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Binding of the Rhesus TRIM5α PRYSPRY Domain to Capsid is Necessary but not Sufficient for HIV-1 Restriction

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Abstract

The PRYSPRY domain of TRIM5α provides specificity and the capsid recognition motif to retroviral restriction. Restriction of HIV-1 by rhesus TRIM5α (TRIM5α_{rh}) has been correlated to its ability to bind to the HIV-1 core, suggesting that capsid binding is required for restriction. This work explores whether the PRYSPRY domain of TRIM5α_{rh} exhibits an additional function besides binding to the HIV-1 core. Using our recently described structure of the PRYSPRY domain, we performed an exhaustive structure-function study of the surface and interior residues of the PRYSPRY domain. Testing retroviral restriction and capsid binding of an extensive collection of 60 TRIM5α_{rh} PRYSPRY variants revealed that binding is necessary but not sufficient for restriction. In support of this hypothesis, we showed that some human tripartite motif proteins bind the HIV-1 capsid but do not restrict HIV-1 infection, such as human TRIM6 and TRIM34. Overall this work suggested that the PRYSPRY domain serves an unknown function, distinct from the binding of TRIM5α_{rh} to the HIV-1 core, to block HIV-1 infection.

Several newly discovered proteins that are endogenously expressed in primates show the ability to dominantly block retroviral infection and cross-species transmission by interfering with the early phase of viral replication (Best et al., 1996; Kirmaier et al.; Sayah et al., 2004; Stremlau et al., 2004). Of particular interest are members of the tripartite motif (TRIM) family of proteins. The splicing variant a of TRIM5 from rhesus macaque (TRIM5α_{rh}) is a ~53 kDa cytosolic protein that potently restricts HIV-1 infection (Stremlau et al., 2004). Expression of TRIM5α_{rh} in mammalian cells blocks HIV-1 and other retroviruses soon after viral entry but prior to reverse transcription (Keckesova et al., 2004; Stremlau et al., 2004). The retroviral capsid protein is the viral determinant for susceptibility to restriction by TRIM5α (Owens et al., 2003). Studies on the fate of the HIV-1 capsid in the cytosol of infected cells have correlated restriction with a decreased amount of cytosolic particulate capsid (Diaz-Griffero et al., 2007a; Perron et al., 2007; Stremlau et al., 2006).

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TRIM5α_{rh} is composed of four distinct domains: RING, B-box 2, coiled-coil and B30.2 (SPRY) (Reymond et al., 2001). The RING domain of TRIM5α_{rh} is an E3 ubiquitin ligase (Diaz-Griffero et al., 2006a; Kar et al., 2008; Kim et al., 2011; Langelier et al., 2008; Li et al., 2013; Lienlaf et al., 2011; Maegawa et al.; Pertel et al., 2011; Yamauchi et al., 2008). The E3-ligase activity of TRIM5α is correlated to the ability of TRIM5α to block HIV-1 (Lienlaf et al., 2011). The B-box 2 domain of TRIM5α and other TRIM proteins, such as TRIM63, self-associates to forming dimeric complexes that are important for TRIM5α higher-order self-association and contribute to capsid binding avidity; these B-box 2 domain functions are essential for full and potent restriction of HIV-1 (Diaz-Griffero et al., 2007b; Diaz-Griffero et al., 2009; Ganser-Pornillos et al.; Javanbakht et al., 2005; Mrosek et al., 2008; Perez-Caballero et al., 2005). The coiled-coil domain enables TRIM5α_{rh} dimerization (Kar et al., 2008; Langelier et al., 2008), which is critical for interaction of the B30.2 (SPRY) domain with the HIV-1 capsid (Sebastian and Luban, 2005; Stremlau et al., 2006).

The B30.2 (SPRY) domain provides the capsid recognition motif that dictates specificity to retroviral restriction (Nakayama et al., 2005; Sawyer et al., 2005; Song et al., 2005; Stremlau et al., 2005; Yap et al., 2005). Restriction of HIV-1 by TRIM5α_{rh} has been correlated to the ability of TRIM5α_{rh} to bind to the HIV-1 capsid, suggesting that capsid binding is required for restriction. An invariant patch on the human TRIM5α (TRIM5α_{hu}) protein has been described as being required for restriction of N-MLV but dispensable for capsid binding (Sebastian et al., 2009). By using a limited number of variants, these experiments showed that binding is necessary but not sufficient for restriction of N-MLV by TRIM5α_{hu} suggesting that the PRYSPRY domain has an additional function. To explore whether the PRYSPRY domain of TRIM5α_{rh} exhibits an additional function besides binding to HIV-1 capsid, we performed structure-function studies using our recently described structure of the PRYSPRY domain (Biris et al., 2012). Analysis of an extensive collection of PRYSPRY variants revealed two surface patches that are dispensable for binding but essential for retroviral restriction.

RESULTS

Mutagenic analysis of the TRIM5α$_{rh}$ PRYSPRY domain

Using the structure of the TRIM5α_{rh} PRYSPRY domain (Biris et al., 2012), we generated a collection of variants to test the hypothesis that the PRYSPRY domain exhibits an additional function besides binding to the HIV-1 capsid. As shown in Figure 1, our mutagenesis studies focused on surface and internal residues of the TRIM5α_{rh} PRYSPRY domain (Table 1 and Figure 1). This work explored most of the charge residues in the surface of the PRYSRY domain, and in cases were changes to alanine resulted in interesting phenotypes, we proceeded to change these amino acids to residues of opposite charge. For simplicity mutations PQIMY327AAAMA, NFNYC345AAAAA and PQIMY327AAAMA/NFNYC345AAAAA were called 327AAAMA, 345AAAAA and 327AAAMA/345AAAAA.

To evaluate expression, we stably transduced dog Cf2Th cells using retroviral vectors expressing HA-tagged mutant and wild type TRIM5α_{rh} proteins. Expression was evaluated by Western blotting using anti-HA antibodies (Figure 2 and S1). As loading control, we blotted cellular extracts using anti-GAPDH antibodies. Most of the variants expressed equally well or better, when compared with to wild type TRIM5α_{rh} (Figure 2 and Table 1). However, we also encountered a number of mutants, including D318E, Y364A, I391A, Y397A, S415A, F417A and R484A that were difficult to stably express in Cf2Th cells.

Ability of TRIM5α$_{rh}$ PRYSPRY domain variants to restrict HIV-1

To test the ability of TRIM5α$_{rh}$ PRYSPRY domain variants to block HIV-1 infection, we challenged Cf2Th cells stably expressing the different variants with increasing amounts of HIV-1 expressing GFP (HIV-1-GFP) as a reporter of infection. These experiments revealed that PRYSPRY domain mutants exhibit a variety of phenotypes ranging from full to the loss of restriction (Figure 3 and S2). In Table 1, our PRYSPRY domain variants are ranked according to their ability to block HIV-1 infection.

Ability of TRIM5α$_{rh}$ PRYSPRY domain variants to bind HIV-1 capsid

To test the ability of PRYSPRY domain variants to interact with the HIV-1 capsid, we tested the ability of the variants to bind *in vitro* assembled HIV-1 CA-NC complexes as previously described (Lienlaf et al., 2011). As in the restriction experiments, binding experiments revealed that the ability of PRYSPRY variants to bind HIV-1 capsid ranged from full binding to complete absence of binding (Figure 4 and S3). In Table 2, PRYSPRY domain variants are ranked according to their ability to bind *in vitro* assembled HIV-1 CA-NC complexes. Of note, we found some variants, e.g. F417A and 327AAAMA/345AAAAA, that had completely lost the ability to bind *in vitro* assembled HIV-1 CA-NC complexes.

Binding of TRIM5α$_{rh}$ to HIV-1 capsid is necessary but not sufficient for restriction

Previous experiments have demonstrated that binding of TRIM5α$_{rh}$ to HIV-1 capsid is necessary for restriction (Diaz-Griffero et al., 2006b; Stremlau et al., 2006). For example, TRIM5α$_{hu}$ does not bind the HIV-1 capsid, and is impaired in its ability to restrict HIV-1 (Li et al., 2006). In addition, mutations or deletions of TRIM5 or TRIMCyp that prevented binding to the HIV-1 capsid were unable to block HIV-1 infection, suggesting that binding is necessary for restriction (Diaz-Griffero et al., 2006b; Javanbakht et al., 2005; Perez-Caballero et al., 2005; Stremlau et al., 2006). To better understand the relationship between the ability of TRIM5α$_{rh}$ to bind capsid and restrict HIV-1 infection, we plotted capsid binding as a function of the restriction abilities of different PRYSPRY variants. As shown in Figure 5, the ability of TRIM5α$_{rh}$ PRYSPRY variants to restrict HIV-1 requires binding; however, binding by itself is not sufficient for restriction since we found a collection of variants that showed wild type levels of binding to the HIV-1 capsid while losing their capacity to restrict HIV-1. These experiments suggested that the PRYSPRY domain is necessary for restriction but serves some function in addition to binding.

Careful analysis of variants that allow binding but lose restriction (binding > 70% and restriction < 40%) revealed two sets of residues that form surface patches important for restriction (Figure 6): Surface patch 1(SP1) is composed of D318, K319 and R320 (Figure 6A); Surface patch 2 (SP2) is composed of N326, P327, Q328, I329, M330 and Y331 (Figure 6B). Even though SP1 and SP2 are located on the surface of the TRIM5α$_{rh}$ PRYSPRY domain structure, we also found that changes in residues located inside the hydrophobic core, such as V405 and P483 depicted as the two black dots surrounded by color dots on Figure 5, resulted in variants that potently bound HIV-1 capsid but did not restrict HIV-1 infection (Figure 6C). In addition, we observed that SP1 and SP2 were conserved in human and some species of monkeys (Figure 6D). Altogether these experiments suggested that binding of TRIM5α$_{rh}$ to the HIV-1 capsid is necessary but not sufficient for restriction, and that the PRYSPRY domain may serve an additional function.

We also observed two variants that exhibit strong HIV-1 restriction but poor binding to capsid (Q393R and T424R). These results suggested that these particular variants are compensating the binding to capsid defect by enhancing the postulated additional function of the PRYSPRY domain in restriction.

Ability of TRIM5α$_{rh}$ PRYSPRY domain variants to shuttle into and out of the nucleus

We have previously described the ability TRIM5α$_{rh}$ to shuttle into and out of the nucleus (Brandariz-Nunez et al., 2013; Diaz-Griffero et al., 2011). To test whether the restriction inability of the PRYSPRY domain variants that bind capsid is related to the shuttling properties of TRIM5α$_{rh}$, we tested the shuttling ability of PRYSPRY variants that bind HIV-1 capsid but do not restrict HIV-1 infection. For this purpose, we studied localization of PRYSPRY domain variants in the presence of leptomycin B (LMB), a specific inhibitor of nuclear export (Fornerod et al., 1997; Kudo et al., 1998; Kuersten et al., 2001; Mohr et al., 2009; Watanabe et al., 1999; Wolff et al., 1997). As shown in Figure 7, all the tested PRYSPRY domain variants, which bind HIV-1 capsid but didn't restrict HIV-1, localized to the nuclear compartment in the presence of LMB. In parallel, we tested the subcellular localization of VI405KK and IL376AA mutants, which are known to lose their ability to shuttle in and out of the nucleus (Brandariz-Nunez et al., 2013; Lukic et al., 2013). Image quantification is shown on Supplementary Figure S4. These results indicated that the PRYSPRY domain variants, which bind HIV-1 capsid but do not restrict infection, have not lost the ability to shuttle in and out of the nucleus.

Tripartite motif proteins that bind *in vitro* assembled HIV-1 CA-NC complexes but do not restrict HIV-1

In this section, we tested the ability of TRIM family members closely related to TRIM5 to bind *in vitro* assembled HIV-1 CA-NC complexes. As shown in Figure 8A, human TRIM6 and TRIM34 binds *in vitro* assembled HIV-1 CA-NC complexes, as previously shown (Li et al., 2007). In agreement with our findings that some TRIM5α$_{rh}$ PRYSPRY variants bind the HIV-1 capsid but do not restrict HIV-1, human TRIM6 and TRIM34 do not restrict HIV-1 infection (Li et al., 2007; Zhang et al., 2006). Furthermore, we tested the ability of different monkey TRIM5α proteins to bind *in vitro* assembled HIV-1 CA-NC complexes. As shown in Figure 8B, TRIM5α proteins that do not restrict HIV-1, such as TRIM5α from chimpanzee and tamarin, bound *in vitro* assembled HIV-1 CA-NC complexes. These results suggested that binding to HIV-1 capsid could occur in the absence of viral restriction.

DISCUSSION

This work represents a comprehensive structure-function study of the PRYSPRY domain of TRIM5α$_{rh}$. Using our recently described structure of the PRYSPRY domain of TRIM5α$_{rh}$ (Biris et al., 2012), we studied a collection of variants that covered most of the surface and core residues of the PRYSPRY domain. These variants were tested for their ability to block HIV-1 infection and binding to *in vitro* assembled HIV-1 CA-NC complexes. Analysis of sixty PRYSPRY domain variants revealed that *in vitro* binding of TRIM5α$_{rh}$ variants to HIV-1 capsid does not correlate with HIV-1 restriction. These findings suggested that binding of TRIM5α$_{rh}$ to the HIV-1 capsid is necessary but not sufficient for restriction, implying that the PRYSPRY domain provides a second function. One possibility is that an intact PRYSPRY domain is required to bind a cellular cofactor, as it has been suggested previously to explain the ability of TRIM5α$_{hu}$ to restrict N-MLV infection (Sebastian et al., 2009). Alternatively, an intact PRYSPRY domain structure might be required to maintain the structural flexibility of the V1 loop. The flexibility of the V1 loop provides structural plasticity (Biris et al., 2012), conferring upon the PRYSPRY domain the ability to bind different epitopes, in agreement with independent findings suggesting that multiple sites on the capsid are important for recognition by TRIM5α proteins (Kono et al., 2010; Ohkura et al., 2011; Ohkura and Stoye, 2013). Therefore, we think that our PRYSPRY domain variants could be affecting structural plasticity, something that might not be detectable via an ordinary binding assay.

This work defines two important surface patches for restriction SP1 and SP2. SP1 is distal from the V1 loop, which has been genetically defined as an important determinant for the ability of TRIM5α_{rh} to restrict HIV-1 (Li et al., 2006; Ohkura et al., 2006; Song et al., 2005; Stremlau et al., 2005; Yap et al., 2005). By contrast, SP2 is located near or within the V1 loop itself, supporting previous observations that the V1 loop is important for restriction. We also showed that SP1 and SP2 are conserved in humans and in monkeys such as African green monkeys, chimps and orangutans.

Interestingly, we also find that changes in residues V40S and P483, which are located in the interior of the protein, resulted in variants that could bind the HIV-1 capsid *in vitro* but did not restrict HIV-1 infection. As previously mentioned, these variants might be affecting the surface of the PRYSPRY domain and/or the flexibility of the V1 loop.

These extensive structure-function studies revealed two PRYSPRY domain variants that potently restricted HIV-1 but poorly bound HIV-1 capsid *in vitro* (Q393R and T424R). One possibility is that these represent gain of function mutants where the defect of binding to capsid is compensated by enhancing the unknown function of the PRYSPRY domain.

One caveat of these studies is that the binding of TRIM5α_{rh} variants to capsid *in vitro* might not reflect the binding of these proteins *in vivo*. One possible approach to measure binding of TRIM5α_{rh} variants to capsid *in vivo* is the use of Förster resonance energy transfer (Day and Davidson, 2012; Padilla-Parra and Tramier, 2012). Infection of cells expressing the different TRIM5α_{rh} variants fused to fluorophore A by HIV-1 viruses where the viral cores contain the capsid protein labeled with fluorophore B will allow the measurement of energy transfer from fluorophore A to B, depending on how close is fluorophore A from B. Energy transfer will be an indication of proximity that could be used as an indirect measure of binding. To test for specificity of this *in vivo* binding assay, we could measure binding of TRIMCyp to HIV-1 viruses containing the capsid mutant G89V or perform the binding in the presence of cyclosporine A, which inhibits the binding of TRIMCyp to *in vitro* assembled HIV-1 CA-NC complexes (Diaz-Griffero et al., 2006b; Stremlau et al., 2006). Even though this is a simple approach, tagging the HIV-1 capsid with a fluorophore in a manner that will not disrupt formation of the mature core is challenging, since the capsid forms the mature HIV-1 core by forming a higher order structure (Ganser-Pornillos et al., 2008).

Because we had previously demonstrated the ability of TRIM5α_{rh} to shuttle in and out of the nucleus (Diaz-Griffero et al., 2011), we tested whether variants that bind HIV-1 capsid but do not restrict the virus are still able to shuttle in and out of the nucleus. Our results demonstrated that shuttling is normal for these variants, suggesting that a different function is lost.

Our extensive mutagenesis supports the hypothesis that binding to HIV-1 capsid could occur in the absence of restriction. To corroborate this hypothesis, we also tested binding of TRIM family members closely related to TRIM5 proteins that do not restrict HIV-1 infection. In agreement, TRIM6 and TRIM34 proteins, which do not restrict HIV-1 infection, bound HIV-1 capsid *in vitro* (Li et al., 2007; Zhang et al., 2006). Similarly, TRIM5α proteins from chimpanzee and tamarin that do not restrict HIV-1 infection bound HIV-1 capsid *in vitro*. Overall this work supports the notion that HIV-1 restriction by TRIM5α_{rh} requires capsid binding and a function provided by the PRYSPRY domain. Future experiments will attempt to elucidate this additional function required for restriction.

Materials and Methods

Creation of cells stably expressing TRIM5α variants. Retroviral vectors encoding wild type or mutant rhesus monkey TRIM5α_{rh} proteins were created using the pLPCX vector. The TRIM5α_{rh} proteins contained an influenza hemagglutinin (HA) epitope tag at the C terminus. Recombinant viruses were produced in 293T cells by cotransfecting the pLPCX plasmids with the pVPack-GP and pVPack-VSV-G packaging plasmids (Stratagene). The pVPack-VSV-G plasmid encodes the vesicular stomatitis virus (VSV) G envelope glycoprotein, allowing efficient entry into a wide range of vertebrate cells. Cf2Th canine thymocytes were transduced and selected in 5 μg/ml puromycin (Sigma).

Protein analysis—Cellular proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris, pH 7.4; 100 mM NaCl; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate [SDS]; 1% NP-40; 2 mg/ml aprotinin; 2 mg/ml leupeptin; 1 mg/ml pepstatin A; 100 mg/ml phenylmethylsulfonyl fluoride). Lysates were analyzed by SDS-PAGE (10% acrylamide), followed by blotting onto nitrocellulose membranes (Amersham Pharmacia Biotech). Protein detection by Western blotting utilized monoclonal antibodies directed against the HA epitope tags (Roche), FLAG epitope tags (Sigma) and monoclonal antibodies to GAPDH (Sigma) directly conjugated to Alexa Fluor 680. Bands were detected by scanning blots with the LI-COR Odyssey Imaging System using both 700 and 800 channels, and integrated intensities were determined using the LI-COR Odyssey band quantitation software with the median top-bottom background subtraction method.

Infection with viruses expressing green fluorescent protein (GFP)—Recombinant HIV-1 expressing GFP was prepared as described (Diaz-Griffero et al., 2008). Recombinant viruses were pseudotyped with the VSV-G glycoprotein. For infections, 3×10^4 Cf2Th cells seeded in 24-well plates were incubated at 37ºC with virus for 24 hours. Cells were washed and returned to culture for 48 hours, and then subjected to FACS analysis with a FACScan (Becton Dickinson). HIV-1 and N-MLV viral stocks were titrated by serial dilution on Cf2Th cells to determine the concentration of infectious viruses.

HIV-1 CA-NC expression and purification. The HIV-1 CA-NC protein was expressed, purified and assembled as previously described (Ganser et al., 1999; Ganser-Pornillos et al., 2004). The pET11a expression 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 hours of induction, the cells were harvested and resuspended in 20 mM Tris-HCl (pH 7.5), 1μM $ZnCl_2$, 10 mM 2-mercaptoethanol and protease inhibitors (Roche). Lysis was performed by sonication and debris was pelleted for 30 minutes at 35,000 × *g*. Nucleic acids were stripped from the solution by using 0.11 equivalents of 2M $(NH_4)_2SO_4$ and the same volume of 10% polyethylenimine. Nucleic acids were removed by stirring and centrifugation at 29,500 × *g* for 15 minutes. Protein was recovered by addition of 0.35 equivalents of saturated $(NH_4)_2SO_4$. The protein was centrifuged at 9,820 × *g* for 15 minutes and resuspended in 100 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 μM $ZnCl_2$ and 10 mM 2-mercaptoethanol. The CA-NC protein was dialyzed against 50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 μM $ZnCl_2$ and 10 mM 2-mercaptoethanol, and stored at −80ºC.

In vitro assembly of CA-NC complexes. HIV-1 CA-NC particles were assembled *in vitro* by diluting the CA-NC protein to a concentration of 0.3 mM in S0 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 8,600 × g for 5 minutes. The pellet was resuspended in assembly buffer (S0 mM Tris-HCl (pH 8.0), 0.5 M NaCl) at a final protein concentration of 0.15 mM (Ganser et al., 1999; Ganser-Pornillos et al., 2004), and stored at 4ºC until needed.

Binding of TRIM5a_{rh} variants to HIV-1 capsid complexes—293T cells were transfected with plasmids expressing wild-type or mutant TRIM5α_{rh} proteins. Forty-eight hours after transfection, cell lysates were prepared as follows: previously washed cells were resuspended in hypotonic lysis buffer (10 mM Tris, pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT). The cell suspension was frozen and thawed, and incubated on ice for 10 minutes. Afterwards, the lysate was centrifuged at maximum speed in a refrigerated Eppendorf micro centrifuge (~ 14,000 × g) for 5 minutes. The supernatant was supplemented with 1/10 volume of 10X PBS and then used in the binding assay. In some cases, samples containing the TRIM5αrh variants were diluted with extracts prepared in parallel from untransfected cells. To test binding, 5 μl of CA-NC particles assembled *in vitro* were incubated with 200 μl of cell lysate at room temperature for 1 hour. A fraction of this mixture was stored (input). The mixture was spun through a 70% sucrose cushion (70% sucrose, 1X PBS and 0.S mM DTT) at 100,000 × *g* in an SW55 rotor (Beckman) for 1 hour at 4°C. After centrifugation, the supernatant was carefully removed and the pellet resuspended in 1X SDS-PAGE loading buffer (pellet). The level of TRIM5αrh proteins was determined by Western blotting with an anti-HA antibody as described above. The level of HIV-1 CA-NC protein in the pellet was assessed by Western blotting with an anti-p24 CA antibody.

Immunofluorescence microscopy in the presence of leptomycin B—Transfections of cell monolayers were performed using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. Transfected cells were incubated at 37 ºC for 24 hours. Cells were treated for 5 hours in the presence of leptomycin B (20 ng/ml) or DMSO for 5 hour. Monolayers were washed twice with PBS and fixed for 15 min with 4% paraformaldehyde in PBS. Fixed cells were washed twice with PBS, incubated for 4 min in permeabilizing buffer (0.5% Triton X-100 in PBS), and then blocked using PBS containing 2% bovine serum albumin for 1 h at room temperature. Subsequently, cells were incubated for 1 h at room temperature with primary antibodies in blocking buffer as described (Brandariz-Nunez et al., 2013). After three washes with PBS, the cells were incubated for 30 min with secondary antibodies. Cellular nuclei were labeled with 1 mg/ml of DAPI (4',6-diamidino-2-phenylindole) in PBS. Subsequently, samples were mounted for fluorescence microscopy using the ProLong Antifade Kit (Molecular Probes, Eugene, OR). Images were obtained with a Zeiss Observer Z1 microscope using a 63X objective, and deconvolution was performed using the software AxioVision V4.8.1.0 (Carl Zeiss Imaging Solutions).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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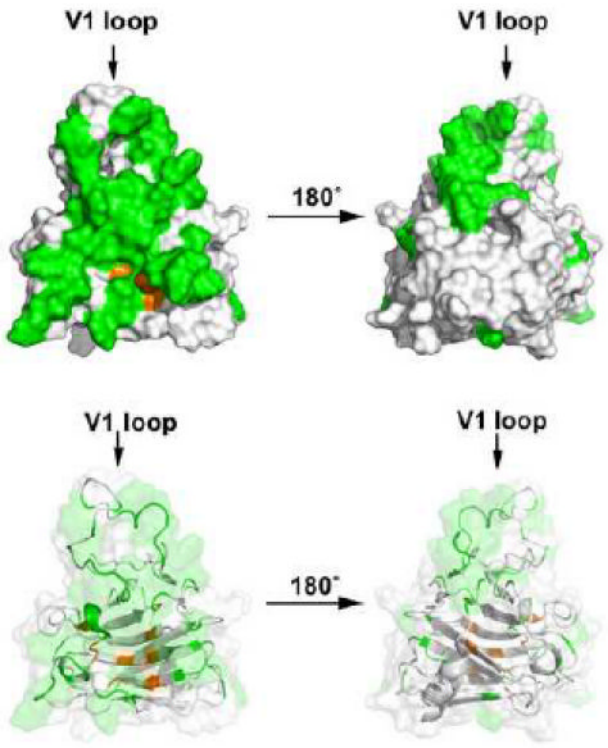


Figure 1. Structure of the PRYSPRY of TRIM5α$_{rh}$ showing the residues targeted in this study

The upper structures show the surface of the PRYSPRY domain of TRIM5α$_{rh}$. Surface and internal residues targeted for mutagenesis are shown in green and orange, respectively. The lower structures show a transparent surface revealing the polypeptide chain of the TRIM5α$_{rh}$ PRYSPRY that similarly labels surface and internal residues targeted for mutagenesis in green and orange, respectively.

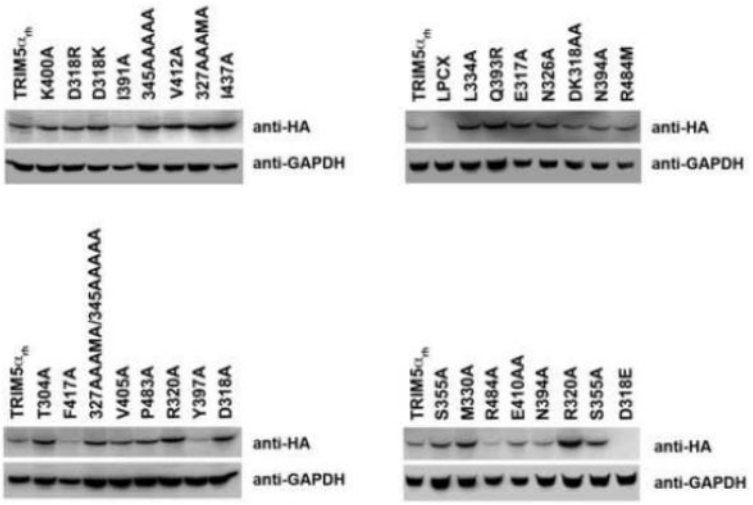


Figure 2. Expression of TRIM5α$_{rh}$ PRYSPRY domain variants

Dog Cf2Th cells were transduced with the LPCX vector expressing HA-tagged wild-type and mutant TRIM5α$_{rh}$ proteins. Stable cell lines were selected with 5 μg/ml of puromycin, and the expression levels of mutant and wild-type TRIM5α proteins were assayed by Western blotting using anti-HA antibodies. To control for protein loading, samples were Western blotted using anti-GAPDH antibodies. The results of three independent experiments were similar, and the results of a single experiment are shown.

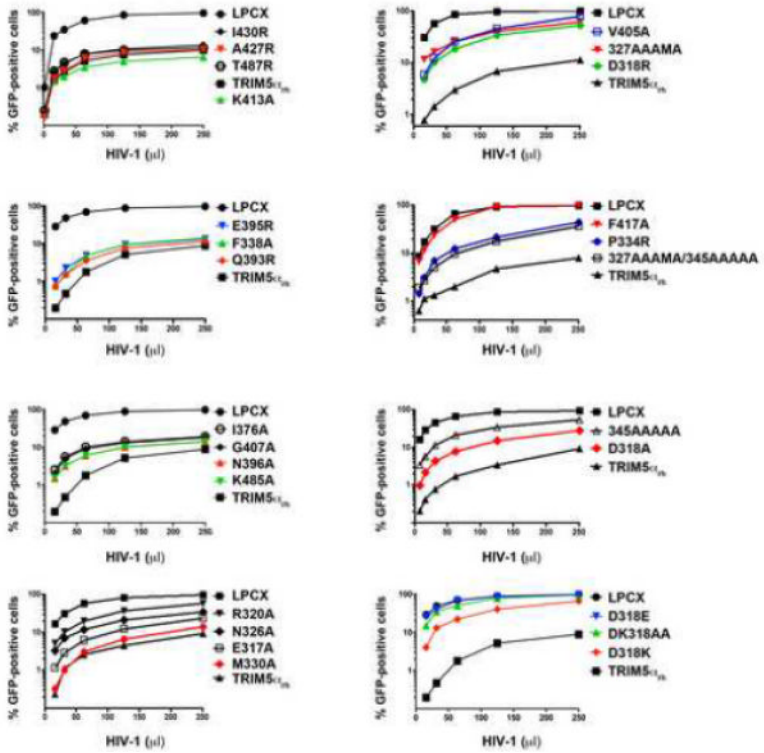


Figure 3. Restriction of HIV-1 infection by TRIM5α$_{rh}$ PRYSPRY variants

Dog Cf2Th cells stably expressing the different TRIM5α$_{rh}$ PRYSPRY variants were challenged with increasing amounts of HIV-1-GFP. Infection was determined by measuring the number of GFP-positive cells 48 hours post-infection using a flow cytometer. The graphs are organized containing mutants from potent to less potent restriction (top to button). Similar results were obtained in three independent experiments, and the result of one experiment is shown.

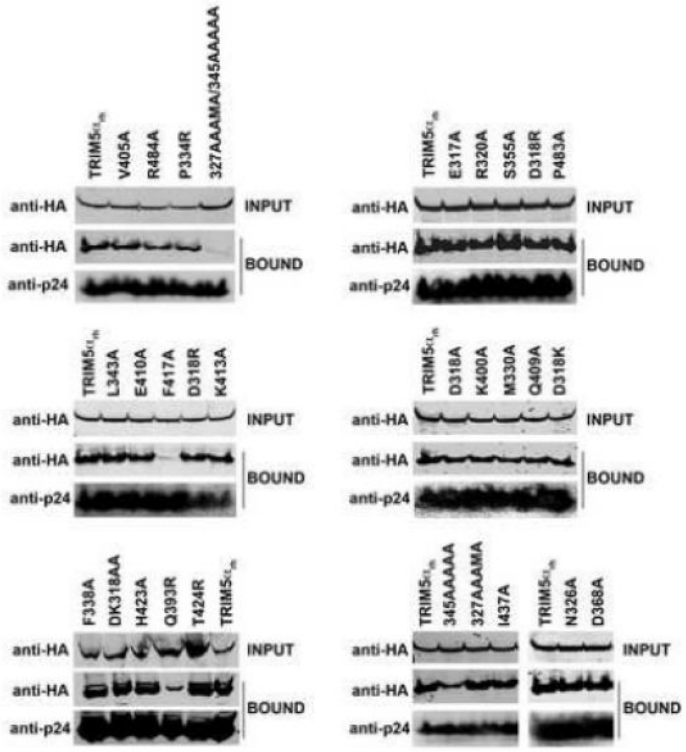


Figure 4. Binding of TRIM5α$_{rh}$ PRYSPRY variants to *in vitro* assembled HIV-1 capsid–nucleocapsid complexes

Human 293T cells were transfected with plasmids expressing the indicated wild-type and mutant HA-tagged TRIM5α$_{rh}$ proteins. Forty-eight hours after transfection, cells were lysed. The lysates were incubated at room temperature for 1 h together with *in vitro* assembled HIV-1 CA-NC complexes. The mixtures were applied to a 70% sucrose cushion and centrifuged. **INPUT** represents the lysates analyzed by Western blotting before being applied to the 70% cushion. The input mixtures were Western blotted using anti-HA antibodies. Similarly, the pellets from the 70% cushion, which represents the **BOUND** fraction**,** were analyzed by Western blotting using anti-HA and anti-p24 antibodies. The results of three independent experiments were similar; the result of a single experiment is shown.

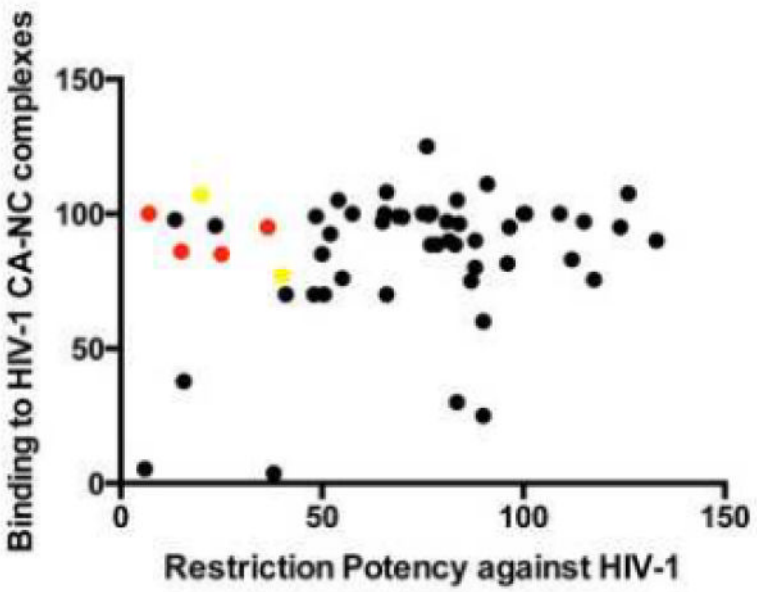
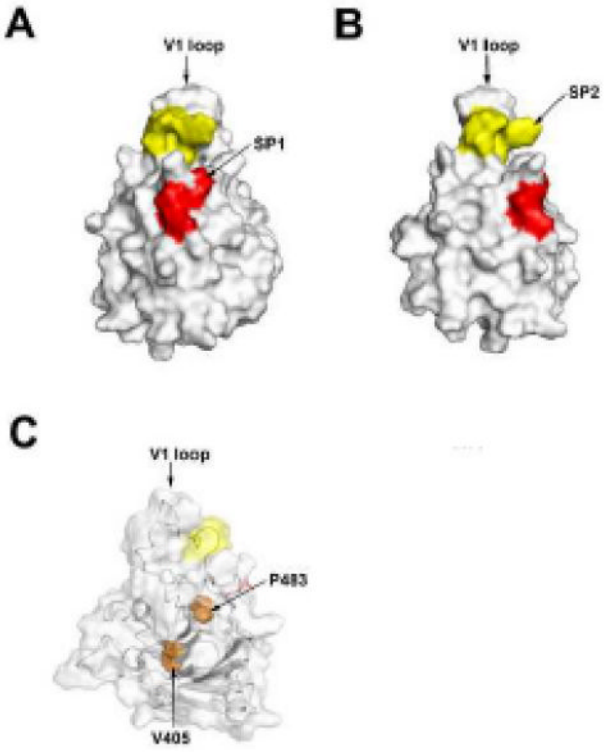


Figure 5. Binding of Trim5α_{rh} to HIV-1 capsid is necessary but not sufficient for restriction

The relative binding of the different TRIM5α_{rh} PRYSPRY domain variants to *in vitro* assembled HIV-1 CA-NC complexes was calculated as described in Table 1. Similarly, the ability of the different TRIM5α_{rh} PRYSPRY domain variants to restrict HIV-1 was calculated as described in Table 1. The values for relative binding to HIV-1 CA-NC complexes were plotted as a function of HIV-1 restriction. Dots in red and yellow a represent TRIM5α_{rh} PRYSPRY domain variants that are located on the surface patches SP1 and SP2, respectively.



	316	SP1						
rhesus m.	A	E	D	K	R	Q	V	S
human	S	E	D	K	R	Q	V	S
agm (pyg)	A	E	D	K	R	Q	V	S
agm (tan)	A	E	D	K	R	Q	V	S
tamarin	S	E	D	E	R	Q	V	R
spider m.	S	E	D	E	R	Q	V	R
squirrel m.	S	E	D	G	R	Q	V	R
orangutan	S	E	D	M	R	Q	V	S
chimpanzee	S	E	D	M	R	Q	V	S

	324		SP2							
rhesus m.	S	R	N	P	Q	I	M	Y	Q	A
agm (tan)	Y	Q	N	P	Q	I	M	Y	Q	A
agm (pyg)	Y	R	N	P	Q	I	M	Y	Q	S
human	S	P	K	P	Q	I	I	Y	G	A
chimpanzee	S	P	K	P	Q	I	I	Y	G	A
orangutan	S	P	E	P	Q	I	T	Y	G	A
squirrel m.	Y	Q	K	P	I	R	H	L	L	V
tamarin	Y	Q	F	Q	I	H	Q	P	S	V
spider m.	Y	Q	E	Q	I	H	Q	P	S	V

Figure 6. TRIM5α_{rh} PRYSPRY domain residues that are important for restriction but dispensable for binding to HIV-1 capsid

(A) The surface patch 1 (SP1), composed of D318, K319 and R320, is shown in red. **(B)** The surface patch 2 (SP2), composed of N326, P327, Q328, I329, M330 and Y331, is shown in yellow. **(C)** The polypeptide chain of the TRIM5α_{rh} PRYSPRY domain showing the internal residues V405 and P483 in orange. In addition the structure shows SP1 and SP2 in red and yellow, respectively. **(D)** The conservation of SP1 and SP2 is shown in human and different species of primates such as rhesus macaques (rhesus m.), African green monkey pyg [agm (pyg)], African green monkey Tantalus [agm (tan)], orangutan, chimpanzee, spider monkey, tamarin monkey and squirrel monkey. The number for the rhesus macaque protein sequence is shown.

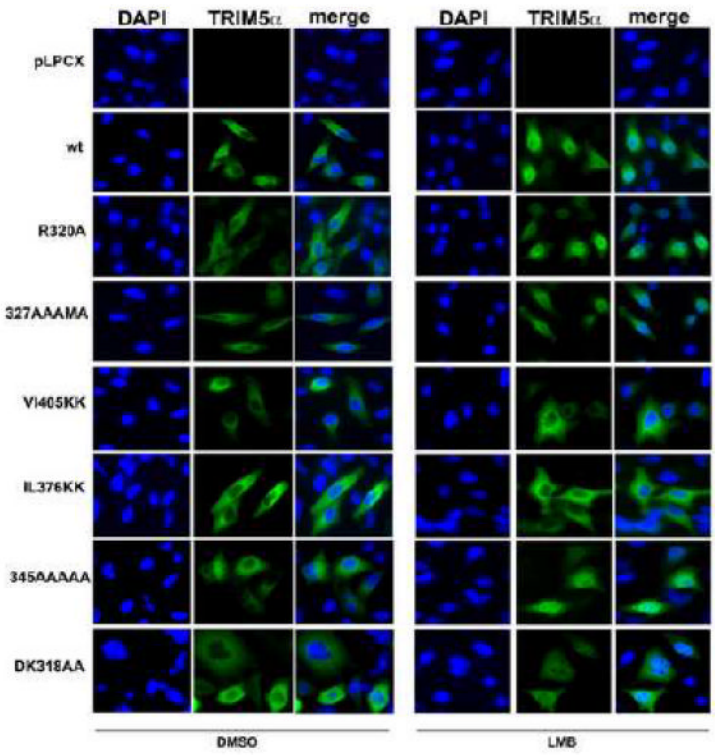


Figure 7. Intracellular distribution of TRIM5α$_{rh}$ PRYSPRY domain variants in the presence of leptomycin B

Human HeLa cells expressing the indicated TRIM5α$_{rh}$ variants were treated with 20 ng/ml of leptomycin B (LMB) or DMSO for 5 h. Treated cells were fixed and immunostained using anti-HA antibodies (green). Nuclei were stained with DAPI (blue). The results of three independent experiments were similar and a representative figure is shown. Quantification is shown in Figure S4.

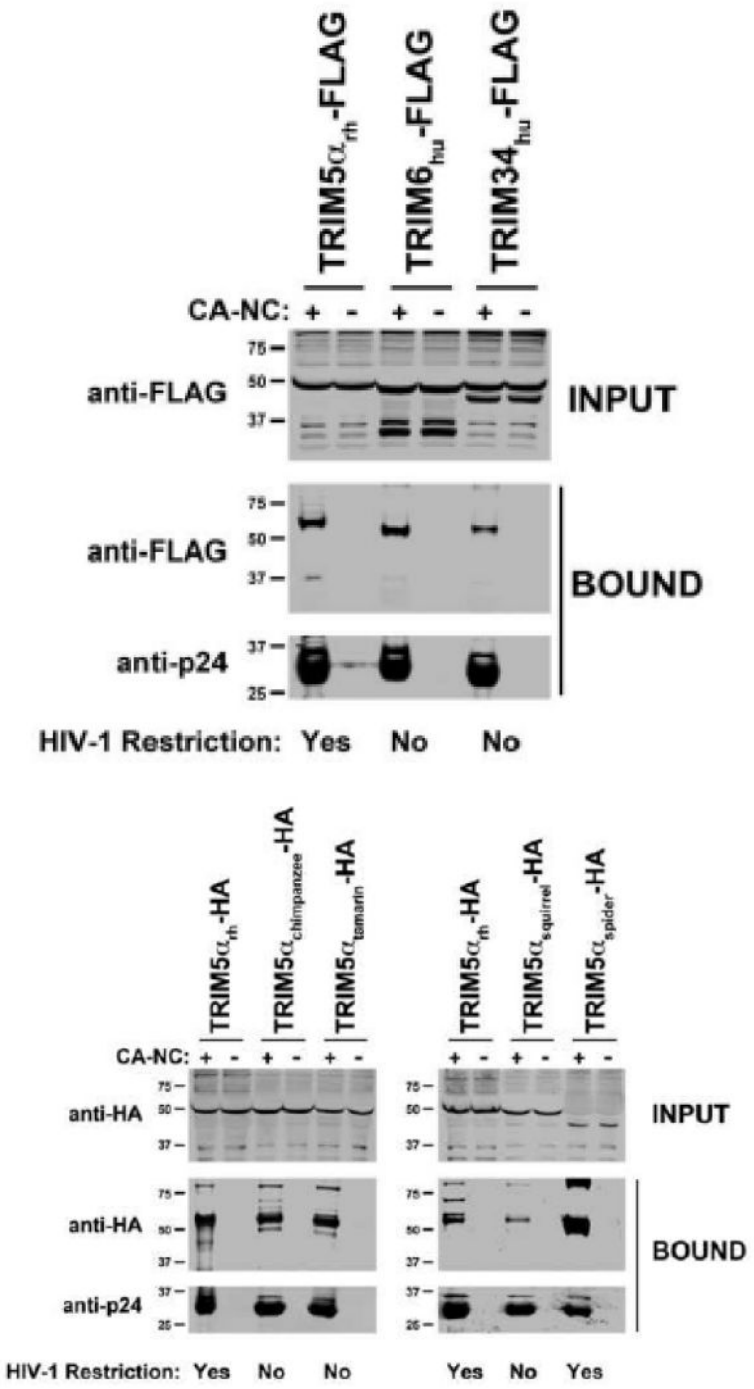


Figure 8. Tripartite motif proteins that bind HIV-1 capsid but do not restrict HIV-1 infection

(A) The ability of TRIM6 and TRIM34 to bind *in vitro* assembled HIV-1 CA-NC complexes was performed, as described in Materials and Methods. Briefly, lysates containing TRIM6-FLAG or TRIM34-FLAG were incubated at room temperature for 1 h together with *in vitro* assembled HIV-1 CA-NC complexes. The mixtures were applied to a 70% sucrose cushion and centrifuged. **INPUT** represents the lysates analyzed by Western blotting before being applied to the 70% cushion. The input mixtures were Western blotted using anti-HA antibodies. Similarly, the pellets from the 70% cushion, which represents the **BOUND**

fraction**,** were analyzed by Western blotting using anti-HA and anti-p24 antibodies. The results of three independent experiments were similar; the result of a single experiment is shown. **(B)** Similarly, we tested the ability of TRIM5α proteins from the indicated monkeys to bind *in vitro* assembled HIV-1 CA-NC complexes. The ability of these proteins to restrict HIV-1 is indicated in the bottom of the figure. The results of three independent experiments were similar and a representative figure is shown.

The PRYSPRY domain of TRIM5α_{rh} provides an unknown function to HIV-1 restriction.

The PRYSPRY is important for the ability of TRIM5α_{rh} to bind HIV-1 capsid.

Human TRIM6 and TRIM34 bind HIV-1 capsid but don’t restrict HIV-1 infection.

Table 1

TRIM5α_{rh} PRYSPRY variants ranked according to restriction ability.

TRIM5α_{rh} Variant	Present at the Surface of the Structure[a]	Expression in Cf2Th Cells[b]	Restriction Potency HIV±SD[c]		Mean Binding to HIV-1 CA-NC Complexes±SD[d]		
Y331A	+	Y	133	1,41	90	7,77	
H423A	+	Y	126	5,65	107,5	6,36	
S373R	+	Y	124	2,82	95	8,48	
K475A	+	Y	117,5	2,12	75,5	4,94	
K413A	+	Y	115	0,11	97	15,55	
R484M	+	Y	112	5,65	83	0,23	
F426A	+	Y	109	5,65	100	4,24	
A427R	+	Y	100,5	3,53	100	5,24	
Wt		Y	100	1,22	100	0,98	
F429A	+	Y	96,5	7,77	95	2,12	
T487R	+	Y	96	1,41	81,5	4,94	
I430R	+	Y	91	7,77	111	0,53	
Q409R	+	Y	90	12,72	60	7,77	
Q393R	+	Y	90	5,65	25	0,7	
F338A	+	Y	88	1,62	90	4,94	
E395R	+	Y	88	7,77	80	1,41	
M330A	+	Y	87	1,41	75	5	
N394A	+	Y	84	5,65	96,3	4,66	
Q409A	+	Y	83,5	3,53	105	5,65	
T424R	+	Y	83,5	3,53	30	7,07	
N394R	+	Y	83	2,82	88,5	6,36	
N396A	-	Y	81,5	4,94	90	5,65	
K485A	+	Y	81	8,48	97	6,36	
K467A	+	Y	78,5	4,94	88,5	2,12	
T304A	+	Y	77	1,41	100	3,44	
G407A	-	Y	77	4,24	88,5	30,4	
1376A	-	Y	76,5	2,12	99,5	4,94	
R325A	+	Y	76	7,07	125	5,65	
S355A	+	Y	75	2,82	100	0,67	
E317A	+	Y	70	3,53	98,75	0,35	
K319A	+	Y	69	5,65	99	1,41	
F340A	+	Y	66	5,65	70	3,04	
E410A	+	Y	66	5,65	108	25,45	
I437A	+	Y	65,5	6,36	100	0,55	
D36SA	+	Y	65	4,24	97	4,24	
V412A	+	Y	57,5	3,53	100	0,32	

TRIM5α$_{rh}$ Variant	Present at the Surface of the Structure[a]	Expression in Cf2Th Cells[b]	Restriction Potency HIV±SD[c]		Mean Binding to HIV-1 CA-NC Complexes±SD[d]		
D318A	+	Y	55	7,07	76	3,18	
D302A	+	N	54	5,65	105	5,65	
L343A	+	Y	52	7,77	92,5	10,6	
R484A	+	N	50,5	7,77	70	8,88	
Y348A	+	Y	50	7,07	85	3,53	
K400A	+	Y	48,5	3,53	99	2,82	
R441A	+	Y	48	2,82	70	0,8	
P334R	+	Y	41	4,24	70	5,65	
N32GA	+	Y	40	8,48	77	0,98	**SP2**
327AAAMA/345AAAAA	+	Y	38	5,65	3,65	1,9	
D318K	+	Y	36,5	0,7	95	4,1	**SP1**
D318R	+	Y	25	4,94	85	7,07	**SP1**
V405A	-	Y	23,5	4,94	95,5	12,02	
327AAAMA	+	Y	20	4,24	107	28,58	**SP2**
345AAAAA	+	Y	15,5	0,7	38	4,03	
R320A	+	Y	15	1,41	86	8,48	**SP1**
P483A	-	Y	13,5	3,53	97,86	18,5	
DK318AA	+	Y	7	5,65	100	1,41	**SP1**
F417A	-	N	6	1	5,3	4,66	
S415A	+	N	4	1,41	ND		
Y397A	-	N	3,66	1,15	ND		
D318E	+	N	3,3	0,42	ND		
Y364A	-	N	3	0,33	ND		
I391A	-	N	3	0,44	23	3,05	

TRIM5α$_{rh}$ PRYSPRY variants that lead to the identification of surface patches SPl and SP2 and shaded in gray, and the particular surface patch is indicated on the right side of the table.

[a] Localization of residues on the surface of the TRlM5α$_{rh}$ PRYSPRY structure indicated as follows:"+" means that the residue is on the surface of the structure;"-" means that the residue is in the interior of the structure.

[b] Expression of each TRlMSarh variant was measured by Western blot using anti-HA antibodies and quantified as described in Materials and Methods. "Y" signifies that the expression of the variant is equal or higher when compared to the wild type (wt); "N" means that the expression is lower relative when comapred to the wt.

[c] Restriction was measured by infecting cells expressing the indicated TRlM5α$_{rh}$ variants with HIV-1 expressing GFP. After 48 hours, the percentage of GFP-positive cells (infected cells) was determined by flow cytometry. Restriction potency was defined here as the fraction of TRlM5α$_{rh}$ PRYSPRY variant fold-restriction relative to the wild-type's fold-restriction when 50% of the control cells are infected. Experiments were performed at least three times and standard deviations (SD) are shown.

[d] Binding to the HIV-1 capsid complexes was determined for each TRlM5α$_{rh}$ variant as described in Materials and Methods. Binding is expressed as the amount of the TRlM5α$_{rh}$ variant bound to HIV-1 capsid complexes divided by the amount of bound wild-type TRlM5α$_{rh}$ at a similar input level. Experiments were repeated at least three times. The means and standard deviations (SD) are shown. Note that because the binding ratios are calculated at input levels at which some binding of the mutant TRlM5α$_{rh}$ protein to the HIV- 1 capsid complexes can be detected, these ratios overestimate the relative capsid-binding affinities of the mutant proteins.

Table 2

TRIM5α_{rh} PRYSPRY variants ranked according to capsid binding ability.

TRIM5α_{rh} Variant	Present at the Surface of the Structure[a]	Expression in Cf2Th Cells[b]	Restriction Potency HIV±sd[c]		Mean Binding to HIV-1 CA-NC Complexes±SD[d]		
R325A	+	Y	76	7,07	125	5,65	
I430R	+	Y	91	7,77	111	0,53	
E410A	+	Y	66	5,65	108	25,45	
H423A	+	Y	126	5,65	107,5	6,36	
327AAAMA	+	Y	20	4,24	107	28,58	**SP2**
Q409A	+	Y	83,5	3,53	105	5,65	
D302A	+	N	54	5,65	105	5,65	
F426A	+	Y	109	5,65	100	4,24	
A427R	+	Y	100,5	3,53	100	5,24	
wt		Y	100	1,22	100	0,98	
T304A	+	Y	77	1,41	100	3,44	
S355A	+	Y	75	2,82	100	0,67	
I437A	+	Y	65,5	6,36	100	0,55	
V412A	+	Y	57,5	3,53	100	0,32	
DK318AA	+	Y	7	5,65	100	1,41	**SP1**
I376A	-	Y	76,5	2,12	99,5	4,94	
K319A	+	Y	69	5,65	99	1,41	
K400A	+	Y	48,5	3,53	99	2,82	
E317A	+	Y	70	3,53	98,75	0,35	
P483A	-	Y	13,5	3,53	97,86	18,5	
K413A	+	Y	115	0,11	97	15,55	
K485A	+	Y	81	8,48	97	6,36	
D368A	+	Y	65	4,24	97	4,24	
N394A	+	Y	84	5,65	96,3	4,66	
V405A	-	Y	23,5	4,94	95,5	12,02	
S373R	+	Y	124	2,82	95	8,48	
F429A	+	Y	96,5	7,77	95	2,12	
D318K	+	Y	36,5	0.7	95	4,1	**SP1**
L343A	+	Y	52	7,77	92,5	10,6	
Y331A	+	Y	133	1,41	90	7,77	
F338A	+	Y	88	1,62	90	4,94	
N396A	-	Y	81,5	4,94	90	5,65	
N394R	+	Y	83	2,82	88,5	6,36	
K467A	+	Y	78,5	4,94	88,5	2,12	
G407A	-	Y	77	4,24	88,5	30,4	
R320A	+	Y	15	1,41	86	8,48	**SP1**

TRIM5α$_{rh}$ Variant	Present at the Surface of the Structure[a]	Expression in Cf2Th Cells[b]	Restriction Potency HIV±sd[c]		Mean Binding to HIV-1 CA-NC Complexes±SD[d]		
Y348A	+	Y	50	7,07	85	3,53	
D318R	+	Y	25	4,94	85	7,07	**SP1**
R484M	+	Y	112	5,65	83	0,23	
T487R	+	Y	96	1,41	81,5	4,94	
E395R	+	Y	88	7,77	80	1,41	
N326A	+	Y	40	8,48	77	0,98	**SP2**
D318A	+	Y	55	7,07	76	3,18	
K475A	+	Y	117,5	2,12	75,5	4,94	
M330A	+	Y	87	1,41	75	5	
F340A	+	Y	66	5,65	70	3,04	
R484A	+	N	50,5	7,77	70	8,88	
R441A	+	Y	48	2,82	70	0,8	
P334R	+	Y	41	4,24	70	5,65	
Q409R	+	Y	90	12,72	60	7,77	
345AAAAA	+	Y	15,5	0,7	38	4,03	
T424R	+	Y	83,5	3,53	30	7,07	
Q393R	+	Y	90	5,65	25	0,7	
I391A	-	N	3	0,44	23	3,05	
F417A	-	N	6	1	5,3	4,66	
327AAAMA/345AAAAA	+	Y	38	5,65	3,65	1,9	
S415A	+	N	4	1,41	ND		
Y397A	-	N	3,66	1,15	ND		
D318E	+	N	3,3	0,42	ND		
Y364A	-	N	3	0,33	ND		

TRIM5α$_{rh}$ PRYSPRY variants that lead to the identification of surface patches SPl and SP2 are shaded in gray, and the particular surface patch is indicated on the right side of the table.

[a] Localization of residues on the surface of the TRIM5arh PRYSPRY structure indicated as follows:"+" means that the residue is on the surface of the structure;"-" means that the residue is in the interior of the structure.

[b] Expression of each TRlM5α$_{rh}$ variant was measured by Western blot using anti-HA antibodies as described in Materials and Methods. "Y" signifies that the expression of the variant is equal or higher when compared to the wild type (wt); "N" means that the expression is lower when compared to the wt.

[c] Restriction was measured by infecting cells expressing the indicated TRlM5α$_{rh}$ variants with HIV-1 expressing GFP. After 48 hours, the percentage of GFP-positive cells (infected cells) was determined by flow cytometry. Restriction potency was defined as the fraction of TRlM5α$_{rh}$ PRYSPRY variant fold-restriction relative to the wild-type's fold-restriction when 50% of the control cells are infected. Experiments were performed at least three times and standard deviations (SD) are shown.

[d] Binding to the HIV-1 capsid complexes was determined for each TRlM5α$_{rh}$ variant as described in Materials and Methods. Binding is expressed as the amount of the TRlM5α$_{rh}$ variant bound to HIV-1 capsid complexes divided by the amount of bound wild-type TRlM5α$_{rh}$ at a similar input level. Experiments were repeated at least three times. The means and standard deviations (SD) are shown. Note that because the binding ratios are calculated at input levels at which some binding of the mutant TRlM5α$_{rh}$ protein to the HIV- 1 capsid complexes can be detected, these ratios overestimate the relative capsid-binding affinities of the mutant proteins.