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Functional Interplay Between the B-box 2 and the B30.2(SPRY) Domains of TRIM5α

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Abstract

The retroviral restriction factors, TRIM5α and TRIMCyp, consist of RING and B-box 2 domains separated by a coiled coil from carboxy-terminal domains. These carboxy-terminal domains (the B30.2(SPRY) domain in TRIM5α and the cyclophilin A domain in TRIMCyp) recognize the retroviral capsid. Here we show that some B-box 2 changes in TRIM5α, but not in TRIMCyp, resulted in decreased human immunodeficiency virus (HIV-1) capsid binding. The phenotypic effects of these B-box 2 changes on the restriction of retroviral infection depended on the potency of restriction and the affinity of the TRIM5α interaction with the viral capsid, two properties specified by the B30.2(SPRY) domain. Thus, some alterations in the TRIM5α B-box 2 domain apparently affect the orientation or conformation of the B30.2(SPRY) domain, influencing capsid recognition.

Keywords

retrovirus; restriction factor; TRIMCyp; cyclophilin; trimer; B-box 2; B30.2(SPRY); HIV-1; N-MLV

Introduction

Retroviruses encounter potent blocks to infection in certain mammalian species. In many cases, the restriction is mediated by dominant host factors, such as Fv1 (Best et al., 1996; Hartley, Rowe, and Huebner, 1970; Lilly, 1967; Lilly, 1970), APOBEC3G (Sheehy et al., 2002) and TRIM5α (Stremlau et al., 2004). TRIM5α blocks retroviral infection at an early post-entry step in a species-specific manner. Rhesus monkey TRIM5α (TRIM5αRh) potently blocks the infection of human immunodeficiency virus-1 (HIV-1) and a range of other retroviruses; by contrast, human TRIM5α (TRIM5αHu) modestly restricts HIV-1 infection and potently blocks infection by N-tropic murine leukemia viruses (N-MLV) (Hatziioannou et al., 2004; Keckesova, Ylinen, and Towers, 2004; Perron et al., 2004; Stremlau et al., 2004; Yap et al., 2004). The mechanism by which TRIM5α blocks retroviral infection has not been clearly defined. Previous studies demonstrated that TRIM5α proteins specifically recognize the viral cores and accelerate the uncoating process (Li et al., 2006c; Perron et al., 2006; Sebastian and...
Luban, 2005; Stremlau et al., 2006), thus potentially interfering with the orderly disassembly of the viral capsid (Forshey et al., 2002).

TRIM5α is a member of the large tripartite motif (TRIM) protein family (Reymond et al., 2001). TRIM proteins contain RING, B-box 2 and coiled-coil domains; many cytoplasmic TRIM proteins, like TRIM5α, also contain a B30.2(SPRY) domain (Meroni and Diez-Roux, 2005; Reymond et al., 2001). The B30.2(SPRY) domain of TRIM5α determines viral specificity and potency of restriction by modulating recognition of the retroviral capsid (Stremlau et al., 2005; Yap, Nisole, and Stoye, 2005; Perez-Caballero et al., 2005a; Li et al., 2006c). For example, in an in vitro binding assay, the B30.2(SPRY) domain was shown to be the major determinant of the association of TRIM5α_R with the assembled HIV-1 capsid-nucleocapsid (CA-NC) complex (Li et al., 2006c; Stremlau et al., 2006).

The role of TRIM5α domains other than the B30.2(SPRY) domain in antiretroviral activity has been investigated. The RING finger domain is the signature of a class of E3 ubiquitin ligases involved in proteasome-mediated protein degradation (Meroni and Diez-Roux, 2005). Indeed, some TRIM proteins have been shown to exhibit ubiquitin ligase activity and modify their target proteins with ubiquitin (Dupont et al., 2005; Horn et al., 2004; Meroni and Diez-Roux, 2005; Trockenbacher et al., 2001; Xu et al., 2003). However, deletion of the TRIM5α RING domain only partially attenuated antiviral activity (Javanbakht et al., 2005; Perez-Caballero et al., 2005a; Stremlau et al., 2004). Moreover, TRIM5α-mediated restriction activity was not affected by modulation of E1 ubiquitin ligase activity in a temperature-dependent cell line (Perez-Caballero et al., 2005b). Finally, proteasome inhibitors did not impair the restriction activity of TRIM5α (Perez-Caballero et al., 2005b; Stremlau et al., 2006; Anderson et al., 2006; Wu et al., 2006). Proteasome inhibitors can rescue viral reverse transcription from the TRIM5α-mediated restriction, but the mechanistic basis for this phenomenon is unknown (Anderson et al., 2006; Wu et al., 2006). The B-box 2 domain plays an important but poorly understood role in the function of TRIM proteins (Meroni and Diez-Roux, 2005; Reymond et al., 2001). Deletion of the B-box 2 domain of TRIM5α eliminated its antiretroviral activities (Javanbakht et al., 2005; Perez-Caballero et al., 2005a). It has been suggested that the RING and B-box 2 domains may specify an “effector” function, either by recruiting an additional cofactor or by participating in particular types of self-association (Diaz-Griffero et al., 2006b; Perez-Caballero et al., 2005a; Stremlau et al., 2006). The coiled-coil domain is known to be essential for both homomultimerization and heteromultimerization of many TRIM proteins (Cao et al., 1997; Javanbakht et al., 2006; Mische et al., 2005; Perez-Caballero et al., 2005a; Reymond et al., 2001). TRIM5α trimerization, which depends on the coiled coil and the L2 linker region connecting the coiled-coil and B30.2 domains, contributes greatly to the avidity for the retroviral capsid and the restriction of viral infection (Javanbakht et al., 2006).

In this study, we altered a combination of residues in the B-box 2 domain of wild-type human and rhesus monkey TRIM5α, as well as chimeric and mutant TRIM5α proteins, and examined the effects of these changes on antiretroviral activity against human immunodeficiency virus-1 (HIV-1), N-tropic Moloney leukemia virus (N-MLV) and simian immunodeficiency virus (SIVmac). The results show that, depending on the restriction potency of the parental proteins, a property determined by the B30.2(SPRY) domain, the same alterations of the B-box 2 domain can confer different phenotypes on retrovirus restriction. Moreover, the B-box 2 alterations used in this study dramatically affected the association of the TRIM5α proteins with in vitro assembled HIV-1 CA-NC complexes, suggesting a functional interplay between the TRIM5α B-box 2 and the B30.2(SPRY) domains.
**Results**

**Differential effects of TRIM5α B-box 2 changes on HIV-1 and N-MLV restriction**

Despite 87% sequence identity, rhesus monkey TRIM5α (TRIM5α_{rh}) and human TRIM5α (TRIM5α_{hu}) exhibit differences in the spectrum of restricted retroviruses due to divergence in the B30.2 domains (Perez-Caballero et al., 2005a; Perron, Stremlau, and Sodroski, 2006; Stremlau et al., 2005; Yap, Nisole, and Stoye, 2005). TRIM5α_{th} more potently restricts HIV-1 infection than TRIM5α_{hu}, whereas TRIM5α_{hu} more potently restricts the infection of N-MLV (Hatziioannou et al., 2004; Keckesova, Ylinen, and Towers, 2004; Perron et al., 2004; Stremlau et al., 2004; Yap et al., 2004). In a previous study, we generated two B-box 2 mutants of TRIM5α_{rh}, each with a few amino acid residues changed to that seen in a relatively distant TRIM protein relative, TRIM21 (Li et al., 2006b). The TRIM21 B-box 2 domain, in contrast to B-box 2 domains of close TRIM5 relatives like TRIM6 or TRIM34, does not effectively substitute for that of TRIM5α_{rh} (Li et al., 2006b). Mutant Cluster I-A exhibits the following changes: Q109E, E110K, V114A, I115L and L118V; Mutant Cluster II-A exhibits the following changes: E120A, R121Q, Q123R and E124K (Figure 1). The wild-type and mutant TRIM5α_{rh} proteins were expressed in HeLa cells (Figure 2A). Both B-box 2 mutants restricted HIV-1 infection nearly as efficiently as the wild-type TRIM5α_{rh} protein (Figure 2B). However, no anti-N-MLV activity was detected for either of the mutants. In fact, N-MLV infected cells expressing the Cluster I-A and Cluster II-A TRIM5α_{rh} mutants more efficiently than cells transduced with the control LPCX vector; these mutants may exert dominant-negative effects on the TRIM5α_{hu} protein endogenously expressed in the HeLa cells. Thus, the B-box 2 changes confer different phenotypes with respect to the anti-HIV-1 and the anti-N-MLV activities of rhesus monkey TRIM5α.

To investigate the phenotypes of the B-box 2 mutants in the context of more potent restriction against N-MLV infection, the same changes were introduced into TRIM5α_{hu}. Interestingly, both TRIM5α_{hu} mutants retained anti-N-MLV activity, but lost the weak restricting activity against HIV-1 associated with wild-type TRIM5α_{hu} (Figure 2B). These results demonstrate that changes in the B-box 2 domain of TRIM5α can affect both HIV-1 and N-MLV restriction. Apparently, if the parental TRIM5α is a potent restrictor of a specific virus, then the restriction activity directed against that virus can better tolerate the changes introduced into the B-box 2 domain.

**The phenotypic effects of the B-box 2 changes are determined by the B30.2(SPRY) domain**

The determinants of species-specific differences in the potency of TRIM5α-mediated restriction to HIV-1 and N-MLV largely reside in the B30.2(SPRY) domain (Perez-Caballero et al., 2005a; Perron, Stremlau, and Sodroski, 2006; Stremlau et al., 2005; Yap, Nisole, and Stoye, 2005). The B30.2 domain from TRIM5α_{rh} is sufficient to confer potent anti-HIV-1 activity on TRIM5α_{hu}, whereas the B30.2 domain from TRIM5α_{hu} further increases the anti-N-MLV potency of TRIM5α_{rh} (Perez-Caballero et al., 2005a; Perron, Stremlau, and Sodroski, 2006; Stremlau et al., 2005; Yap, Nisole, and Stoye, 2005). To examine whether the effects of the B-box 2 changes on restriction are influenced by the B30.2(SPRY) domain, the B-box 2 changes were introduced into two previously established chimerae, TRIM5α R(H286-493) and TRIM5α H(R286-493) (Stremlau et al., 2005). TRIM5α R(H286-493) is rhesus monkey TRIM5α containing a human TRIM5α B30.2(SPRY) domain; TRIM5α H(R286-493) is human TRIM5α with a rhesus monkey TRIM5α B30.2(SPRY) domain. The residue numbering scheme is based on the human TRIM5α sequence (Stremlau et al., 2005). The TRIM5α R (H286-493) and H(R286-493) proteins, with wild-type or mutant B-box 2 domains, were expressed in HeLa cells (Figure 3A). As expected (Stremlau et al., 2005), HeLa cells overexpressing TRIM5α R(H286-493) were more susceptible to HIV-1 infection than cells expressing wild-type TRIM5α_{rh}; however, compared to cells transduced with the empty vector.
control (LPCX), the TRIM5α R(H286-493)-expressing cells still exhibited a strong restriction against HIV-1 infection (Figure 3B). TRIM5α R(H286-493) restricted N-MLV infection slightly more potently than TRIM5αrh for the range of viral doses used. Introduction of the Cluster I-A and Cluster II-A changes into the TRIM5α R(H286-493) protein resulted in restriction phenotypes different from those seen in the TRIM5αrh background. The TRIM5α R(H286-493)(I) and TRIM5α R(H286-493)(II) proteins did not detectably block HIV-1 infection, whereas both mutants were almost as potent as the parent TRIM5α R(H286-493) protein in restricting N-MLV infection.

As previously reported (Stremlau et al., 2005), human TRIM5α gained potent restriction activity against HIV-1 upon acquiring the B30.2(SPRY) domain from TRIM5αrh (see TRIM5α H(R286-493) in Figure 2B); the N-MLV-blocking activity of this chimera was slightly diminished compared to that of TRIM5αhu. When the Cluster I-A and Cluster II-A changes were introduced into TRIM5α H(R286-493), restriction activity against N-MLV was completely lost, whereas the anti-HIV-1 activities of these mutants were maintained (Figure 3B). These results demonstrate that, in the presence of a B30.2(SPRY) domain that specifies a strong restriction against a certain target virus, the effects of the Cluster I-A and Cluster II-A B-box 2 alterations can be functionally tolerated. On the other hand, if the B30.2(SPRY) domain confers relatively weak restricting ability to the TRIM5α protein, the two sets of B-box 2 alterations completely eliminate detectable antiviral activity.

**Effects of B-box 2 alterations on the restriction activities of human TRIM5α R332P**

A single amino acid change (R332P) in the B30.2(SPRY) domain of human TRIM5α is able to confer potent restricting ability against both HIV-1 and SIVmac infection, even though strong SIV-restricting activity is not a characteristic of either TRIM5αth or TRIM5αhu (Li et al., 2006c; Stremlau et al., 2005; Yap, Nisole, and Stoye, 2005). To examine how changes in the B-box 2 domain affect the multiple restriction activities of TRIM5αth R332P, the Cluster I-A and Cluster II-A alterations were combined with the R332P change in human TRIM5α; the antiviral activities of these TRIM5αth variants were tested in HeLa cells overexpressing these proteins (Figure 4A). The two sets of B-box 2 changes completely abolished the ability of the proteins to block HIV-1 and SIVmac infection (Figure 4B). Interestingly, the Cluster II-A changes also greatly attenuated the ability of TRIM5αhu R332P to restrict N-MLV infection, whereas the TRIM5αth R332P variant with the Cluster I-A changes efficiently blocked N-MLV infection (Figure 4C). Thus, many but not all of the antiviral activities of TRIM5αhu R332P are sensitive to the B-box changes studied here.

**The B-box 2 alterations decrease the capsid-binding abilities of TRIM5α**

TRIM5α variants from Old World monkeys specifically associate with HIV-1 capsid complexes; this interaction depends on the TRIM5α B30.2 domain (Stremlau et al., 2006). Human and New World monkey TRIM5α proteins associate less efficiently with HIV-1 capsid complexes, accounting for the lack of HIV-1 restriction in cells of these species (Stremlau et al., 2006; Li et al., 2006c). Because the B-box 2 changes in this study affected TRIM5α restriction activity in a potency-dependent manner determined by the B30.2 domain, the B-box 2 changes might affect the capsid-binding abilities of TRIM5α. To test this hypothesis, an in vitro HIV-1 CA-NC binding assay was employed (Li et al., 2006c; Stremlau et al., 2006). As expected (Li et al., 2006c; Stremlau et al., 2006), TRIM5αth associated with the in vitro assembled HIV-1 CA-NC complexes efficiently; TRIM5αhu only weakly associated with the complexes (Figure 5). TRIM5α H(R286-493), which has a B30.2 domain from TRIM5αth, associated with the HIV-1 capsid complexes as efficiently as the wild-type TRIM5αth. Conversely, TRIM5α R(H286-493), with the B30.2 domain of TRIM5αhu, bound the HIV-1 capsid complexes inefficiently. To our surprise, both sets of B-box 2 alterations dramatically reduced the binding capabilities of TRIM5αth, TRIM5α H(R286-493)
The Cluster I-A and Cluster II-A changes did not cause any perceptible reduction in the binding of TRIM5α(R286-493) to HIV-1 CA-NC complexes, possibly due to the low binding level seen for the unmodified TRIM5α(R286-493) protein and the sensitivity of this assay. These results indicate that the Cluster I-A and Cluster II-A changes in the B-box 2 domain generally affect the capsid-binding affinity of TRIM5α proteins.

The B-box 2 alterations do not affect trimerization and subcellular localization of TRIM5α

On the linear sequence of TRIM5α the B-box 2 domain is separated from the B30.2 domain by the coiled coil. One possible explanation for how changes in the B-box 2 domain could affect capsid binding is that the B-box 2 domain may be required to correctly orient the coiled-coil domain for trimerization, which in turn increases the avidity of TRIM5α for retroviral capsids (Javanbakht et al., 2006). To evaluate this possibility, TRIM5αH(R286-493) and their derived B-box 2 mutants were subjected to crosslinking with glutaraldehyde. As shown in Figure 6, the Cluster I-A and Cluster II-A B-box 2 alterations do not significantly affect the ability of TRIM5α proteins to trimerize.

Deletions or alterations of the TRIM5α RING and B-box 2 domains are in some cases associated with changes in the subcellular localization of the mutant protein (Diaz-Griffero et al., 2006a; Diaz-Griffero et al., 2006b; Javanbakht et al., 2005; Reymond et al., 2001). However, neither of the B-box 2 alterations studied here caused redistribution of the TRIM5α proteins (Figure 7). Within the limits of our ability to detect, the subcellular localization of the Cluster I-A and Cluster II-A mutants exhibited cytoplasmic localizations similar to those of the parental TRIM5α variants.

Phenotype of B-box 2 changes in owl monkey TRIMCyp

A retrotransposition that occurred near the root of the owl monkey lineage of New World monkeys resulted in a replacement of the TRIM5 gene with TRIMCyp (Nisole et al., 2004; Ribeiro et al., 2005; Sayah et al., 2004). The encoded TRIMCyp protein consists of a fusion of the RING, B-box 2 and coiled coil of TRIM5 with cyclophilin A. Because cyclophilin A interacts with the capsid proteins of HIV-1 and at least a few other retroviruses (Franke, Yuan, and Luban, 1994; Gamble et al., 1996; Lin and Emerman, 2006; Yoo et al., 1997), TRIMCyp can restrict infection by these viruses (Diaz-Griffero et al., 2006b; Nisole et al., 2004; Sayah et al., 2004; Zhang et al., 2006). Because cyclophilin A can bind with low affinity to monomeric HIV-1 capsid proteins (Yoo et al., 1997), the interaction of TRIMCyp with retroviral capsids may be less sensitive to B-box 2 changes than TRIM5α-capsid interaction. To test this hypothesis, the abilities of wild-type TRIMCyp and B-box 2 Cluster I-A and Cluster II-A mutants to bind HIV-1 CA-NC complexes were compared. Figure 8A shows that neither of the B-box 2 alterations detectably affected the binding affinity of TRIMCyp for the assembled HIV-1 capsid complexes.

The effect of the Cluster I-A and Cluster II-A changes in the TRIMCyp B-box 2 domain on the ability to restrict HIV-1 infection was examined. The B-box 2 TRIMCyp mutants blocked HIV-1 infection nearly as efficiently as wild-type TRIMCyp (Figure 8B). None of the TRIMCyp variants inhibited N-MLV infection, as expected from the inability of cyclophilin A to interact with MLV capsids (Braaten, Franke, and Luban, 1996). We conclude that the Cluster I-A and Cluster II-A alterations do not appreciably affect TRIMCyp capsid interaction or antiviral activity.

Phenotype of B-box 2 changes in the context of a TRIM5-Cyp chimera

The different phenotypes associated with the Cluster I-A and Cluster II-A B-box 2 changes in the contexts of TRIM5α and TRIMCyp proteins could formally be attributed to the use of cyclophilin A to bind the retroviral capsid or to the differences between the TRIM domains of TRIM5α and TRIM5αH. The Cluster I-A and Cluster II-A changes did not cause any perceptible reduction in the binding of TRIM5α(R286-493) to HIV-1 CA-NC complexes, possibly due to the low binding level seen for the unmodified TRIM5α(R286-493) protein and the sensitivity of this assay. These results indicate that the Cluster I-A and Cluster II-A changes in the B-box 2 domain generally affect the capsid-binding affinity of TRIM5α proteins.
TRIM5α and TRIMCyp. To distinguish these possibilities, we created and tested rhRBCC-Cyp, in which the cyclophilin A domain from owl monkey TRIMCyp replaces the B30.2 (SPRY) domain of TRIM5αrh (Figure 1). The Cluster I-A and Cluster II-A B-box 2 changes were introduced into the rhRBCC-Cyp protein. These B-box 2 changes did not affect the ability of the rhRBCC-Cyp protein to bind HIV-1 CA-NC complexes (Figure 9A). The abilities of the rhRBCC-Cyp Cluster I-A and Cluster II-A mutants to restrict HIV-1 infection were as great as that of the unmodified rhRBCC-Cyp protein (Figure 9B). The rhRBCC-Cyp proteins did not efficiently inhibit N-MLV infection (Figure 9C). We conclude that the Cluster I-A and Cluster II-A B-box 2 changes do not affect capsid binding and HIV-1 restriction when the capsid-binding moiety is cyclophilin A.

Discussion

Changes in the B-box 2 domains of TRIM5α and TRIMCyp proteins have been shown to diminish or abolish antiretroviral activity (Diaz-Griffero et al., 2006b; Javanbakht et al., 2005; Li et al., 2006b; Perez-Caballero et al., 2005a). Some of these changes do not appreciably decrease expression levels, alter subcellular localization or affect the capsid-binding ability of the TRIM5α/TRIMCyp proteins (Diaz-Griffero et al., 2006b; Stremlau et al., 2006). These B-box 2 changes apparently influence an “effector” function of TRIM5α/TRIMCyp that operates after capsid binding. The nature of this effector function is not known; it may be involved in the accelerated uncoating of the retroviral capsid that is strongly correlated with TRIM5α restriction of viral infection (Perron et al., 2006; Stremlau et al., 2006).

Here we studied two clusters (Cluster I-A and Cluster II-A) of TRIM5α B-box 2 changes involving residues that differ between TRIM5 and the distant relative TRIM21. The TRIM21 B-box 2 domain very inefficiently substitutes for that of TRIM5α, compared with the B-box 2 domains of the closer TRIM5 relatives, TRIM6 and TRIM34 (Li et al., 2006b). On models of the B-box 2 domain based upon the TRIM29 B-box 2 structure, most of the residues involved in the Cluster I-A and Cluster II-A changes are predicted to be surface-exposed (data not shown). A key observation of our studies is that the viral restriction phenotypes of the Cluster I-A and Cluster II-A B-box 2 mutants of TRIM5α are conditional, influenced by both the TRIM5α background and the restricted virus. The phenotypes of the B-box 2 mutants are generally correlated with the potency of the restriction against the particular viruses by the TRIM5α parent with unmodified B-box 2 sequences. By analyzing recombinant TRIM5α proteins, we show that the determinant of the conditional B-box 2 phenotype is the TRIM5α B30.2(SPRY) domain, which makes major contributions to capsid binding (Stremlau et al., 2006). Direct analysis of binding to HIV-1 capsid-nucleocapsid complexes revealed the surprising finding that both Cluster I-A and Cluster II-A B-box changes caused substantial reductions in capsid-binding affinity of TRIM5α proteins. These results indicate that the decreases in TRIM5α antiretroviral function associated with the Cluster I-A and Cluster II-A changes, by definition, do not result from disruption of an effector function. This conclusion is consistent with the expectation that TRIM5α mutants with true effector function defects will exhibit a restriction phenotype regardless of the high affinity of TRIM5α for the capsid, as has been previously observed (Diaz-Griffero et al., 2006b; Stremlau et al., 2006).

We considered several possible explanations for the unexpected effect of the Cluster I-A and Cluster II-A B-box 2 changes on TRIM5α binding to the retroviral capsid:

1. Because TRIM5α trimerization contributes greatly to the avidity for assembled HIV-1 capsid complexes (Javanbakht et al., 2006) and because of the proximity of the B-box 2 and coiled-coil domains, it is possible that the alterations introduced into the B-box 2 domain affected TRIM5α trimer formation or stability. However, we observed no differences in trimerization between the TRIM5α proteins with and without Cluster I-A and Cluster II-A changes.
2. The Cluster I-A and Cluster II-A changes could hypothetically disrupt B-box 2 contributions to capsid-binding affinity that are mediated by B-box 2-capsid contacts or by B-box 2 interactions with cellular cofactors that promote higher avidity. However, such models are incompatible with previous observations that the TRIM5α B-box 2 domain can be disrupted or deleted with no apparent decrease in capsid-binding affinity (Stremlau et al., 2006).

3. The Cluster I-A and Cluster II-A B-box 2 changes might alter capsid-binding avidity by influencing the orientation of the B30.2(SPRY) domains. The available data are compatible with this explanation. In particular, our observations with TRIMCyp and rh5RBCC-Cyp mutants indicate the specificity of the effects of these B-box 2 changes for TRIM5 proteins with B30.2(SPRY) domains. The Cluster I-A and Cluster II-A B-box 2 changes exerted no detectable effect on the ability of TRIMCyp or rh5RBCC-Cyp to bind HIV-1 capsid complexes. In the TRIMCyp and rh5RBCC-Cyp proteins, the capsid-binding moiety of TRIM5α, the B30.2(SPRY) domain, is replaced by cyclophilin A. Thus, the disruptive effects of the Cluster I-A and Cluster II-A B-box 2 changes on HIV-1 capsid binding are apparently dependent on the B30.2(SPRY) domains being the direct capsid-binding entity; these disruptive effects are completely eliminated when the capsid recognition function is mediated by the cyclophilin A domain. These observations support a model in which the B-box 2 and B30.2 domains are spatially related on TRIM5α proteins. Presumably the spatial relationships and potential packing contacts involving the B-box 2 and B30.2(SPRY) domains on the TRIM5α trimer differ from those of the B-box 2 and cyclophilin A domains on the TRIMCyp trimer. Thus, in this model, the effects of Cluster I-A and Cluster II-A B-box 2 changes on capsid binding are expected to differ for TRIM5α and TRIMCyp proteins.

The observed effects of the Cluster I-A and Cluster II-A B-box 2 changes on capsid binding could be mediated by direct B-box 2 - B30.2(SPRY) contacts or by indirect alteration of subunit relationships on the trimer. Precise understanding of these relationships awaits a TRIM5α structure.

We note that the binding of the Cluster I-A B-box 2 mutants to HIV-1 CA-NC complexes was generally very low, and did not demonstrate an increase even for TRIM5α proteins (TRIM5α<sub>hu</sub> (I) and TRIM5α H(R286-493) (I)) that exhibited HIV-1-restricting ability. These observations suggest that, at least for TRIM5α proteins like the Cluster I-A mutants whose level of expression is low, the HIV-1 capsid-binding assay is not sensitive enough to allow precise assessment of affinities that, although low, can still influence biological phenotypes.

Although replacement of the TRIM5α<sub>hu</sub> B30.2(SPRY) domain with the complete B30.2 (SPRY) domain of TRIM5α<sub>rh</sub> reverted the HIV-1-restriction phenotype of the Cluster I-A and Cluster II-A B-box 2 changes, the replacement of arginine 332 in the B30.2(SPRY) domain did not. This is consistent with previous observations suggesting that, although the presence of arginine at residue 332 is the major determinant of the poor anti-HIV-1 potency of TRIM5α<sub>hu</sub>, its replacement is not sufficient to achieve the same levels of HIV-1 capsid binding and restriction as those of TRIM5α<sub>rh</sub> (Li et al., 2006c). Thus, TRIM5α<sub>hu</sub> R332P has apparently gained sufficient affinity for the HIV-1 (and possibly SIV<sub>mac</sub>) capsids to allow restriction, but not enough to counteract the influence of these particular B-box 2 alterations.

Further studies of TRIM5α and TRIMCyp may provide an understanding of the structural basis of the functional inter-domain relationships elucidated in this work.
Materials and Methods

Plasmid construction

Mutations affecting the B-box 2 domain of the wild-type human and rhesus monkey TRIM5α proteins were introduced into pLPCX-TRIM5α expression plasmids, using overlapping PCR extension with primers carrying the corresponding nucleic acid changes (Horton et al., 1993; Horton et al., 1989). The PCR-amplified TRIM5 fragments were digested and cloned into the EcoRI and ClaI sites of the pLPCX vector, as previously described (Stremlau et al., 2004). The chimeric constructs containing B-box 2 alterations were created by exchanging fragments generated by BsmI and ClaI digestion of the above constructs and similarly digested plasmids expressing TRIM5α R(H286-493), TRIM5α H(R286-493), TRIM5αhu R332P or TRIMCyp (Stremlau et al., 2005). The rh5RBCC-Cyp protein contains residues 1-296 of TRIM5αrh (MASGIL...FRELTDAD296) fused to the Cyp A domain from owl monkey TRIMCyp. The junction of the TRIM5αrh and TRIMCyp moieties in the rh5RBCC-Cyp has the following sequence: ...FRELTDADQRYWDAAWDLVASAMVNPTV... All constructs contain C-terminal epitope tags derived from influenza hemagglutinin (HA).

Creation of cells stably expressing TRIM proteins

Recombinant viruses were produced in 293FT cells (Invitrogen) by cotransfecting the pLPCX plasmids expressing TRIM proteins with the pVPack-GP and pVPack-VSV-G packaging plasmids (Stratagene). The pVPack-VSV-G plasmid encodes the vesicular stomatitis virus (VSV) G envelope glycoprotein, which allows efficient entry into a wide range of vertebrate cells (Yee, Friedmann, and Burns, 1994). The resulting virus particles were used to transduce 2 × 10^5 HeLa cells in six-well plates. The transduced HeLa cells were then selected in 1 μg/ml puromycin (Sigma).

Immunoblotting

HeLa cells stably expressing the transduced proteins were lysed with phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH₂PO₄, 138 mM NaCl, 8 mM Na₂PO₄) containing 1% NP40 and protease inhibitor cocktail (Roche). The lysates were resolved by SDS-PAGE and Western blotted with horseradish peroxidase (HRP)-conjugated anti-HA antibody (Roche) and anti-β-actin antibody (Sigma).

Infection with viruses expressing GFP

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

Crosslinking of TRIM proteins

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

Subcellular localization using immunofluorescence confocal microscopy

HeLa cells stably expressing TRIM5α variants were cultured on 8-well chamber slides. Twenty-four hours later, the cells were fixed, permeabilized, and incubated with rat anti-HA 3F10 antibody (1:200, Roche) followed by secondary anti-rat IgG conjugated with FITC.
The processed cells were analyzed using a confocal microscope (Nikon Eclipse E800) with laser (Bio-Rad MRC 1024), and the images were obtained using Bio-Rad Lasersharp 2000 software.

HIV-1 capsid-binding assay

Purification of recombinant HIV-1 CA-NC protein from *Escherichia coli* was carried out as previously described (Ganser et al., 1999). For a source of TRIM5 proteins, HeLa cells stably expressing the TRIM5 variants were lysed by freeze-thawing in hypotonic lysis buffer (10 mM Tris, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT) containing protease inhibitor (Roche). The lysates were then mixed with the *in vitro* assembled HIV-1 CA-NC complexes and the binding assay was carried out as previously described (Li et al., 2006a; Li et al., 2006c; Stremlau et al., 2006).

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References


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Figure 1. TRIM5 and TRIMCyp constructs
The human and rhesus monkey TRIM5α proteins, TRIM5α<sub>hu</sub> and TRIM5α<sub>rh</sub> chimeric proteins, and the owl monkey TRIMCyp protein are depicted. A chimeric rh5RBCC-Cyp protein in which the amino-terminal portion of TRIM5α<sub>rh</sub> was fused with the cyclophilin A domain of owl monkey TRIMCyp was also studied. The changes introduced into the B-box 2 domain of the proteins to create the Cluster I-A and II-A mutants are shown. The carboxy-terminal HA tag is shown.
Figure 2. Expression and antiretroviral activity of TRIM5αB-box 2 mutants

(A). The expression level of TRIM5α proteins with C-terminal HA tags in HeLa cells stably expressing these proteins was determined by Western blotting comparable amounts of cell lysate with an anti-HA antibody. The blot was also probed for β-actin to control for loading amount. Rh5, wild-type rhesus monkey TRIM5α; hu5, wild-type human TRIM5α; the mutants labeled X(I) contain the Cluster I-A B-box 2 domain changes Q109E, E110K, V114A, I115L and L118V; the mutants labeled X(II) contain the Cluster II-A B-box 2 domain changes E120A, R121Q, Q123R and E124K, where X represents the TRIM5 backbone (X = rh5, rhesus monkey TRIM5α; X = hu5, human TRIM5α) in which the changes were made. (B). The effects of the TRIM5α proteins described above on retroviral infection were assessed. HeLa cells stably expressing the wild-type and mutant TRIM proteins, or control HeLa cells transduced with the empty LPCX vector, were incubated with various amounts of HIV-1-GFP or N-MLV-GFP. Infected GFP-positive cells were counted by FACS. Twenty microliters of the preparation of HIV-1-GFP used in these experiments correspond to ~3,000 cpm reverse transcriptase units; 100 microliters of N-MLV-GFP correspond to ~400 cpm reverse transcriptase units.
Figure 3. Expression and antiretroviral activity of TRIM5α<sub>rh</sub>-TRIM5α<sub>hu</sub> chimerae with B-box 2 changes
(A). Expression of the unmodified (wt) TRIM5α R(H286-493) and H(R286-493) proteins and the Cluster I-A (I) and Cluster II-A (II) mutants was examined by Western blotting lysates from HeLa cells with an anti-HA antibody (Roche). Lysates were Western blotted for β-actin as a control. Rh5 = wild-type TRIM5α<sub>rh</sub>. (B). Infection with HIV-1-GFP and N-MLV-GFP of HeLa cell lines stably expressing the indicated parental and mutant proteins. The results shown are typical of those obtained in two repeat experiments. (rh5 = TRIM5α<sub>rh</sub>; hu = TRIM5α<sub>hu</sub>).
Figure 4. Effects of B-box 2 domain changes on the antiretroviral activity of human TRIM5α R332P (A). Expression of the mutant proteins in HeLa cells was analyzed by Western blotting, as described in the Figure 1 legend. Lysates were also blotted with an antibody against β-actin as a control. (hu5 R332P = TRIM5αhu R332P). (B and C). HeLa cells expressing the indicated proteins were incubated with HIV-1-GFP and SIVmac-GFP (B) and N-MLV-GFP (C), then analyzed for GFP expression by FACS. Ten dose units of this preparation of SIVmac correspond to ~3000 cpm reverse transcriptase units. The experiment was repeated twice with results similar to those shown.
Figure 5. Effects of the B-box 2 changes on the association of TRIM5 proteins with assembled HIV-1 capsid complexes

Cell lysates of HeLa cells stably expressing the C-terminally HA-tagged TRIM5 variants were used in the HIV-1 CA-NC binding assay. The top and middle panels show the amounts of TRIM5 protein in the input and pellet, respectively; the bottom panel shows the amount of HIV-1 CA-NC protein that was pelleted through the 70% sucrose cushion. None of the TRIM5 protein variants were able to pellet through the 70% sucrose cushion in the absence of added HIV-1 CA-NC complexes (data not shown). (hu5 = human TRIM5α; rh5 = TRIM5α_{rh}; wt = wild-type; I = Cluster I-A mutant; II = Cluster II-A mutant).
Figure 6. Oligomerization of TRIM5α protein variants
Lysates from HeLa cells stably expressing the indicated TRIM5α proteins were crosslinked with increasing concentrations of glutaraldehyde (0, 0.2, 0.4, 0.8 and 2.0 mM). The crosslinked products were resolved by SDS-PAGE and visualized by Western blotting with an anti-HA antibody. The position of the molecular weight markers is indicated on the figure.
Figure 7. Localization of TRIM5α protein variants
HeLa cells stably expressing the indicated HA-tagged TRIM5α proteins (or the empty LPCX vector) were fixed and permeabilized. Cells were stained with an anti-HA antibody followed by a secondary anti-rat IgG antibody conjugated with FITC. Stained cells were examined using a confocal fluorescent microscope. The unmodified TRIM5α proteins (wt) are shown in the left column, and the Cluster I-A and Cluster II-A variants of each protein in the middle and right columns, respectively.
Figure 8. Effects of B-box 2 changes on TRIMCyp function

(A). The indicated TRIM5α and TRIMCyp variants were expressed stably in HeLa cells. Cell lysates were incubated with HIV-1 CA-NC complexes and the mixtures were layered onto a 70% sucrose cushion and centrifuged. The TRIM5α and TRIMCyp proteins in the input and pellet were detected by Western blotting with an anti-HA antibody. The CA-NC protein in the pellet was detected by Western blotting with an anti-p24 antibody. (B and C). HeLa cells stably expressing the indicated TRIM5α or TRIMCyp variants were exposed to the indicated amounts of HIV-1-GFP (B) or N-MLV-GFP (C). GFP-positive cells were counted. The results shown are those of a typical experiment; the experiment was repeated with similar results.
Figure 9. Effects of B-box 2 changes on the chimeric rh5RBCC-Cyp protein

(A) The binding of the unmodified rh5RBCC-Cyp (wt) protein and the Cluster I-A (I) and Cluster II-A (II) derivatives to HIV-1 CA-NC complexes was assessed on 70% sucrose cushions. TRIM5αrh and TRIM5αhu were included as controls. The steady-state expression level of the unmodified (wt) rhRBCC-Cyp protein was significantly lower than that of TRIM5αrh, and the Cluster I-A and II-A derivatives of rh5RBCC-Cyp were expressed at approximately 5-fold higher levels than that of the wt rhRBCC-Cyp protein (data not shown). In the binding assay, an attempt was made to adjust the amount of input lysate to offset these different levels of expression. The TRIM5 proteins were detected by Western blotting with an anti-HA antibody, and the CA-NC protein by an anti-p24 antibody. (B and C) HeLa cells
stably expressing the indicated proteins were exposed to HIV-1-GFP (B) or N-MLV-GFP (C). GFP-positive cells were counted. The results of typical experiments are shown.