were then sorted out by FACS, and 0.18% of intact cells were recovered. These cells were individually distributed into 2× 96-wells plates and the surviving clones were challenged again with HIV-1TRIP-CMV-GFP. Cell clones that expressed mutated versions of TRIM5 α _{Hu} and that were confirmed as being more than 2-times resistant to transduction by HIV-1TRIP-CMV-GFP were analyzed further.

2.4. Site-directed mutagenesis

The R332G, R335G and R332G–R335G mutations were described before (Pham et al., 2010). Other mutations were introduced in MIP-TRIM5 α _{Hu} by using the following primers through the same “sewing PCR” procedure as before (the modified codons are underlined):

K324Q_{fwd}, GAGCTCTCCGCAACCACAGATAATA;

K324Q_{rev}, ATCTGTGGTTGCGGAGAGCTCACTT;
 I328S_{fwd}, ACCACAGATATCATATATGGGGCACGAG;
 I328S_{rev}, TGCCCCATATGATATCTGTGGTTTCG;
 Y329C_{fwd}, CAGATAATATGTGGGGCACGAGGGA;
 Y329C_{rev}, CCTCGTGCCCCACATATTATCTGT;
 G330E_{fwd}, ATAATATATGAGGCACGAGGGACAA;
 G330E_{rev}, CCCTCGTGCCCTCATATATTATCTGT;
 G333V_{fwd}, GGGGCACGAGTGACAAGATAACCAGA;
 G333V_{rev}, GGTATCTTGTCACCTCGTGCCCCAT;
 Y336C_{fwd}, GGGACAAGATGCCAGACATTTGTGA;
 Y336C_{rev}, AAATGTCTGGCATCTTGTCCTCGT;
 Q337P_{fwd}, GGACAAGATACCCGACATTTGTGAA;
 Q337P_{rev}, TTCACAAATGTCGGGTATCTTGTC;
 T338P_{fwd}, CAAGATACCAGCCATTTGTGAATTTC;
 T338P_{rev}, TTCACAAATGGCTGGTATCTTGTC;
 G330E-R332G_{fwd}, CAGATAATATATGAGGCAGGAGGGACAAGATACC;
 G330E-R332G_{rev}, GGTATCTTGTCCTTCCTGCCTCATATATTA;
 G330E-R335G_{fwd}, ATAATATATGAGGCACGAGGGACAGGATACCAGAC;
 G330E-R335G_{rev}, GTCTGGTATCCTGTCCCTCGTGCCCTCATATATTA;
 G330E-R332G-R335G_{fwd}, ATAATATATGAGGCAGGAGGGACAGGATACCAGAC;
 G330E-R332G-R335G_{rev}, GTCTGGTATCCTGTCCCTTCCTGCCTCATATATTA.

TE671, CRFK and Sup-T1 cells stably expressing these TRIM5 α _{Hu} variants were generated by transduction with the MLV-based vector MIP-TRIM5 α _{Hu} followed by puromycin selection, exactly as described previously (Pham et al., 2010).

2.5. Viral challenges

Cells expressing various TRIM5 α orthologs or mutants were challenged with single doses of HIV-1TRIP-CMV-GFP or HIV-1NL-GFP as described previously (Pham et al., 2010). The viral dose used was typically set so that approximately 10% of control permissive cells are infected, thus allowing reliable quantification of infection when the magnitude of restriction is up to 100-fold. Two days post-infection, adherent cells were trypsinized and fixed in 2% formaldehyde-phosphate-buffered saline while Sup-T1 cells were PBS-washed and then treated with 2% formaldehyde. All cells were subjected to fluorescence-activated cell sorting analysis on an FC500 MPL instrument (Beckman Coulter, Mississauga, ON, USA) using the MXP/CXP software. On the basis of light scatter profiles, only intact cells were included in the analysis. GFP-positive cells were gated and counted as a percentage of total intact cells following analysis of 10,000–25,000 cells. For the propagation assay, 2×10^6 Sup-T1 cells were infected with 50 μ l of supernatant from 293T cells transfected with pNL4-3, containing approximately 10 ng of CAp24 as measured by ELISA. The next day, cells were washed to remove input virus and then kept in culture for 4 weeks. Cell-free supernatants were collected every 2 or 3 days for analysis. p24 (CA) concentrations in the supernatants were determined by a home-made ELISA assay using the 183 CA monoclonal antibody (Bounou et al., 2002).

2.6. HIV-1 CA–NC expression and purification

The HIV-1 capsid–nucleocapsid (CA–NC) protein was expressed, purified and assembled as previously described (Ganser-Pornillos et al., 2004; Ganser et al., 1999). Briefly, a pET11a-based vector (Novagen) expressing the CA–NC protein of HIV-1 was used to transform BL-21(DE3) *E. coli*. CA–NC expression was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) when the culture reached an optical density of 0.6 at 600 nm. After 4 h of induction, cells were harvested and resuspended in 20 mM Tris–HCl (pH 7.5), 1 μ M ZnCl₂, 10 mM 2-mercaptoethanol and protease inhibitors (Roche). Lysis was performed by sonication, and debris were pelleted for 30 min at 35,000 $\times$ g. Nucleic acids were precipitated by adding 0.11 equivalents of 2 M (NH₄)₂SO₄ and the same volume of 10% polyethylenimine and then pelleted by centrifugation at 29,500 $\times$ g for 15 min. Proteins were recovered by adding 0.35 equivalents of saturated (NH₄)₂SO₄ and centrifuging at 9820 $\times$ g for 15 min. Pellets were resuspended in 100 mM NaCl, 20 mM Tris–HCl (pH 7.5), 1 μ M ZnCl₂ and 10 mM 2-mercaptoethanol. Finally the CA–NC protein was dialyzed against 50 mM NaCl, 20 mM Tris–HCl (pH 7.5), 1 μ M ZnCl₂ and 10 mM 2-mercaptoethanol, and stored at –80°C.

2.7. In vitro assembly of HIV-1 CA–NC complexes and binding to TRIM5 α variants

HIV-1 CA–NC particles were assembled in vitro by diluting the CA–NC protein to a concentration of 0.3 mM in 50 mM Tris–HCl (pH 8.0), 0.5 M NaCl and 2 mg/ml DNA oligo-(TG)₅₀. The mixture was incubated at 4°C overnight and centrifuged at 8600 $\times$ g for 5 min. The pellet was resuspended in assembly buffer (50 mM Tris–HCl, pH 8.0, 0.5 M NaCl) at a final protein concentration of 0.15 mM and stored at 4°C until needed. 293T cells were transfected with plasmids expressing wild-type or mutant TRIM5 α proteins. 48 h after transfection, cell lysates were prepared as follows. Cells were PBS-washed and resuspended in capsid-binding buffer (10 mM Tris, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT). Cells were disrupted by freeze–thawing, and incubated on ice for 10 min. Lysates were centrifuged in a refrigerated microcentrifuge (~14,000 $\times$ g) for 5 min. Supernatants were supplemented with 1/10 volume of 10 $\times$ PBS and then used in the binding assay. In some cases, samples containing the TRIM5 α variants were diluted with extracts prepared in parallel from untransfected cells. To assay binding, 5 μ l of CA–NC particles assembled in vitro were incubated with 200 μ l of cell lysate at room temperature for 1 h. A portion of this mixture, referred to as “input” was stored. The mixture was spun through a 70% sucrose cushion (70% sucrose, 1 $\times$ PBS and 0.5 mM DTT) at 100,000 $\times$ g in an SW55 rotor (Beckman) for 1 h at 4°C. After centrifugation, the supernatant was carefully removed and the pellet was resuspended in 1 $\times$ SDS-PAGE loading buffer. Levels of TRIM5 α proteins were determined by Western blotting with an anti-FLAG antibody as described above. Levels of HIV-1 CA–NC protein in the pellet were assessed by Western blotting with an anti-p24 capsid antibody.

2.8. Structural models of the PRYSPRY domain

Prediction structures were generated using the web-based I-TASSER system (Roy et al., 2010; Zhang, 2008) with default parameters. Molecular graphics and analyses were performed with the UCSF Chimera 1.6.2 package (Pettersen et al., 2004).

3. Results

3.1. A v1-focused mutagenic screen

Previously published data pointed to v1 as the major region determining retroviral restriction specificity (Ohkura et al., 2006; Sawyer et al., 2005; Stremlau et al., 2005). We thus developed a novel mutagenic screen specifically targeting this domain. A megaprimer was used to introduce mutations at residues 323–341 of TRIM5 α _{Hu} (Fig. 1A). A library of more than 20,000 clones was generated, each carrying about 3 mutations on average. The library was retrovirally transduced in human TE671 cells and we applied the same functional screen that was used previously by us (Pham et al., 2010) and others (Stremlau et al., 2004) to isolate cells with decreased permissiveness to transduction by the GFP-encoding, VSV G-pseudotyped HIV-1 vector TRIP-CMV-GFP. Following two rounds of functional selection to retain only the clones that restricted HIV-1 by 2-fold or more compared to the control transduced with the “empty” vector, we recovered 5 cell lines in which GFP transduction by HIV-1TRIP-CMV-GFP was inhibited about 5-fold (Fig. 1B). Sequencing of the TRIM5 α _{Hu} expressed from the retroviral vector showed that 3 of them contained mutations at position 332 (Fig. 1B). Two of those were the R332G mutation that has been well-characterized by others (Li et al., 2006). These three clones were not analyzed any further as their restriction potential was entirely explained by Arg332 mutations.

Each of the 7 substitutions found in clones B3 and G4 was introduced in the parental retroviral vector expressing TRIM5 α _{Hu} by site-directed mutagenesis and then transduced into TE671 cells. Permissiveness to GFP transduction by HIV-1TRIP-CMV-GFP was then analyzed for each of the mutants. Only the mutation G330E of G4 conferred a strong level of HIV-1 resistance, close to 10-fold (Fig. 1C). The fact that G330E was more restrictive than the parental clone G4 was probably due to the presence of mitigating mutations in clone G4, such as Y329C. This mutation alone abolishes the slight level of restriction (2-fold) conferred by over-expression of the wild-type (WT) TRIM5 α _{Hu}, and thus it is likely that it would also decrease restriction when combined with G330E, although we did not test this. Thus, HIV-1 restriction in TE671-B3 cells was due to a combination effect involving 2 or 3 of the substitutions found in this clone, while restriction in TE671-G4 cells was explained by mutation G330E. In this preliminary analysis, G330E was found to be as efficient at inhibiting HIV-1 as previously described mutation R332G. However, it was approximately 2-times less restrictive than the double mutation R332G–R335G and 10-times less restrictive than the Rhesus macaque ortholog (Fig. 1C).

3.2. Effect of alternative mutations at Gly330

Previous mutations modulating the capacity of TRIM5 α _{Hu} to inhibit restriction of HIV-1 affected the charge of the protein. Removal of the positive charge conferred by Arg332 or Arg335 was, indeed, key to HIV-1 restriction (Li et al., 2006; Pham et al., 2010). The negatively charged glutamic acid brought by the G330E mutation likewise affects the protein charge. In order to test whether restriction was primarily due to the removal of the glycine or to the introduction of negatively charged residue, we tested the restriction potential of other substitutions at position Gly330. We found that introduction of an aspartic acid significantly (3- to 4-fold) inhibited HIV-1 replication, while G330A or G330S had

little or no effect (Fig. 2). These data suggest that introducing a negative charge at that position was important to inhibit HIV-1. However, G330D was not as restrictive as G330E, and only the latter mutant was retained for further investigation.

3.3. Combining G330E with mutations at Arg332 or Arg335

Previously, we found that R332G and R335G had additive effects on HIV-1 replication (Pham et al., 2010). Thus, we aimed at analyzing whether the inhibition of HIV-1 conferred by G330E was, similarly, additive to that of R332G, R335G, or the double mutant R332G–R335G. As shown in Fig. 3, however, any combination including G330E was no more restrictive, and even slightly less restrictive, than the relevant controls lacking it. Therefore, G330E has a mitigating effect on HIV-1 restriction stemming from removing positive charges in the v1 region.

3.4. In vitro binding analysis

Mutations at Arg332 of TRIM5 α_{Hu} have been demonstrated to promote HIV-1 restriction through increased binding of TRIM5 α_{Hu} to its molecular target, the CA protein of HIV-1 (Li et al., 2006). Presumably, mutations at positions 330 and 335 would also increase CA recognition, but this had not been demonstrated for Arg335 mutations. We analyzed the potential of TRIM5 α_{Hu} mutants to bind in vitro assembled HIV-1 capsid–nucleocapsid (CA–NC) complexes, as described before (Diaz-Griffero et al., 2009). TRIM5 α_{Rh} and both R332G–R335G and G330E–R332G–R335G mutants of TRIM5 α_{Hu} clearly bound in vitro assembled CA–NC complexes, and the strength of the interaction roughly correlated with the magnitude of restriction (Fig. 4). G330E–R332G–R335G was less efficient than R332G–R335G at binding in vitro assembled HIV-1 CA–NC complexes, supporting the finding that the G330E mutation does not increase restriction when it is combined with Arg332 and Arg335 mutations. Single mutants G330E and R335G were not observed to significantly bind in vitro assembled HIV-1 CA–NC complexes in these experimental conditions, but this may be simply due to a lack of sensitivity of our assay.

3.5. Restriction in other viral and cellular environments

T lymphocytes are the main host cells in natural HIV-1 infection. To analyze whether G330E and other mutants would inhibit HIV-1 when stably expressed in lymphocytes, Sup-T1 cells were transduced with the MIP-TRIM5 α vectors. Like before, untransduced cells were eliminated by antibiotic treatment and cells were then challenged with a single dose of HIV-1NL-GFP. This HIV-1 vector is similar to HIV-1TRIP-CMV-GFP in that it is derived from a subtype B HIV-1 strain and it does not encode a viral envelope. However, HIV-1NL-GFP resembles wild-type HIV-1 since all viral proteins except for Nef and Env are expressed following infection. As shown in Fig. 5A, the infectivity data obtained closely mirrored those generated with HIV-1TRIP-CMV-GFP in TE671 cells. Although the overall level of restriction was smaller than in TE671 cells, as observed before (Pham et al., 2010), it is worth noting that expression of either R332G–R335G or G330E–R332G–R335G in Sup-T1 cells inhibited HIV-1 almost as efficiently as TRIM5 α_{Rh} . Like before, G330E inhibited HIV-1 as efficiently as mutations in Arg332 or Arg335, but G330E–R332G–R335G was not more efficient than R332G–R335G.

Human cells express low levels of endogenous TRIM5 α that do not significantly affect HIV-1 replication, and over-expressed transgenic TRIM5 α is known to be dominant over its endogenous counterpart (Berthoux et al., 2005a). However, it was still possible that some of the mutants constructed might behave differently in the absence of endogenous TRIM5 α . To test this directly, we transduced feline cells, which do not express any TRIM5, with the same TRIM5 α orthologs and mutants that had been tested in human cells. These cells were then challenged with HIV-1NL-GFP and the percentages of infected cells were determined. We found that the restriction pattern in feline cells (Fig. 5B) closely resembled what had been obtained in human TE671 and Sup-T1 cells (Fig. 5A). Specifically, G330E restricts HIV-1 in these cells about as efficiently as R332G and R335G do (about 10-fold compared with the empty vector control), and G330E does not potentiate HIV-1 restriction by the R332G–R335G double mutant.

3.6. Restriction of a propagating infection

Experiments using VSV G-pseudotyped HIV-1 vectors may not always faithfully recapitulate TRIM5 α -mediated restriction of WT viruses. Indeed, TRIM5 α may also interfere with late (assembly) steps of the retroviral cycle (Sakuma et al., 2007) although this conclusion has been rebutted by others (Zhang et al., 2008). In addition, HIV-1 propagation occurs mostly through so-called viral synapses forming between cells, rather than by transmission of cell-free viruses (Groot et al., 2008; Haller and Fackler, 2008; Rudnicka et al., 2009). It is unclear whether TRIM5 α restricts HIV-1 originating from viral synapses the same way it would restrict infection by cell-free viruses, but there are some indications that it does not (Richardson et al., 2008). In order to test whether G330E TRIM5 α_{Hu} would efficiently disrupt the spread of HIV-1 in susceptible cells, we infected Sup-T1 cells expressing WT, G330E or R332G–R335G TRIM5 α_{Hu} with HIV-1NL4-3 (Fig. 6A). Over-expression of WT TRIM5 α_{Hu} slightly delayed the peak of replication from ~5 to ~7 days post infection, an effect seen before (Pham et al., 2010). Spreading of HIV-1 in cells expressing G330E or R332G–R335G TRIM5 α_{Hu} was apparent but no clear peak was visible during the course of the experiment. Indeed, while the p24 concentration in the supernatants of these two cultures was between 10 and 100 pg/ml from day 5 to day 16 of the propagation assay, these concentrations were more than 10,000-times smaller than those measured at the peak of replication in control cells and in cells over-expressing WT TRIM5 α_{Hu} . Yet, viral replication did occur in these cells, as evidenced by the presence of syncytia (Fig. 6B) followed by widespread cell death (not shown). Therefore, transduction of G330E or R332G–R335G TRIM5 α_{Hu} is able to efficiently attenuate the spread of HIV-1 in susceptible cells, without completely disrupting it. That HIV-1 was roughly equally inhibited in G330E and R332G–R335G cells, despite the fact that the latter is more efficient at inhibiting HIV-1 single-cycle vector transduction, indicates that restriction of vector transduction may not always be a faithful model for the restriction of HIV-1 propagation.

3.7. Modeling the effect of v1 mutations on the TRIM5 α_{Hu} PRYSPRY domain

The 4 hyper-variable regions of TRIM5 α are present at the surface of the protein and constitute the binding interface for interactions with retroviral capsids, as has been confirmed recently (Biris et al., 2012). We used an *in silico* assay to predict the impact of mutations at positions 330, 332 and 335 on this CA-binding interface (Fig. 7). The I-

TASSER program integrated more than 10 published structures of domains similar to the WT TRIM5 α _{Hu} PRYSPRY domain. Among those was the recently published structure of the Rhesus macaque TRIM5 α PRYSPRY (Biris et al., 2012). According to this prediction tool, introduction of the R332G and R335G mutations caused significant changes in all 3 main variable regions (v1, v2, v3). This was most apparent in the surface representation (Fig. 7B). Interestingly, the triple mutant (G330E-R332G-R335G) had a surface structure that was very different from that of the R332G-R335G mutant and, in fact, more closely resembled that of the WT protein. These observations help explain why G330E-R332G-R335G was slightly less restrictive than the 332-335 double mutant.

4. Discussion

Our data reveal that a novel mutation in the v1 region of the human TRIM5 α results in a 10-fold decrease in permissiveness to HIV-1 vector transduction, similar to what was previously achieved by other point mutations in this region (Li et al., 2006; Pham et al., 2010; Yap et al., 2005). This mutation, G330E, was isolated using a degenerated oligonucleotide, a technique which to our knowledge had never been applied to generate anti-HIV-1 restriction factors. The change in charge associated with the substitution is crucial to G330E inhibitory activity, as was previously found to be the case for other mutants in this region (Li et al., 2006; Pham et al., 2010). Interestingly, the inhibitory effect of G330E is not additive to that of R332G or R335G, while R332G and R335G do have additive effects. In fact, G330E slightly decreased restriction stemming from Arg332 and/or Arg335 mutations and seemed to decrease binding of R332G-R335G to in vitro assembled HIV-1 CA-NC complexes. The molecular interactions between TRIM5 α and its molecular target, the N-terminal region of CA, have not been elucidated at the structural level despite some recent advances (Biris et al., 2012; Ganser-Pornillos et al., 2011). Future progress in that field is a prerequisite to the full understanding of phenotypes associated with mutations in the v1 domain of TRIM5 α _{Hu}. However, our structure prediction analysis suggests that discrete mutations in v1 can have long-range effects on the whole CA-interaction surface of the PRYSPRY domain. This explains how single mutations in TRIM5 α _{Hu} v1 can modulate the affinity of TRIM5 binding to CA even though this binding depends on multiple weak interactions involving all the variable regions (Biris et al., 2012; Ohkura et al., 2006).

Neither E330 nor D330 are found in TRIM5 α of primates that do or do not restrict HIV-1 (Song et al., 2005); therefore, the mutation found in our screen could not have been predicted from the available literature, an indication of the usefulness of random mutagenesis approaches. However, TRIM5 α from Rhesus macaques and from African green monkeys, two orthologs that strongly restrict HIV-1, bear the following residues at the corresponding positions: Q330, P332, L335. Because this particular combination is different from what we have been testing, it would be valuable to test all the possible combinations (Q330-G332-G335, E330-P332-L335, etc.) in order to isolate (i) the most potent anti-HIV-1 TRIM5 α _{Hu} variants and (ii) the least likely to allow HIV-1 escape. In addition, our spreading infection experiment suggests that assays with fully replicative viruses cannot be circumvented in the search for the most efficient HIV-1 restriction factors. HIV-1 vector transduction assays are convenient and informative but do not provide definitive predictions of antiviral activity.

5. Conclusions

An innovative random mutagenesis screen has led to the isolation of a novel determinant of HIV-1 restriction by TRIM5 α_{Hu} with potential applications in gene therapy and genome editing.

Acknowledgements

We thank Michel J. Tremblay and Réjean Cantin for sharing their BSL3 facility and the p24 ELISA assay. This work was supported by the Canadian Institutes for Health Research and the Canada Research Chairs program (L.B.). M.V. was supported by a FRQS Master's Training Award. Work in F.D.-G.'s laboratory was supported by NIH R01 AI087390 to F.D.-G. and a K99/R00 Pathway to Independence Award from the National Institutes of Health.

References

- Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA. Production of acquired immunodeficiency syndrome-associated retro-virus in human and nonhuman cells transfected with an infectious molecular clone. *Journal of Virology*. 1986; 59(2):284–291. [PubMed: 3016298]
- Anderson J, Akkina R. TRIM5 α (rh) expression restricts HIV-1 infection in lentiviral vector-transduced CD34(+)-cell-derived macrophages. *Molecular Therapy*. 2005; 12(4):687–696. [PubMed: 16081321]
- Anderson JL, Campbell EM, Wu X, Vandegraaff N, Engelman A, Hope TJ. Proteasome inhibition reveals that a functional preintegration complex intermediate can be generated during restriction by diverse TRIM5 proteins. *Journal of Virology*. 2006; 80(19):9754–9760. [PubMed: 16973579]
- Battivelli E, Migraine J, Lecossier D, Yeni P, Clavel F, Hance AJ. Gag cytotoxic T lymphocyte escape mutations can increase sensitivity of HIV-1 to human TRIM5 α , linking intrinsic and acquired immunity. *Journal of Virology*. 2011; 85(22):11846–11854. [PubMed: 21917976]
- Berthoux L, Sebastian S, Sayah DM, Luban J. Disruption of human TRIM5 α antiviral activity by nonhuman primate orthologs. *Journal of Virology*. 2005a; 79(12):7883–7888. [PubMed: 15919943]
- Berthoux L, Sebastian S, Sokolskaja E, Luban J. Lv1 inhibition of human immunodeficiency virus type 1 is counteracted by factors that stimulate synthesis or nuclear translocation of viral cDNA. *Journal of Virology*. 2004; 78(21):11739–11750. [PubMed: 15479815]
- Berthoux L, Sebastian S, Sokolskaja E, Luban J. Cyclophilin A is required for TRIM5{ α }-mediated resistance to HIV-1 in Old World monkey cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2005b; 102(41):14849–14853. [PubMed: 16203999]
- Berthoux L, Towers GJ, Gurer C, Salomoni P, Pandolfi PP, Luban J. As(2)O(3) enhances retroviral reverse transcription and counteracts Ref1 antiviral activity. *Journal of Virology*. 2003; 77(5):3167–3180. [PubMed: 12584341]
- Bérubé J, Bouchard A, Berthoux L. Both TRIM5 α and TRIMCyp have only weak antiviral activity in canine D17 cells. *Retrovirology*. 2007; 4:68. [PubMed: 17892575]
- Biris N, Yang Y, Taylor AB, Tomashevski A, Guo M, Hart PJ, Diaz-Griffero F, Ivanov DN. Structure of the rhesus monkey TRIM5 α PRYSPRY domain, the HIV capsid recognition module. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109(33):13278–13283. [PubMed: 22847415]
- Black LR, Aiken C. TRIM5 α disrupts the structure of assembled HIV-1 capsid complexes in vitro. *Journal of Virology*. 2010; 84(13):6564–6569. [PubMed: 20410272]
- Bounou S, Leclerc JE, Tremblay MJ. Presence of host ICAM-1 in laboratory and clinical strains of human immunodeficiency virus type 1 increases virus infectivity and CD4(+)-T-cell depletion in human lymphoid tissue, a major site of replication in vivo. *Journal of Virology*. 2002; 76(3):1004–1014. [PubMed: 11773376]

- Campbell EM, Perez O, Anderson JL, Hope TJ. Visualization of a proteasome-independent intermediate during restriction of HIV-1 by rhesus TRIM5 α . *Journal of Cell Biology*. 2008; 180(3):549–561. [PubMed: 18250195]
- Chakrabarti LA, Simon V. Immune mechanisms of HIV control. *Current Opinion in Immunology*. 2010; 22(4):488–496. [PubMed: 20650621]
- Cowan S, Hatzioannou T, Cunningham T, Muesing MA, Gottlinger HG, Bieniasz PD. Cellular inhibitors with Fv1-like activity restrict human and simian immunodeficiency virus tropism. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99(18):11914–11919. [PubMed: 12154227]
- Cullen BR. Role and mechanism of action of the APOBEC3 family of antiretroviral resistance factors. *Journal of Virology*. 2006; 80(3):1067–1076. [PubMed: 16414984]
- Diaz-Griffero F, Li X, Javanbakht H, Song B, Welikala S, Stremlau M, Sodroski J. Rapid turnover and polyubiquitylation of the retroviral restriction factor TRIM5. *Virology*. 2006; 349(2):300–315. [PubMed: 16472833]
- Diaz-Griffero F, Perron M, McGee-Estrada K, Hanna R, Maillard PV, Trono D, Sodroski J. A human TRIM5 α B30.2/SPRY domain mutant gains the ability to restrict and prematurely uncoat B-tropic murine leukemia virus. *Virology*. 2008; 378(2):233–242. [PubMed: 18586294]
- Diaz-Griffero F, Qin XR, Hayashi F, Kigawa T, Finzi A, Sarnak Z, Lienlaf M, Yokoyama S, Sodroski J. A B-box 2 surface patch important for TRIM5 α self-association, capsid binding avidity, and retrovirus restriction. *Journal of Virology*. 2009; 83(20):10737–10751. [PubMed: 19656869]
- Evans DT, Serra-Moreno R, Singh RK, Guatelli JC. BST-2/tetherin: a new component of the innate immune response to enveloped viruses. *Trends in Microbiology*. 2010; 18(9):388–396. [PubMed: 20688520]
- Forshey BM, Shi J, Aiken C. Structural requirements for recognition of the human immunodeficiency virus type 1 core during host restriction in owl monkey cells. *Journal of Virology*. 2005; 79(2):869–875. [PubMed: 15613315]
- Ganser-Pornillos BK, Chandrasekaran V, Pornillos O, Sodroski JG, Sundquist WI, Yeager M. Hexagonal assembly of a restricting TRIM5 α protein. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108(2):534–539. [PubMed: 21187419]
- Ganser-Pornillos BK, von Schwedler UK, Stray KM, Aiken C, Sundquist WI. Assembly properties of the human immunodeficiency virus type 1 CA protein. *Journal of Virology*. 2004; 78(5):2545–2552. [PubMed: 14963157]
- Ganser BK, Li S, Klishko VY, Finch JT, Sundquist WI. Assembly and analysis of conical models for the HIV-1 core. *Science*. 1999; 283(5398):80–83. [PubMed: 9872746]
- Goffinet C, Allespach I, Homann S, Tervo HM, Habermann A, Rupp D, Oberbremer L, Kern C, Tibroni N, Welsch S, Krijnse-Locker J, Banting G, Krausslich HG, Fackler OT, Keppler OT. HIV-1 antagonism of CD317 is species specific and involves Vpu-mediated proteasomal degradation of the restriction factor. *Cell Host Microbe*. 2009; 5(3):285–297. [PubMed: 19286137]
- Groot F, Welsch S, Sattentau QJ. Efficient HIV-1 transmission from macrophages to T cells across transient virological synapses. *Blood*. 2008; 111(9):4660–4663. [PubMed: 18296630]
- Haller C, Fackler OT. HIV-1 at the immunological and T-lymphocytic viro-logical synapse. *Biological Chemistry*. 2008; 389(10):1253–1260. [PubMed: 18713012]
- Ikeda Y, Ylinen LM, Kahar-Bador M, Towers GJ. Influence of gag on human immunodeficiency virus type 1 species-specific tropism. *Journal of Virology*. 2004; 78(21):11816–11822. [PubMed: 15479823]
- Kambal A, Mitchell G, Cary W, Gruenloh W, Jung Y, Kalomoiris S, Nacey C, McGee J, Lindsey M, Fury B, Bauer G, Nolta JA, Anderson JS. Generation of HIV-1 resistant and functional macrophages from hematopoietic stem cell-derived induced pluripotent stem cells. *Molecular Therapy*. 2011; 19(3):584–593. [PubMed: 21119622]
- Kar AK, Diaz-Griffero F, Li Y, Li X, Sodroski J. Biochemical and biophysical characterization of a chimeric TRIM21-TRIM5 α protein. *Journal of Virology*. 2008; 82(23):11669–11681. [PubMed: 18799572]
- Langelier CR, Sandrin V, Eckert DM, Christensen DE, Chandrasekaran V, Alam SL, Aiken C, Olsen JC, Kar AK, Sodroski JG, Sundquist WI. Biochemical characterization of a recombinant

- TRIM5alpha protein that restricts human immunodeficiency virus type 1 replication. *Journal of Virology*. 2008; 82(23):11682–11694. [PubMed: 18799573]
- Li Y, Li X, Stremlau M, Lee M, Sodroski J. Removal of arginine 332 allows human TRIM5alpha to bind human immunodeficiency virus capsids and to restrict infection. *Journal of Virology*. 2006; 80(14):6738–6744. [PubMed: 16809279]
- Lienlaf M, Hayashi F, Di Nunzio F, Tochio N, Kigawa T, Yokoyama S, Diaz-Griffero F. Contribution of E3-ubiquitin ligase activity to HIV-1 restriction by TRIM5alpha(rh): structure of the RING domain of TRIM5alpha. *Journal of Virology*. 2011; 85(17):8725–8737. [PubMed: 21734049]
- Mangeat B, Gers-Huber G, Lehmann M, Zufferey M, Luban J, Piguet V. HIV-1 Vpu neutralizes the antiviral factor Tetherin/BST-2 by binding it and directing its beta-TrCP2-dependent degradation. *PLoS Pathogens*. 2009; 5(9):e1000574. [PubMed: 19730691]
- Mariani R, Chen D, Schrofelbauer B, Navarro F, Konig R, Bollman B, Munk C, Nymark-McMahon H, Landau NR. Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell*. 2003; 114(1):21–31. [PubMed: 12859895]
- Marin M, Rose KM, Kozak SL, Kabat D. HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation. *Nature Medicine*. 2003; 9(11):1398–1403.
- Mehle A, Strack B, Ancuta P, Zhang C, McPike M, Gabuzda D. Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin–proteasome pathway. *Journal of Biological Chemistry*. 2004; 279(9):7792–7798. [PubMed: 14672928]
- Naviaux RK, Costanzi E, Haas M, Verma IM. The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *Journal of Virology*. 1996; 70(8):5701–5705. [PubMed: 8764092]
- Ohkura S, Yap MW, Sheldon T, Stoye JP. All three variable regions of the TRIM5alpha B30.2 domain can contribute to the specificity of retrovirus restriction. *Journal of Virology*. 2006; 80(17):8554–8565. [PubMed: 16912305]
- Owens CM, Yang PC, Gottlinger H, Sodroski J. Human and simian immunodeficiency virus capsid proteins are major viral determinants of early, postentry replication blocks in simian cells. *Journal of Virology*. 2003; 77(1):726–731. [PubMed: 12477877]
- Perez-Caballero D, Hatzioannou T, Yang A, Cowan S, Bieniasz PD. Human tripartite motif 5alpha domains responsible for retrovirus restriction activity and specificity. *Journal of Virology*. 2005a; 79(14):8969–8978. [PubMed: 15994791]
- Perez-Caballero D, Hatzioannou T, Zhang F, Cowan S, Bieniasz PD. Restriction of human immunodeficiency virus type 1 by TRIM-CypA occurs with rapid kinetics and independently of cytoplasmic bodies, ubiquitin, and proteasome activity. *Journal of Virology*. 2005b; 79(24):15567–15572. [PubMed: 16306627]
- Perron MJ, Stremlau M, Lee M, Javanbakht H, Song B, Sodroski J. The human TRIM5alpha restriction factor mediates accelerated uncoating of the N-tropic murine leukemia virus capsid. *Journal of Virology*. 2007; 81(5):2138–2148. [PubMed: 17135314]
- Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera—a visualization system for exploratory research and analysis. *Journal of Computational Chemistry*. 2004; 25(13):1605–1612. [PubMed: 15264254]
- Pham QT, Bouchard A, Grutter MG, Berthoux L. Generation of human TRIM5alpha mutants with high HIV-1 restriction activity. *Gene Therapy*. 2010; 17(7):859–871. [PubMed: 20357830]
- Richardson MW, Carroll RG, Stremlau M, Korokhov N, Humeau LM, Silvestri G, Sodroski J, Riley JL. Mode of transmission affects the sensitivity of human immunodeficiency virus type 1 to restriction by rhesus TRIM5alpha. *Journal of Virology*. 2008; 82(22):11117–11128. [PubMed: 18768965]
- Rold CJ, Aiken C. Proteasomal degradation of TRIM5alpha during retrovirus restriction. *PLoS Pathogens*. 2008; 4(5):e1000074. [PubMed: 18497858]
- Rossi JJ, June CH, Kohn DB. Genetic therapies against HIV. *Nature Biotechnology*. 2007; 25(12):1444–1454.
- Roy A, Kucukural A, Zhang Y. I-TASSER: a unified platform for automated protein structure and function prediction. *Nature Protocols*. 2010; 5(4):725–738.

- Rudnicka D, Feldmann J, Porrot F, Wietgreffe S, Guadagnini S, Prevost MC, Estaquier J, Haase AT, Sol-Foulon N, Schwartz O. Simultaneous cell-to-cell transmission of human immunodeficiency virus to multiple targets through polysynapses. *Journal of Virology*. 2009; 83(12):6234–6246. [PubMed: 19369333]
- Sakuma R, Noser JA, Ohmine S, Ikeda Y. Rhesus monkey TRIM5alpha restricts HIV-1 production through rapid degradation of viral Gag polyproteins. *Nature Medicine*. 2007; 13(5):631–635.
- Sawyer SL, Wu LI, Emerman M, Malik HS. Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102(8):2832–2837. [PubMed: 15689398]
- Sebastian S, Luban J. TRIM5alpha selectively binds a restriction-sensitive retroviral capsid. *Retrovirology*. 2005; 2:40. [PubMed: 15967037]
- Sebastian S, Sokolskaja E, Luban J. Arsenic counteracts human immunodeficiency virus type 1 restriction by various TRIM5 orthologs in a cell type-dependent manner. *Journal of Virology*. 2006; 80(4):2051–2054. [PubMed: 16439561]
- Shi J, Aiken C. Saturation of TRIM5 alpha-mediated restriction of HIV-1 infection depends on the stability of the incoming viral capsid. *Virology*. 2006; 350(2):493–500. [PubMed: 16624363]
- Song B, Gold B, O'Huigin C, Javanbakht H, Li X, Stremlau M, Winkler C, Dean M, Sodroski J. The B30.2(SPRY) domain of the retroviral restriction factor TRIM5alpha exhibits lineage-specific length and sequence variation in primates. *Journal of Virology*. 2005; 79(10):6111–6121. [PubMed: 15857996]
- Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature*. 2004; 427(6977):848–853. [PubMed: 14985764]
- Stremlau M, Perron M, Lee M, Li Y, Song B, Javanbakht H, Diaz-Griffero F, Anderson DJ, Sundquist WI, Sodroski J. Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103(14):5514–5519. [PubMed: 16540544]
- Stremlau M, Perron M, Welikala S, Sodroski J. Species-specific variation in the B30.2(SPRY) domain of TRIM5alpha determines the potency of human immunodeficiency virus restriction. *Journal of Virology*. 2005; 79(5):3139–3145. [PubMed: 15709033]
- Uchil PD, Quinlan BD, Chan WT, Luna JM, Mothes W. TRIM E3 ligases interfere with early and late stages of the retroviral life cycle. *PLoS Pathogens*. 2008; 4(2):e16. [PubMed: 18248090]
- Wu X, Anderson JL, Campbell EM, Joseph AM, Hope TJ. Proteasome inhibitors uncouple rhesus TRIM5alpha restriction of HIV-1 reverse transcription and infection. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103(19):7465–7470. [PubMed: 16648264]
- Yap MW, Nisole S, Stoye JP. A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction. *Current Biology*. 2005; 15(1):73–78. [PubMed: 15649369]
- Zhang F, Perez-Caballero D, Hatzioannou T, Bieniasz PD. No effect of endogenous TRIM5alpha on HIV-1 production. *Nature Medicine*. 2008; 14(3):235–236. (author reply 236–8).
- Zhang Y. I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics*. 2008; 9:40. [PubMed: 18215316]
- Zhao G, Ke D, Vu T, Ahn J, Shah VB, Yang R, Aiken C, Charlton LM, Gronen-born AM, Zhang P. Rhesus TRIM5alpha disrupts the HIV-1 capsid at the inter-hexamer interfaces. *PLoS Pathogens*. 2011; 7(3):e1002009. [PubMed: 21455494]
- Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nature Biotechnology*. 1997; 15(9):871–875.

Fig. 1. Isolation of amino acid 330 of TRIM5 α _{Hu} as a determinant of HIV-1 restriction. (A) A megaprimer approach to introduce mutations in the v1 region of TRIM5 α _{Hu}. The nucleotide sequence of the region targeted by the primer is shown in the box under the corresponding amino acid sequence. Partly degenerated primer synthesis was used to introduce random mutations at the 38 nucleotide positions shown in capital letters, and the mix of mutated megaprimers were cloned into the retroviral vector construct pMIP-TRIM5 α _{Hu} by sewing PCR, and then transduced into TE671 cells. (B) HIV-1 restriction potential of 5 TE671 clones isolated in a functional screen. Presumed HIV-1-resistant cell clones were grown and tested for HIV-1 permissiveness. The left panel shows percentages of infected (GFP-positive) cells two days after challenging the 5 clones with a single dose of HIVTRIP-CMV-GFP. Control cells transduced with the empty MIP vector or cells transduced with the weakly restrictive wild-type TRIM5 α _{Hu} or with restrictive TRIM5 α _{Rh} were included. The right panel lists mutations found in the TRIM5 α _{Hu} variants expressed by each of the TE671 cell clones isolated. (C) HIV-1 restriction potential of individual point mutations found in cell clones B3 and G4. Mutations were introduced into pMIP-TRIM5 α _{Hu} and TE671 cells transduced with the constructed vectors were challenged with HIVTRIP-CMV-GFP as above. Previously characterized restrictive TRIM5 α _{Hu} mutants R332G and R332G-R335G were included in the assay.

Fig. 2.

Restriction of HIV-1 by TRIM5 α_{Hu} mutants of Gly330. TE671 cells were transduced to express the indicated TRIM5 α and then challenged with a single dose of HIVTRIP-CMV-GFP exactly as in Fig. 1. The percentages of infected cells were determined by FACS 2 days later. Shown are average values from triplicate infections with standard deviations.

Fig. 3.

Antiretroviral effect conferred by G330E when combined with R332G or R335G. The indicated single, double or triple TRIM5 α _{Hu} mutants were constructed and retrovirally transduced into TE671 cells. The cell lines created were then challenged with HIVTRIP-CMV-GFP as before. Bars show averages of percent infected cells after single-dose infections in triplicates, with standard deviations.

Fig. 4.

In vitro TRIM5α binding to HIV-1 CA. CA-NC complexes were assembled in vitro and mixed with lysates from 293T cells transfected with the relevant TRIM5α-FLAG-expressing plasmid constructs. CA-NC complexes were separated from soluble proteins by ultracentrifugation through a sucrose cushion and analyzed by Western blotting using CA and FLAG antibodies (pellet). A fraction of the pre-centrifugation mix was analyzed by Western blot for CA content (input). The graph on the right shows relative CA-bound TRIM5α as determined by the ratio between pellet-associated and total (input) TRIM5α, relative to that obtained for TRIM5α_{Rh}. Bars represent the average of 3 experiments with standard deviations.

Fig. 5.

Restriction of HIV-1 replication in human lymphocytic cells and in nonhuman cells. Human Sup-T1 cells (A) and feline fibroblast CRFK cells (B) were retroviral transduced with the “empty” MIP vector or with MIP expressing the indicated TRIM5α. The cells were then challenged in triplicates with a single dose of HIV-1NL-GFP. Percentages of infected (GFP-positive) cells were determined 2 days later by FACS.

Fig. 6.

HIV-1 propagation in human lymphocytic cells expressing G330E TRIM5 α_{Hu} . Sup-T1 cells transduced with WT or mutated TRIM5 α_{Hu} or untransduced (vector) were then infected with the replication-competent HIV-1 clone NL4-3. Unbound virus was eliminated 16 h later and infection was allowed to proceed for 24 days. (A) CA content in the supernatant of infected cells and of uninfected cells as a control were determined periodically by ELISA. (B) Micrograph of R332G-R335G TRIM5 α_{Hu} -expressing cells 14 days post-infection, showing the presence of syncytia.

Fig. 7. Model representation of the TRIM5 α_{Hu} PRYSPRY domain. The predicted impact of v1 mutants on the structure of the PRYSPRY domain was modeled in silico based on the published structures of similar domains. (A) Ribbon model and (B) surface representation of WT, R332G-R335G and G330E-R332G-R335G TRIM5 α_{Hu} . The v1, v2 and v3 regions are shown in green, blue and cyan, respectively. The aminoacids at positions 330, 332 and 335 are shown in orange.