

Susceptibility and adaptation to human TRIM5 α alleles at positive selected sites in HIV-1 capsid

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ABSTRACT

Numerous in vitro studies attribute to human TRIM5 α some modest anti-HIV-1 activity and human population studies suggest some differential effect of TRIM5 α polymorphisms on disease progression. If the activity of TRIM5 α were relevant in vivo, it could result in positive selection on the viral capsid. To address this issue, we identified 10 positively selected sites in HIV-1 capsid from multiple viral strains and generated 17 clade B viruses carrying a minor (i.e. low frequency) residue or an alanine at those positions. All recombinant viruses were susceptible to the modest effect of common human TRIM5 α and allelic variants R136Q, and H419Y; H43Y and G249D TRIM5 α were generally inactive. Increased sensitivity to TRIM5 α was observed for some capsid variants, suggesting that minor residues are selected against in human populations. On the other hand, the modest potency of human TRIM5 α does not translate in escape mutations in the viral capsid.

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Introduction

Human immunodeficiency virus type 1 (HIV-1) has a great potential for adaptation as a result of genetic variability due to high mutation rates, recombination events and short generation times (Ho et al., 1995; Mansky, 1998; Zhuang et al., 2002). Between hosts, evolution of HIV-1 is primarily shaped by neutral selection associated with stochastic events; within hosts, HIV-1 is subjected to natural selection mediated by diverse selective forces (Rambaut et al., 2004). On one hand, constraining forces are exerted at the RNA and protein level to preserve viral fitness, therefore limiting the plasticity of the HIV-1 genome (Holmes, 2003; Sanjuan and Borderia, 2011; Snoeck et al., 2011; Woo et al., 2010). On the other hand, immune response and drugs impose positive selection on HIV-1 leading to the emergence of viral escape mutants (Snoeck et al., 2011; Telenti, 2005). HIV-1 escape mutants are generated in response to adaptive immune defense mediated by antibodies, cytotoxic T lymphocytes (CTL) and to a lesser extent by T helper lymphocytes (Goulder and Watkins, 2008; Henn et al., 2012; Meddows-Taylor et al., 2004; Paul and Piontkivska, 2010; Richman et al., 2003; Wei et al., 2003). Recently, killer inhibitory

receptors (KIR) from natural killer cells were also shown to exert pressure on HIV-1 and to induce escape mutants (Alter et al., 2011). The identification of positively selected sites outside recognized antibody, CTL and helper epitopes suggests that HIV-1 may be exposed to other selective pressures such as innate immunity host factors (Snoeck et al., 2011).

The restriction factor tripartite motif 5 alpha (TRIM5 α) targets lentiviruses in a species-specific manner through recognition of the capsid (CA) (Stremlau et al., 2004b). Rhesus macaques express allelic forms of TRIM5 α , i.e. TRIM5^{TFP} (TFP339-341), TRIM5^Q (Q339) and TRIM5^{CypA}, that restrict efficiently HIV-1 and provide different degrees of protection from infection by simian immunodeficiency viruses (SIV) (Kirmaier et al., 2010; Newman et al., 2006). In particular, TRIM5^{TFP} and TRIM5^{CypA} alleles protect against cross-species transmission of SIV from the Sooty Mangabey (SIVsm) and these protective alleles are associated with the selection of escape CA mutations (Kirmaier et al., 2010). The rhesus TRIM5 α variants retain some modest antiviral activity—2- to 3-fold at most—against the adapted SIVmac239 and SIVmac251 strains in vitro (Kirmaier et al., 2010; Lim et al., 2010; Newman et al., 2006; Stremlau et al., 2004b). Rhesus macaques carrying two protective TRIM5^{TFP} alleles present lower viral loads of SIVmac251, less depletion of CD4+T cells and a higher survival rate than TRIM5^Q homozygous macaques (Letvin et al., 2011; Lim et al., 2010).

A modest 2- to 3-fold activity of human TRIM5 α against HIV-1 can also be observed in vitro by overexpressing human TRIM5 α in

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various cell lines (Li et al., 2007, 2006; Pham et al., 2010; Sebastian et al., 2006; Song et al., 2005; Stremlau et al., 2004a) and in primary CD4 cells (Richardson et al., 2008). Knock down of endogenous TRIM5 α in human cells results in diminished restriction (Sebastian et al., 2006; Sokolskaja et al., 2006). Primary HIV-1 isolates have been identified that display increased sensitivity to human TRIM5 α -mediated restriction (Battivelli et al., 2010; Kaumanns et al., 2006). There is controversy about the ability of several human TRIM5 alleles to influence disease progression in vivo (Goldschmidt et al., 2006; Javanbakht et al., 2006; Liu et al., 2011; Nakajima et al., 2009; Price et al., 2010; Rahm and Telenti, 2012; Sawyer et al., 2006; Speelman et al., 2006; van Manen et al., 2008).

If the activity of TRIM5 α were important in vivo, or if different human alleles differed in restricting capacity, these could translate in the selection of escape variants in HIV-1. Positive selection pressure on the HIV-1 CA would thus serve as a proof of measurable TRIM5 α activity in vivo. To this end, we identified amino acids under significant positive selection in CA sequences from various HIV-1 subtypes. We constructed viruses carrying the minor amino acid represented at each position or an alanine mutation and tested their susceptibility to different human allelic TRIM5 α .

Results

Identification of positively selected sites in HIV-1 CA

A total of 113 curated clade B HIV-1 *gag* sequences were used for detection of positive selection using Bayes Empirical Bayes analysis. Separate analyses were run on 244 sequences from other viral clades. HIV-1 CA was globally under strong purifying selection with Ka/Ks ratios ranging from 0.13 to 0.18 for the various subtypes. However, we identified 6 amino acids under significant positive selection (posterior probability > 0.95) in the N-terminal and C-terminal domains of clade B HIV-1, and 4 additional residues in other clades (Table 1 and Fig. 1A): L6 (in β -hairpin); A14 (between β -hairpin and helix 1); L83 (in helix 4); I91 (in Cyclophilin A (CypA) binding loop); N120 and I124 (between helix 6 and 7); T148 (in interdomain linker); T171 (in helix 8); N183 (in helix 9); T210 (between helix 10 and 11) and G225 (in unstructured part). In addition, H87, that has been associated with modulation of TRIM5 α activity (Hatzioannou et al., 2004a; Ikeda et al., 2004; Owens et al., 2004) and identified in multiple subtypes with posterior probabilities of 0.702 in subtype B and 0.668 in nonBC subtypes (Fig. 1A), had a posterior probability of 0.984 under the M7/M8 model, and it was retained for subsequent analyses. In many cases, the consensus at these positions reflects the ancestral HIV-1 M and the consensus SIV_{cpz} residues (Table 1) suggesting that those residues were already fixed in the virus transmitted to humans. Most study positions mapped to described CTL and T-helper epitopes (<http://www.hiv.lanl.gov/content/immunology/products.html>) (Table 1) and position L6 has been associated with selection pressure exerted by KIR (Alter et al., 2011).

Determinants of TRIM5 α recognition localize in the N-terminal domain of HIV-1 CA constituting a binding interface on the outer CA lattice (Ganser-Pornillos et al., 2011; Hatzioannou et al., 2004a; Owens et al., 2003). We mapped each positively selected position onto the structure of CA in its hexameric form (PDB: 3H4E) (Pornillos et al., 2009). Apart from positions 6 and 14, the study positions were not found at binding interfaces of the hexamer. Additionally, most of the positively selected positions were not part of well-defined secondary structure elements, with the exception of positions 6 and 171 (Fig. 1B). Five of the identified residues (L6, H87, I91, N120, I124) belong to structures exposed at the surface of CA, i.e., the β -hairpin, the CypA binding loop and the

loop between helix 6 and helix 7, or in proximity (A14 and L83) (Fig. 1A, B). The three exposed structures in the N-terminus of CA were reported to modulate TRIM5 α restriction of HIV-1 (Hatzioannou et al., 2004a; Kuroishi et al., 2009; Maillard et al., 2011), SIV (Kono et al., 2010) and N-tropic murine leukemia virus (N-MLV) (Lassaux et al., 2005; Maillard et al., 2007; Ohkura et al., 2011). Four residues (T148, T171, N183, G225) localize in the C terminus of CA. Amino acid replacements in the C-terminus of CA that impact the stability of the viral core may affect TRIM5 α sensitivity (Shi and Aiken, 2006; Zhao et al., 2011).

Characterization of recombinant viruses

To test the importance of positively selected sites of HIV-1 CA on viral fitness and TRIM5 α -mediated restriction, we built 17 recombinant viruses carrying an alanine or a minor amino acid represented in subtype B at the study position in N- and C-termini (Table 1). Of note, some alanine mutations existed naturally as minor variants in subtype B or other subtypes while four alanine mutants (L83A; H87A; I124A; N183A) are unobserved in nature. The control virus carried the common residue at the ten CA positions.

p24 titers of minor variants ranged from 12% (T148A) to 186% (I91V), and the infectious titers ranged from 10% (T148A) to 365% (I91V) compared to the control virus (Fig. 1C). Thus, with the exception of T148A and T171A viruses (having 10- and 5-fold lower titers than control virus respectively), minor CA variants had no major deleterious defects in 293T used as producer cells and in CrFK as target cells. Three viruses with alanine mutations (not observed as minor variants), L83A, I124A and N183A displayed a decrease in both p24 and infectious titers. In particular, I124A mutant, but not the minor variant I124V, displayed a 10-fold reduction of p24 production and a 100-fold decrease of infectivity indicating a major defect. Infectious titers were not correlated to the frequency of the minor amino acid in the population (data not shown).

Restriction by human and rhesus allelic TRIM5 α variants

The sensitivity of recombinant viruses to TRIM5 α -mediated restriction was assessed by infection of CrFK stable lines expressing the most common human allelic TRIM5 α (H43, R136, G249, H419) or a restrictive rhesus TRIM5 α variant, referred to as TRIM5^{TFP} in (Kirmaier et al., 2010) (Fig. 2A, B). Consistent with previous observations in CrFK cells (Hatzioannou et al., 2004b; Rahm et al., 2011; Sebastian et al., 2006), human TRIM5 α marginally restricted the control CA HIV-1 with approximately two-fold effect (Fig. 2B). Of note, all TRIM5 α variants blocked N-MLV infection; human H43Y TRIM5 α was the least active. All recombinant viruses were at least equally blocked, with some minor variants, specifically L6M, I91V, T171A, G225A and the H87A mutant, reaching four to 6-fold restriction (Fig. 2C). Rhesus macaque TRIM5 α strongly inhibited all HIV-1 viruses; the L6A variant was the least restricted (Fig. 2D).

We next tested the restriction mediated by four frequent human TRIM5 α variants that carry non-synonymous polymorphisms: RING domain H43Y, coiled-coil R136Q, intervening sequence G249D, and the v3 variant H419Y (Goldschmidt et al., 2006; Javanbakht et al., 2006; Sawyer et al., 2006) (Fig. 2E–H). These allelic variants were less active (R136Q > H419Y > G249D > H43Y) than the common human TRIM5 α . As previously described (Goldschmidt et al., 2006; Javanbakht et al., 2006; Sawyer et al., 2006), the H43Y variant was inactive against all HIV-1 constructs (Fig. 2E). No viral construct singly escaped from one or more TRIM5 α alleles compared to other CA constructs.

Table 1
Sites of the HIV-1 CA under positive selective pressure.

| Sites ^a | | Structural location ^b | Amino acid content ^c | | | Posterior probability ^d | Ancestral reference ^e | | Epitopes ^f | Mutants ^g |
|--------------------|-----|----------------------------------|--|---|--|------------------------------------|----------------------------------|-----------------------|------------------------|-----------------------------|
| Capsid | Gag | | Subtype B | Subtype C | nonBC | | HIV-1 M | SIVcpz _{Ptt} | | |
| L6 | 138 | β-Hairpin | L:75.2% I:18.6% M:6.2% | L:83.1% I:5.2% A:4.1% H:2.3% S:2.3% V:1.2% P:1.2% M:0.6% | A:53.4% L:42.5% Y:1.4% I:1.4% M:1.3% | 0.984 (B) 0.999 (C) | L6 | A6 | CTL T-helper KIR | L6A ^h L6M |
| A14 | 146 | Interdomain β-hairpin- helix 1 | A:70.8% P:26.5% S:2.7% | A:72.5% P:17.5% S:7.0% N:1.8% T:0.6% K:0.6% | A:53.4% S:28.7% P:9.6% N:5.5% T:1.4% V:1.4% | 1 (C) 1 (nonBC) | A14 | A14 P14 | CTL | A14P |
| L83 | 215 | Helix 4 | L:88.4% V:10.7% | L:80.7% V:8.8% | L:80.5% V:5.6% | 0.97 (B) 1 (C) | L83 | L83 | CTL T-helper | L83A ⁱ L83V |
| H87 | 219 | CypA loop | H:80.4% Q:19.6% | H:84.8% Q:11.1% P:4.1% | H:71.9% Q:26.7% P:1.4% | 0.702 (B) 0.668 (nonBC) | H87 | H87 | CTL T-helper | H87A ⁱ H87Q |
| I91 | 223 | CypA loop | I:73.2% V:23.2% A:1.8% N:0.9% H:0.9% | I:49.1% V:39.1% N:4.7% A:3.5% M:1.2% T:1.2% H:0.6% Y:0.6% | I:72.6% V:20.5% F:2.7% P:1.4% A:1.4% N:1.4% | 1 (B and C) 0.942 (nonBC) | I91 | I91 | CTL T-helper | I91A I91V |
| N120 | 252 | Interdomain helix 6–helix 7 | N:59.8% S:20.5% H:17.0% G:0.9% Q:0.9% Y:0.9% | S:56.7% G:17.4% N:17.0% A:5.9% H:1.2% R:0.6% T:0.6% D:0.6% | S:68.5% G:28.8% N:2.7% | 1 (B and C) 0.965 (nonBC) | S120 | A120 | T-helper | N120A ^h N120H |
| I124 | 256 | Interdomain helix 6–helix 7 | I:93.8% V:4.4% T:1.8% | V:50.8% I:48.0% M:1.2% | I:78.1% V:21.9% | 0.989 (C) | I124 | I124 V124 | CTL T-helper | I124A ⁱ I124V |
| T148 | 280 | Interdomain linker | T:69.0% V:13.3% S:8.0% I:6.2% A:3.5% | V:94.7% S:1.2% T:2.9% A:0.6% Q:0.6% | V:100.0% | 1 (B) | V148 | V148 | CTL T-helper | T148A |
| T171 | 303 | Helix 8 | T:96.4% V:3.6% | T:93.0% V:3.5% C:1.7% A:1.2% I:0.6% | T:79.5% A:12.3% V:6.8% C:1.4% | 0.988 (nonBC) | T171 | T171 | CTL T-helper | T171A ^h |
| N183 | 315 | Helix 9 | N:97.3% T:0.9% H:0.9% G:0.9% | N:97% G:2.4% D:0.6% | N:49.4% G:47.9% S:2.7% | 0.996 (nonBC) | N183 | N183 | CTL T-helper | N183A ⁱ |
| G225 | 357 | Unstructured | G:75.2% S:21.2% A:2.7% H:0.9% | G:47.4% S:52.0% A:0.6% | G:52.1% S:46.6% Q:1.3% | 1 (B and C) 0.993 (nonBC) | G225 | S225 | CTL | G225A |

The analysis of 357 HIV-1 CA sequences from different subtypes (B, C and other) identified 10 sites under positive selection detected by Bayes Empirical Bayes (BEB) analysis using the M2a model. H87 variants were included based on previous reports and M7/M8 analyses.

^a Site of the HIV-1 CA under positive selection according to the subtype B reference and corresponding numbering in Gag.

^b Structural location of the sites according to Gamble et al. (1996, 1997). CypA: Cyclophilin A.

^c Amino acid frequency of the site residue according to subtype B (113 sequences), C (171 sequences) or other (73 sequences). The minor amino acid in subtype B considered for in vitro reconstruction is represented in bold.

^d Probability value upon data analysis for each subtype indicated in brackets using a maximum likelihood tree.

^e The corresponding residues in the capsids of the ancestral HIV-1 group M and SIVcpz are indicated for each study position. A consensus gag was built from fifteen SIVcpz sequences originating from *Pan troglodytes troglodytes* subspecies.

^f HIV-1 CA sites correspond to CD8 T cell (CTL) and CD4 T cell (T-helper) epitopes based on the HIV Immunology database provided on the Los Alamos National Laboratory (<http://www.hiv.lanl.gov/content/immunology/products.html>). The KIR epitope was described in Alter et al. (2011).

^g Mutation of the residue under positive selection with the minor variant or an alanine built in a B subtype CA background (from HIV-1_{NL4.3}).

^h Alanine variants that were observed in non-B subtypes.

ⁱ Alanine variants that were not observed naturally in any subtype.

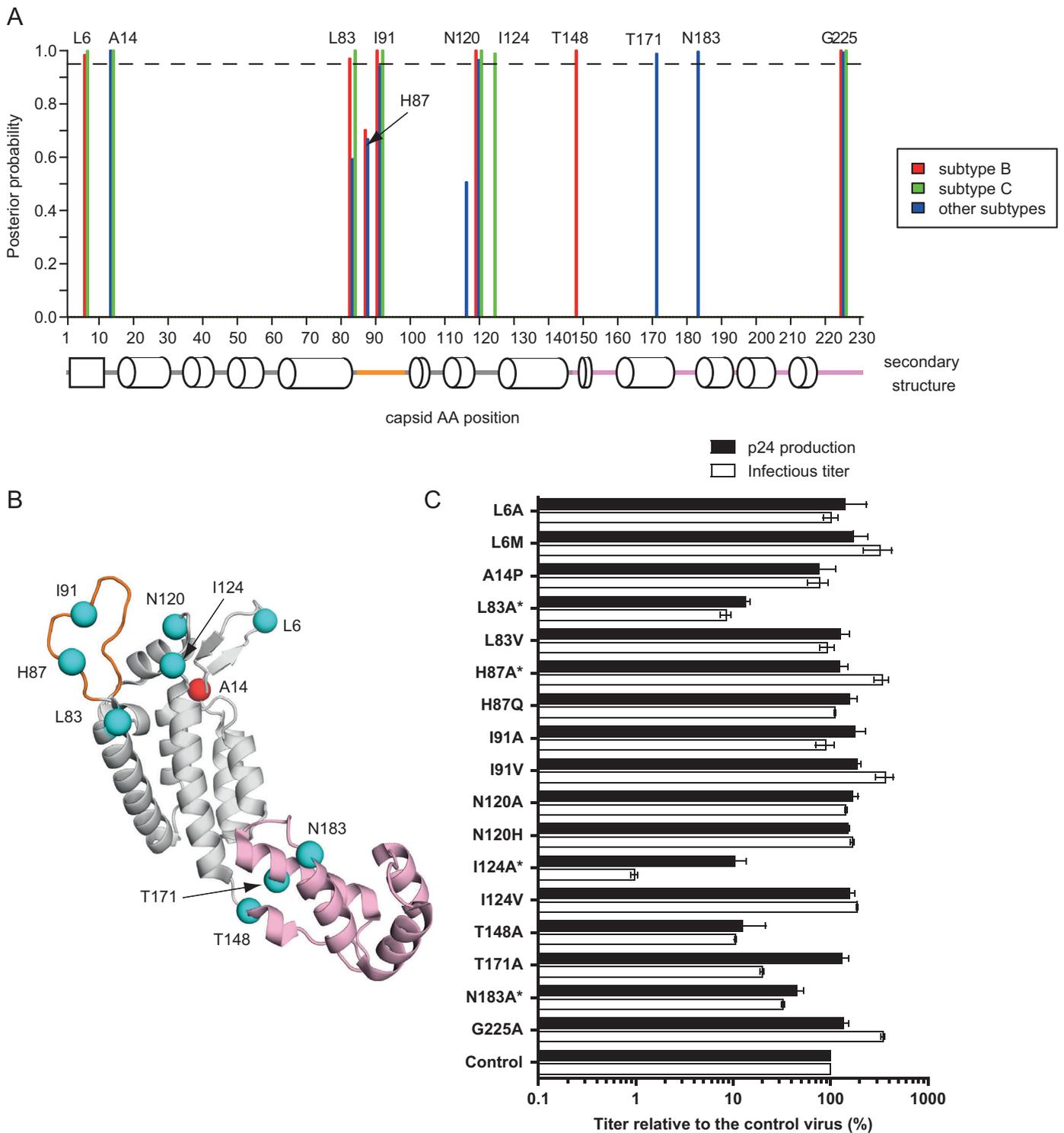
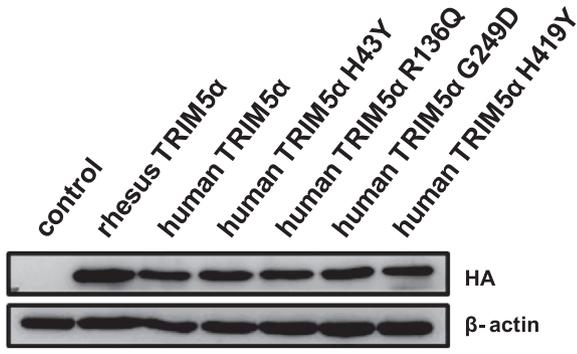
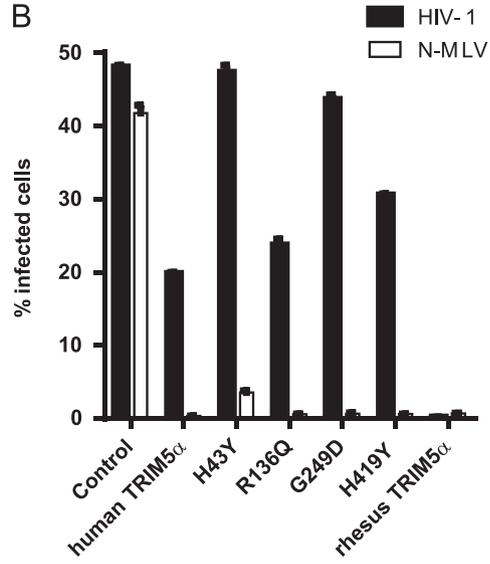


Fig. 1. Positive selection, structural position and viral titers of HIV-1 CA mutants. **A.** Amino acids of HIV-1 CA under positive selection. X axis: amino acid numbering. Y axis: vertical lines correspond to the value of the posterior probability; residues with a significant posterior probability (> 0.95) correspond to positively selected positions. The secondary structure of CA is shown below the graph: cylinders show the position of α -helices and the box shows the position of the β -hairpin. The N-terminal domain is represented by the gray line, the C-terminal domain is symbolized by the pink line, the CypA binding loop is shown in orange. Except for L6 and T171, none of the positively selected positions occur in the middle of well-defined secondary structure elements. **B.** Mapping of the positively selected positions on the structure of HIV-1 CA (PDB: 3H4E). The N-terminal domain is shown in gray and the C-terminal domain in pink. The CypA binding loop is shown in orange. Cyan spheres represent the different positively selected positions. Position 14 is displayed with a red sphere to indicate that a cysteine was used at this position in the crystallized protein. **C.** p24 and infectious titers of HIV-1 CA mutants. Titers of VSV-G pseudotyped GFP-reporter viruses were determined by ELISA detection of p24 HIV-1 CA protein (using polyclonal antibodies). Results are presented as the percent of p24 concentration (pg/ml) for each mutant virus compared to the control CA-containing virus set to 100%. Infectious titers were determined on the same viral stocks by infection of wild-type CrFK cells using serial dilution of viral supernatants in duplicate. Two days after infection, the percent of GFP-positive cells was measured by flow cytometry, thereby providing infectious titers in transducing units per milliliter (TU/ml) of virus. Results are presented as the percent of infectious titer (TU/ml) for each mutant virus compared to the virus with control CA set to 100%. Error bars represent the standard deviation from the mean of two independent viral productions. Stars indicate viruses carrying non-natural alanine mutations.

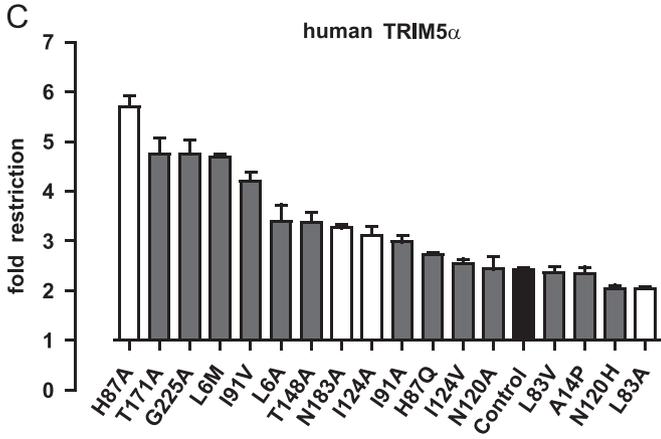
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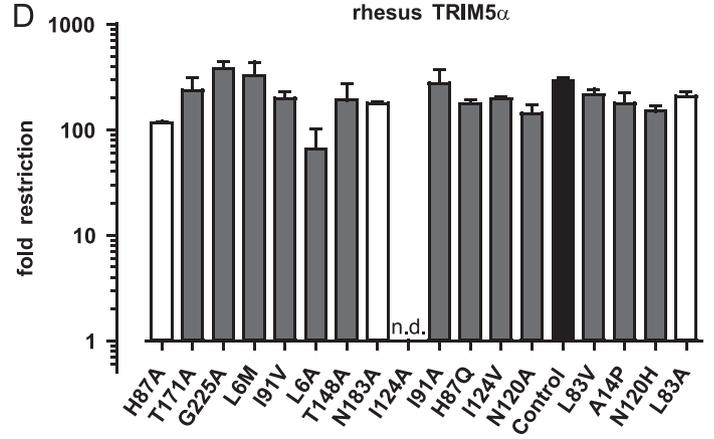
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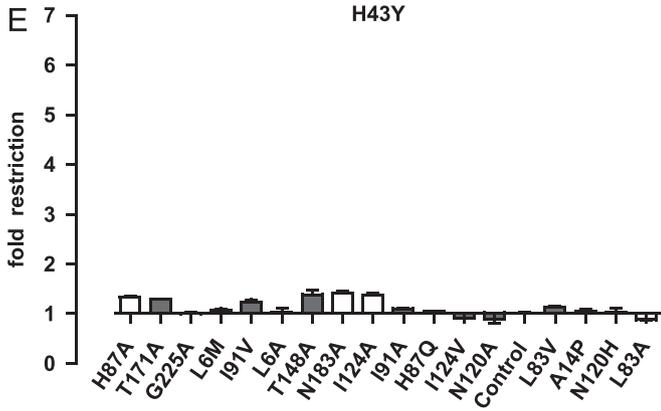
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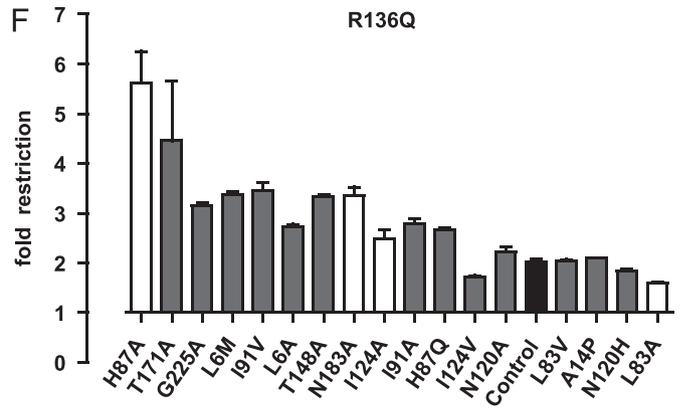
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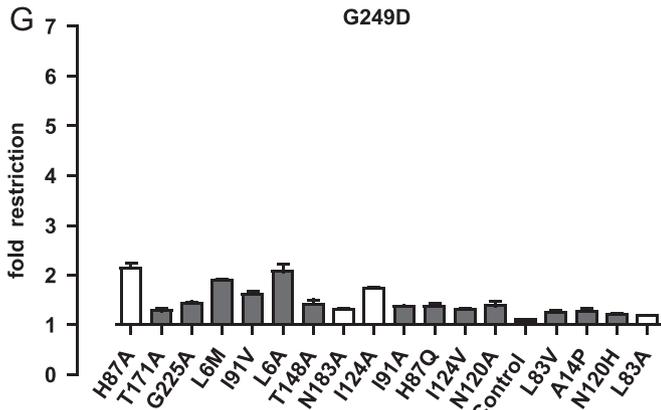
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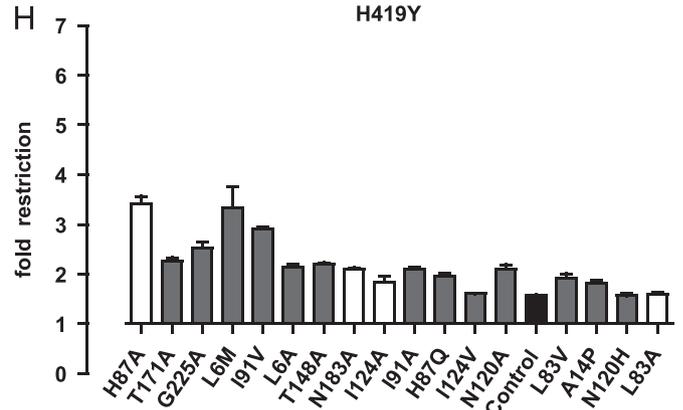
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Discussion

The HIV-1 CA is highly conserved, and *in vitro* mutations of conserved residues generally result in impaired particle production or infectivity (Ganser-Pornillos et al., 2004). We analyzed amino acids under selective pressure in CA sequences from HIV-1 infected individuals and identified six positions in clade B, and four additional sites in other clades to be under significant positive selection. Recombinant CA viruses carrying minor amino acid variants at these sites were competent for viral particle production and infection. All viral constructs were highly sensitive to the inhibition by the prototypical rhesus macaque *TRIM5^{TRP}* and modestly inhibited by several human allelic *TRIM5 α* variants. However, we did not observe unique CA viral variants selectively escaping restriction from the human allelic *TRIM5 α* tested. In agreement with previous reports *TRIM5 α* H43Y was impaired (Goldschmidt et al., 2006; Javanbakht et al., 2006; Sawyer et al., 2006). We interpret these data as indicating that the majority of circulating strains represent an optimum of adaptation to human *TRIM5 α* . Indeed, many of the majority residues are already present in the ancestral HIV-1M CA and SIVcpz_{Ptt}.

Interestingly, viral constructs carrying some minority variants (L6M, H87A, I91V, T171A, G225A) were slightly more—not less—restricted than the control CA virus. L6 lies in the β -hairpin; mutations of this exposed structure have been previously associated with modest changes in susceptibility to human *TRIM5 α* (then referred as Ref1) restriction (Hatzioannou et al., 2004a). H87 and I91 reside in the CypA binding loop, where the cellular CypA interacts with HIV-1 CA (Luban, 2007). This loop is a determinant region of *TRIM5 α* specificity (Hatzioannou et al., 2004a; Kootstra et al., 2003; Owens et al., 2004; Ylinen et al., 2005). H87Q mutation was associated with reduced susceptibility to rhesus *TRIM5 α* (2- to 3-fold effect) in the presence of CypA in the target cells (Ikeda et al., 2004; Owens et al., 2004; Pacheco et al., 2010). In our dataset, H87Q mutation provided a limited benefit (1.7-fold) to HIV-1 infection in the presence of rhesus *TRIM5 α* , but no escape from human allelic *TRIM5 α* variants. In contrast, the H87A mutant and I91V increased HIV-1 sensitivity to human *TRIM5 α* . It remains unclear how T171A and G225A mutations in the C-terminal domain of CA affect *TRIM5 α* restriction. One possible explanation is that these variants modify the stability of the core favoring accelerated uncoating triggered by *TRIM5 α* . Thus, major residues of HIV-1 CA represent adaptation to *TRIM5 α* while minor residues may be selected against (purifying selection) in humans.

What are then the forces that result in signs of positive selection at those residues? L6, H87, I91, T171, and G225 map to recognized CTL epitopes: L6 (VV9 epitope); H87 and I91 (B7-HA9 epitope), T171 (A24-RL11 epitope) and G225 (GL9 epitope). Mutations H87Q and I91V correspond to compensatory mutations for the common CTL-escape T110N mutation (T242N in Gag) in the TW10 epitope restricted by B*57:01 that inflict a cost in viral fitness (Brockman et al., 2007; Martinez-Picado et al., 2006).

Position T110 itself was not identified as harboring positive selected variants; however 29 T110N variants were identified in this study. The selective pressure posed by the acquired immunity might lead to escape mutants that render the virus more susceptible to human *TRIM5 α* *in vivo*, partially explaining their large fitness cost. This scenario has been proposed by Battivelli et al. (2011) who reported that recombinant viruses carrying *gag* from clinical subtype B isolates were more sensitive to human *TRIM5 α* because of multiple CTL-escape and compensatory mutations in CypA binding loop, TW10 epitope (helix 6) and KK10 epitope (helix 7) of HIV-1 CA (Battivelli et al., 2011).

We investigated positions in HIV-1 CA that were positively selected at statistically significant level, though this does not cover all the mutational space. Since the initial decision to build the identified residues, we have extended clade B evolutionary analysis to 635 clade B CA sequences (Snoeck et al., 2011). This larger dataset confirmed L6, L83, I91, N120 and T148 as residues under positive selection, and identified two more residues, G116 and L136, that are not included in the present work. Thus, our study does not exclude the possibility of additional escape CA sites, rare variants, or combination of variants resulting in escape. At position R100 in HIV-1 CA, we only identified three R100T and one R100S variant strains. This position corresponds to R97 in SIVsm, where R97S represents an escape mutant from rhesus *TRIM5 α* (Kirmaier et al., 2010). HIV-1 CA R100S, that was previously built (Owens et al., 2004), infected human and monkey cells expressing endogenous *TRIM5 α* as efficiently as WT CA virus. Context specific effects could be an issue for non-clade B residues built in a clade B background. However, 4 of 5 residues discussed above to be associated with increased susceptibility to *TRIM5 α* represented clade B minority species in a clade B CA context.

We also limited the study to well recognized human *TRIM5* polymorphisms. Genome-wide association analyses did not identify additional common variants influencing HIV-1 infection in the *TRIM5* locus; however, other rare alleles may exist (Fellay et al., 2007; Pereyra et al., 2010).

Conclusions

The majority of CA sequences in HIV-1 clade B circulating in the human population reflect optimal adaptation to human *TRIM5 α* . The modest restriction consistently observed *in vitro* may not translate into relevant activity *in vivo*; in fact, there is no agreement on differences in disease progression among individuals carrying the various alleles despite documented differences in *in vitro* activity. However, while different human *TRIM5 α* alleles may not impose further escape at population level; it may contribute to restriction in the context of escape from the acquired immunity.

Fig. 2. Restriction of HIV-1 CA mutants by human and rhesus *TRIM5 α* variants. (A) Western blot analysis of HA-tagged *TRIM5 α* and β -actin in stable CrFK cell lines expressing rhesus macaque *TRIM5 α* , the common human *TRIM5 α* variant and four allelic forms of human *TRIM5 α* (H43Y, R136Q, G249D, H419Y) using antibodies directed against the HA tag or β -actin. Control cells contain an empty pLPCX vector. (B) Infection of CrFK expressing the common human *TRIM5 α* , four human *TRIM5 α* variants (H43Y, R136Q, G249D, H419Y) and rhesus macaque *TRIM5 α* (*TRIM5^{TRP}* allele) using 5000 pg equivalent of p24 of the control CA HIV-1 reporter GFP virus and comparable infectious dose of N-MLV GFP virus. The percentage of infected cells was measured by flow cytometry two days post-infection. Control cells were transfected with empty pLPCX vector. Error bars represent the standard error of the mean from duplicates. (C–H) Restriction activity expressed as fold changes after infection of CrFK expressing (C) the common human *TRIM5 α* , (D) rhesus macaque *TRIM5 α* (*TRIM5^{TRP}* allele), (E) human H43Y *TRIM5 α* , (F) human R136Q *TRIM5 α* , (G) human G249D *TRIM5 α* , and (H) human H419Y *TRIM5 α* . Duplicate infections were performed using 5000 pg equivalent of p24 of each HIV-1 reporter GFP virus carrying mutations in the CA as indicated on X-axis. The percentage of infected cells was measured by flow cytometry 2 days post-infection. The fold restriction activity (i.e. inhibition of viral infection) was determined by dividing the percent of GFP-positive cells for CrFK controls cells by the percent of GFP-positive cells for CrFK-*TRIM5 α* cells. CA mutants are organized on X-axes in decreasing order of the fold restriction obtained for the common human *TRIM5 α* . Black bar: virus with control CA; gray bars: viruses carrying mutations naturally observed in B, C and other (nonBC) subtypes; white bars: viruses carrying non-natural alanine mutations. Error bars represent the standard error of the mean from duplicates. n.d.: not determined; the reduced infectivity of the mutant virus I124A did not allow sufficient readout to quantify the restriction activity by rhesus *TRIM5 α* . Results from a single experiment are shown; similar results were obtained in two independent experiments.

Materials and methods

Sequence analysis

HIV-1 CA sequences from subtype B, and non-B subtypes were retrieved from the Los Alamos database (<http://www.hiv.lanl.gov/content/index>) for identification of positively selected sites. Maximum likelihood trees were built for each subtype using the HKY85 substitution model implemented in Mega v5 with estimated ts/tv ratio. Bayes Empirical Bayes analysis implemented in PAML v4.5 applied the M2a to identify amino acids under positive selection. All residues identified under positive selection were confirmed in model M7/M8. For in vitro reconstruction of CA minor variants in subtype B, we built clade B variants with a frequency of 2.7–26.5%. We avoided rare mutants that could result from sequencing errors. The ancestral group M HIV-1 gag sequence was retrieved from Los Alamos website (<http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html>). Fifteen SIVcpz gag from *Pan troglodytes troglodytes* (SIVcpz_{Ptt}) from the Los Alamos repository were used to build a consensus SIVcpz_{Ptt} gag sequence.

Molecular modeling

The X-ray structure of HIV-1 CA in its hexameric form was used for structural modeling (PDB: 3H4E) (Pornillos et al., 2009). In the experimental structure, a cysteine is found at position 14 making a disulfide bridge with its neighbor in the hexamer. Histidine is found at position 120 in the published CA structure, while we used a reference strain containing asparagine at this position in all experiments. Finally, position 225 is located in a disordered region that is not resolved in the structure and therefore could not be modeled.

Cell lines and stable lines

Human embryonic kidney 293T cells and Crandell feline kidney (CrFK) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin). pLPCX vectors encoding HA-tagged human TRIM5α variants and rhesus macaque TRIM5α, as well as puromycin resistance, were previously described (Goldschmidt et al., 2006; Rahm et al., 2011). 293T cells were cotransfected with pLPCX or each pLPCX–TRIM5α plasmid, the packaging MLV Gag–Pol plasmid, and the VSV-G plasmid using polyethylenimine (Polysciences). Supernatants were harvested 48 h post-transfection, filtered (0.45 µm), concentrated (Centricon units, Millipore) and used to transduce 10⁶ CrFK cells in the presence of 10 µg/ml of polybrene. After 72 h, CrFK cells were selected in the presence of 5 µg/ml of puromycin. The expression of TRIM5α from CrFK stable cell lines was confirmed by western blot using anti-HA antibodies.

Western blots

Whole-cell lysates of CrFK cells were prepared in RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with Complete Mini protease inhibitor cocktail (Roche) and centrifuged at 12,000g for 10 min. The equivalent of 10 µg total protein was subjected to SDS-PAGE and immunoblotted with rat monoclonal anti-HA antibody (Roche) and mouse monoclonal anti-β-actin (Sigma).

Recombinant viruses

The sequence of gag from a pNL4.3Δenv proviral clone was inserted into a cloning vector and single mutations were introduced in the CA sequence by site-directed mutagenesis using the QuickChange protocol (Stratagene). The sequence of NL4-3 carries a H120 that was modified to 120 N to reflect the consensus sequence prior to introducing subsequent mutations. Mutated gag fragments were cloned back into a pNL4.3-based HIV-1 Gag–Pol vector. The resulting recombinant Gag–Pol vectors were used to produce viruses by co-transfection of 293T with an HIV-1 GFP genomic vector (pSIN.cPPT.CMV.GFP.WPRE) and VSV-G envelope vector using calcium phosphate. Transfection medium was replaced by fresh medium 20 h post-transfection. Viral supernatants were harvested 48 h after transfection and filtered through 0.45 µm filters. Viral production was assessed by p24 quantification by ELISA (Innogenetics). For determination of infectious titers, 4 × 10⁴ CrFK cells were infected with serial dilutions of viral supernatant in 48-well plates and GFP-positive cells were quantified by fluorescence-activated cell sorter (FACS) analysis two days after infection. If required, viral supernatants were concentrated by using Centricon centrifugal filter devices (Millipore).

Infectivity assays

CrFK stable cell lines expressing the various TRIM5α were infected by spinoculation of 5000 pg (p24 equivalent) of concentrated virus onto 5 × 10⁴ cells in 48-well plates for 1 h at 1500 g in the presence of 10 µg/ml polybrene. Spinoculation allowed use of less virus to maintain standard single stocks for the infection of 18 viruses in 7 cell lines in duplicate (252 conditions). Virus-containing medium was removed and replaced by fresh medium after spinoculation. Two days after infection, GFP-positive cells were quantified by FACS analysis.

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Author contributions

NR, AC and AT conceived the study.
NR, MO and RM performed experiments.
JS, DG and PJM performed analyses.
NR and AT wrote the main draft, edited by all authors.

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