The Retroviral Restriction Factor TRIM5α

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Retroviruses are obligate intracellular parasites that have coevolved with their hosts for millions of years. It is therefore not surprising that retroviruses take advantage of numerous host factors during their life cycle. In addition to positive cellular factors that are of use to the virus, host cells have also evolved intracellular proteins to antagonize the retroviral replication cycle. Such inhibitory cellular factors have been called retroviral restriction factors. Recently, several such restriction factors have been cloned, including Friend virus susceptibility factor 1, apolipoprotein B mRNA-editing enzyme catalytic proteins 3F and 3G, and ZAP. Here, we review the explosion of publications from the past 2 years concerning TRIM5, a host factor that potently inhibits HIV-1 and other retroviruses.

Introduction

The first restriction factor to be described was the murine Friend virus susceptibility factor 1 (Fv1). It was discovered as an inhibitory activity against certain strains of murine leukemia virus (MLV). In 1996, Fv1 was cloned and revealed to most closely resemble the *gag* gene of endogenous retroviruses [1]. Fv1 exists as several alleles, each of which possesses a unique restriction specificity. Mice carrying the Fv1-n allele, for example, are resistant to infection by B-tropic strains of MLV but are susceptible to N-tropic strains (N-MLV). The block is also apparent in cultured cells from these mice. The viral determinant for this inhibition is the capsid (CA) protein, which forms a protective core structure around the viral RNA genome. The difference between a restricted and an unrestricted virus in the case of Fv1, for example, is one amino acid change in MLV CA at position 110. The Fv1 gene product is capable of blocking the restricted

virus after the reverse transcription step and before integration of the viral genome, but its exact mechanism of action is still unknown.

Certain members of the family of apolipoprotein B mRNA-editing enzyme catalytic proteins (APOBEC) have also been shown to possess intracellular antiviral activity against retroviruses. Most notably, human APOBEC3G and APOBEC3F show potent activity against HIV-1 [2–4]. The restriction factor is packaged into virions in producer cells, but does not exert its negative influence on viral replication until the viral RNA is reverse transcribed in the target cell. At this time, APOBEC proceeds to hypermutate the viral genome, leading to aborted infection. Recent data indicate, however, that APOBEC proteins act against the virus by blocking accumulation of complete viral reverse transcripts rather than through their enzymatic cytidine deaminase activity [5,6]. Interestingly, HIV-1 has evolved a strategy to prevent the deleterious actions of APOBEC3G: the HIV-1 Vif protein apparently binds to APOBEC3G in the producer cell and prevents its packaging into the virus by targeting it to the proteasomal degradation pathway [7,8].

Several other retroviral restriction factors have been identified. Among these is ZAP, a zinc finger protein that inhibits cytoplasmic viral RNA accumulation, specifically that of MLV and several alphaviruses, by binding defined RNA sequences and presumably targeting the viral RNAs to the exosome for degradation [9,10]. Also on the growing list of antiviral cellular activities is a presently unknown human factor that was shown to trap HIV-1 particles at the plasma membrane in certain human cell lines, preventing viral particle release. It was demonstrated that the HIV-1 Vpu protein counteracts this factor and thus ensures proper viral egress [11,12].

TRIM5α was discovered in screens aimed at identifying the dominant inhibitory activity against HIV-1 in rhesus macaque cells [13•] and in owl monkey cells [14•]. The alpha isoform of rhesus TRIM5 is a cytoplasmic protein that very effectively blocks HIV-1, after entry into the cell but before it is able to complete reverse transcription of its genome, and like Fv1, it was found to be CA-specific. Subsequently, it was found that the
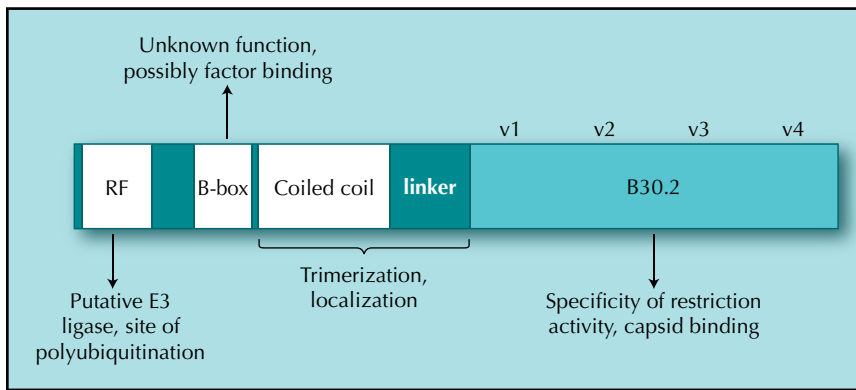
Figure 1. TRIM5α schematic showing protein domains and their functions. RF—RING finger domain; v1–v4—variable regions.

Table 1. Virus specificities of TRIM5α proteins from various primate species

TRIM5α originating species	Restricted viruses
Human *(Homo sapiens)*	N-MLV, EIAV, HIV-1 (weak)
Chimpanzee *(Pan troglodytes)*	N-MLV
Orangutan *(Pongo pygmaeus)*	N-MLV, SIVmac
Rhesus macaque *(Macaca mulatta)*	HIV-1, N-MLV, SIVagm, EIAV, SIVmac (weak)
African green monkey *(Cercopithecus tantalus)*	HIV-1, SIVmac, N-MLV, EIAV
African green monkey *(Cercopithecus aethiops)*	HIV-1, N-MLV, EIAV, SIVagm (weak)
Owl monkey TRIMCyp *(Aotus trivirgatus)*	HIV-1, FIV, SIVagm (weak)
Squirrel monkey *(Saimiri* sp)	SIVmac
Spider monkey *(Ateles geoffroyi)*	HIV-1, SIVmac, SIVagm
EIAV—equine infectious anemia virus; N-MLV—N-tropic murine leukemia virus; SIV—simian immunodeficiency virus.	

human TRIM5α protein possesses modest activity against HIV-1 and that it potently inhibits certain strains of MLV [15•,16–19]. This review focuses on the restriction factor TRIM5α, its specificity, and its proposed mechanism of action.

TRIM5α Background

TRIM5α is a member of the large TRIM family of proteins, named for their tripartite motif consisting of RING finger, one or two B-boxes, and a coiled coil region [20] (Fig. 1). This multigene family comprises at least 68 members, most of which are uncharacterized and have unknown cellular functions. Mutations in several TRIM family members have been implicated in diseases, such as familial Mediterranean fever (TRIM20), Mulibrey nanism (TRIM37), and Opitz syndrome (TRIM18). In addition to the three conserved motifs, many TRIM proteins contain a C-terminal B30.2 domain. TRIM5α, which possesses such a B30.2 domain, is one splice variant of the TRIM5 gene, and the only isoform to exhibit restriction activity.

To date, the TRIM5α cDNAs from a large number of primates and cows have been cloned and tested for antiviral activity against retroviruses [21–25]. Interestingly, a species-dependent specificity has been observed with respect to which virus is inhibited (Table 1). Rhesus

TRIM5α potently restricts HIV-1, whereas the TRIM5α orthologs from African green monkey species have a much broader restriction range. The African green monkey *Cercopithecus tantalus*, for example, blocks HIV-1, SIVmac, N-MLV, and equine infectious anemia virus. The human TRIM5α ortholog was found to potently restrict N-MLV as well as equine infectious anemia virus. The owl monkey, a New World monkey species, presents a special case: instead of TRIM5α, it expresses a gene fusion termed TRIMCyp, in which the B30.2 domain is replaced with cyclophilin A. This gene is the result of a retrotransposition event of a cyclophilin A cDNA into the owl monkey TRIM5 locus [14•,26]. The TRIMCyp protein potently restricts HIV-1, which is partially explained by the well-described binding of cyclophilin A to the HIV-1 capsid [27].

Studies by many laboratories have sought to elucidate the importance of each of the four domains of TRIM5α (RING, B-box, coiled coil, and B30.2) for restriction activity (Fig. 1). Although no domain seems to be completely dispensable for the full inhibitory effect, mutants lacking the RING domain have been found to retain partial restriction activity [28,29]. The B-box, coiled coil, and B30.2 domains, however, have all been shown to be essential for restriction [28–30]. The following discusses the proposed function of each domain in restriction, specifically the RING finger and

the C-terminal B30.2 domain, and their roles in the overall mechanism of retroviral antagonism.

The B30.2 domain
Structure
The prototype of the B30.2 domain is found in the human major histocompatibility complex (MHC) class I region comprising an exon that was initially termed B30-2 [31]. Independently, using computer algorithms, a closely related domain named SPRY was discovered in the kinase splA (from *Dictyostelium discoideum*) and in mammalian ryanodine receptors [32]. Sequence alignments revealed the SPRY domain to be contained within the slightly larger B30.2 domain; and in the case of TRIM5α, the B30.2 domain is actually composed of a PRYSPRY domain (the PRY part constituting a 60 amino acid stretch N-terminal of the SPRY domain). The crystal structures of three different B30.2 domains have been solved recently [33–35], based on which the TRIM5α PRYSPRY domain can be modeled reasonably well. PRYSPRY forms one compact domain of a 13-stranded β-sandwich, containing a hydrophobic core and a putative ligand-binding pocket. The deletion of the B30.2 domain in several proteins abolishes interaction with their respective binding partners, which has lead to the assumption that B30.2 acts as a protein-interacting module. This observation is supported by the evidence that the B30.2 domain in TRIM5α is essential for restriction activity and is the determinant for virus specificity [13•,15•,16–18,21,22,28,36–39]. TRIM5α hybrid proteins, for example, that contain the human TRIM domains fused to the rhesus B30.2 domain are able to restrict HIV-1, thereby taking on the restriction phenotype of rhesus TRIM5α [28].

Mapping restriction specificity
One approach to determine essential regions for anti–HIV-1 activity in the B30.2 domain of rhesus TRIM5α is via evolutionary sequence analysis. Using this strategy, a 13-amino-acid patch of positively selected residues was identified in the SPRY domain by comparing the TRIM5α sequences from 17 primate genomes [37]. By assessing the restriction activity of chimeric rhesus-human TRIM5α proteins, the ability to restrict HIV-1 was narrowed down to a few amino acid residues in SPRY (the evolutionary hot spot, also termed v1 for variable region 1) [28,38]. Then, it was pinpointed more precisely to one specific amino acid: the mutation of residue R332 in human TRIM5α to any uncharged amino acid results in anti–HIV-1 activity [40].

The mapping of regions important for restriction of N-MLV has been more challenging. Both rhesus and human TRIM5α proteins are able to block N-MLV, though the human form is more potent, which makes the restriction phenotype of chimeric proteins difficult to interpret. Some data from chimerae nevertheless point toward the importance of two of the four variable, surface-exposed loops in the B30.2 domain (v1 and v3) in the restriction of N-MLV [41]. It was proposed that the presence of negatively charged residues at two specific positions in the v3 region of human TRIM5α SPRY potentiate the interaction with the positive charge of arginine in the MLV CA that determines N-tropism (R110) [41].

Sequence analysis revealed that the three variable regions in the TRIM5α SPRY domain exhibit significant length polymorphism from species to species [21]. The broad range of retroviruses restricted by African green monkey TRIM5α is proposed to be attributable to a 20-amino acid insertion (sequence duplication) in the v1 region of the SPRY domain [21]. Another species, the spider monkey, was also found to restrict a wide variety of retroviruses, and its SPRY domain shows evidence of a tandem triplication in the v3 region. Based on these findings, it is reasonable to assume that these loops of variable length impart on the B30.2 domain a flexible surface for interaction with diverse ligands (eg, capsid or other unknown structures).

Capsid binding
Using virus chimerae, it was determined early on that sensitivity to TRIM5-mediated restriction is determined by the viral capsid protein. For example, rhesus macaque cells, which display a TRIM5α-mediated block to HIV-1, show much less restriction of an HIV-1 chimera bearing a SIVmac capsid [42]. Based on this observation of capsid-specificity, it was reasonable to assume that TRIM5α directly bound to the restricted retroviral capsid. However, demonstrating such a direct interaction proved to be difficult. Conventional coimmunoprecipitations failed, and expression of capsid monomers in a restrictive cell was not able to saturate restriction activity [43]. This led to the hypothesis that TRIM5α recognized a higher order structure of capsid multimers, possibly a hexameric unit [44], and based on this reasoning, two different capsid-binding assays were developed that recapitulate restriction specificity. In the first assay, detergent-stripped MLV virions were mixed with cell lysates containing glutathione-S-transferase–tagged human TRIM5α. Pull-down of TRIM5α on glutathione-conjugated sepharose beads resulted in the cosedimentation of restricted N-tropic but not of unrestricted B-tropic MLV [45•]. This interaction was shown to be dependent on the SPRY domain. Unfortunately, most likely due to the relative instability of HIV-1 cores, this assay could not be replicated with rhesus TRIM5α and HIV-1 CA.

The second approach to show TRIM5α interaction with capsid constitutes the reverse strategy. Here, HIV-1 capsid complexes assembled from purified CA-NC protein were incubated with lysates containing TRIM5α and then accelerated through a sucrose cushion. The capsid pellet was then probed for TRIM5α. Using this assay, it was

shown that the ability of TRIM5α proteins from different species to associate with HIV-1 capsid correlates with their ability to restrict HIV-1 [46•].

In summary, the variable B30.2 domain of TRIM5α proved to be the specificity determinant for retroviral restriction and associates in a complex with the viral capsid structure.

The RING domain

The RING domain of TRIM5α is located at the N-terminus of the protein and spans about 40 amino acids. Via cysteine residues at positions 15 and 18, it binds two zinc atoms, forming a "cross-brace" motif. RING fingers of numerous proteins (in the context of the tripartite motif) have been implicated in the control of substrate levels via their E3 ubiquitin ligase activity and subsequent proteasomal degradation. The RING finger was demonstrated to play a role in the transfer of ubiquitin to substrate molecules as well as to the TRIM proteins themselves. A biochemical interaction between ubiquitin-conjugating (E2) enzymes and the RING motif has also been shown. In the case of TRIM5, the splice variant TRIM5α interacts with the E2 enzyme UbcH5B and was found to possess auto-E3 ligase activity [47]. It is therefore conceivable that the TRIM5α RING finger possesses a similar activity, mediating the transfer of ubiquitin to substrate molecules and/or itself, followed by proteasomal targeting.

Naturally, one proposed target of TRIM5α-mediated ubiquitination is the retroviral capsid, resulting in its degradation by the proteasome. It was demonstrated for TRIM5α that a mutant lacking the RING domain is more stable than the wild-type protein, which is rapidly turned over via a proteasome-dependent mechanism [48]. In addition, it was shown that although TRIM5α can be polyubiquitinated, the ΔRING mutant has lost this characteristic [48]. (Mono- and diubiquitinated forms could still be detected, however.) This data suggests that the rapid turnover of TRIM5α is mediated via polyubiquitination of the RING domain and proteasomal degradation.

However, the importance of the RING domain for TRIM5α-mediated restriction is not clear. RING finger mutants retain partial restriction activity [13•,18,29,49], demonstrating that the RING domain is not absolutely required for antiviral potential. It has been argued that the observed decrease in activity for the deletion mutant results from its mislocalization in the cell rather than from a lack of RING-domain functionality [48]. Another study supports the idea that ubiquitin ligase activity via the RING finger is not part of the restriction mechanism; it showed that the E1 ubiquitin-activating enzyme is not required for TRIM5-mediated restriction activity [49]. The same study did, however, acknowledge the general importance of the RING domain and the fact that it was required for full restriction activity.

Recently, another group reported that proteasome inhibitors were able to counteract restriction and increase viral cDNA levels in the cytoplasm, even though the block to infectivity itself was not overcome, and nuclear viral DNA levels were unchanged and low [50,51]. It was proposed that the TRIM5α-mediated restriction mechanism is composed of two pathways, one of which is mediated by the proteasome and can be overcome by proteasome inhibitors. The second mechanism, however, still prevents viral cDNA transport into the nucleus, so that overall infectivity is not affected, even if one pathway fails. For reasons explained earlier, the RING domain is a likely candidate for mediating the former pathway, via its E3 ligase activity.

B-box and coiled coil domains

B-box and coiled coil domain are located C-terminal of the RING finger in TRIM proteins. The B-box motif is exclusively found in the TRIM family of proteins; it binds one zinc atom via conserved cysteine and histidine residues, but the function of B-boxes remains unknown. The coiled coil domain, which follows the B-box motif, is less conserved on the amino acid sequence level, but the region is always predicted to pack into the characteristic coiled coil formation.

Interestingly, the TRIM5α B-box was shown to be essential for restriction [28,29]. Deletion of the RING/B-box part of TRIM5α as well as mutation of the zinc-coordinating residues in the B-box cause a complete loss of antiviral activity. Undoubtedly, studies are underway to determine the function of the TRIM5α B-box, which most likely occurs via interaction with an as of yet unknown factor.

The coiled coil domain is thought to mediate homo- and hetero-interaction between TRIM proteins [20], which in the case of TRIM5α was demonstrated by the dominant negative activity of mutants lacking the RING/B-box domains (leaving the coiled coil/B30.2 region intact) [29]. The activity of human TRIM5α was also shown to be inhibited by exogenous expression of TRIMCyp or rhesus TRIM5α, presumably via dominant-negative coiled coil interactions [52]. In addition, the coiled coil region was shown to be required for self-association of TRIM5α in coimmunoprecipitation experiments. Cross-linking studies furthermore demonstrated that TRIM5α exists as a trimer [53] and that the coiled coil and its C-terminal linker region are necessary and sufficient for trimerization [30]. The self-association of TRIM5α is thought to have a part in the formation of cytoplasmic speckles that are characteristic of the TRIM5α intracellular localization pattern when overexpressed. It was demonstrated, however, that these cytoplasmic bodies are not required for the restriction activity of TRIM5α [54]. Interestingly, replacement of the coiled coil domain with a heterologous trimerization domain (GCN4) recapitulated the trimerization of

TRIM5α but did not restore restriction activity [30]. This observation suggests that the coiled coil domain is responsible for more than oligomerization during the antiviral activity of TRIM5α.

Mechanism of Restriction

In the short time since its discovery in 2004, intense studies of the restriction factor TRIM5α have led to numerous publications, but its exact mechanism of action is still unclear. It has been established that TRIM5α binds to the retroviral capsid via its B30.2 domain, and a model has been proposed in which a trimeric TRIM5α cluster interacts with a hexameric capsid subunit [30,53]. After capsid binding, it is very likely that a subsequent, second step is required for antiviral potential, whether it be an enzymatic activity of TRIM5α itself via its RING finger, or the recruitment of other factors via its B-box.

One study supports the idea that TRIM5α intervenes with the normal uncoating step of the viral capsid, and that it leads to an accelerated and therefore deleterious disassembly of the capsid structure, leaving the viral RNA unprotected. In a recent publication, the authors track the fate of incoming capsid in infected, restrictive cells. They demonstrate that after ultracentrifugation in the presence of TRIM5α, the particulate, intact capsid in the pellet is reduced, and monomeric capsid molecules accumulate in the supernatant [46•]. Although this study seems very convincing, other possible restriction mechanisms include the sequestering of the incoming reverse transcription complex [55], or an inhibition of the virus via TRIM5α-mediated detrimental modification of the viral capsid (eg, ubiquitination or sumoylation). Naturally, a combination of these possibilities is also conceivable. In addition, restriction seems to involve several pathways, each resulting in the inhibition of a different step in the early retroviral lifecycle. Evidence for this multistep model is provided by proteasome inhibitor studies as mentioned earlier [50,51], TRIMCyp chimerae experiments [55], and studies using drugs that counteract restriction activity [56].

Conclusions

The TRIM5α-mediated restriction activity is likely to be a complex mechanism consisting of more than one pathway. TRIM5α recognizes the incoming capsid structure via its B30.2 domain, and the domains of the tripartite motif take part in one or more subsequent step(s) that result in the inhibition of the virus.

Recently, four studies have been conducted to determine whether polymorphisms within the human TRIM5α gene show a correlation with susceptibility to HIV-1 infection [57–60]. Of the several nonsynonymous single nucleotide polymorphisms found, none showed a correlation with infectibility or progression towards AIDS. This does not seem surprising, since HIV-1 has been present in the human population for too short a period to give rise to detectable TRIM5α variants. However, two studies identified the importance of an allele resulting in the amino acid change R136Q. Although one study found an association of this allele with an increased susceptibility to HIV-1 infection in European Americans [57], the other study found a slight protective effect of R136Q in African Americans [60]. In addition, one nonsynonymous single nucleotide polymorphism that results in the amino acid change H43Y was shown to have decreased overall restriction activity (against both N-MLV and HIV-1) [58]. It is not clear why such a defective TRIM5α variant has persisted in the human population, but it is thought that a population bottleneck and a diminished selective pressure are at least partially responsible.

Retroviral inhibition by cellular proteins is proving to be an important and expanding field of research. Uncovering the mechanism of action of these restriction factors will help elucidate poorly understood stages of the retroviral life cycle. Most importantly, these factors have the potential to become clinically relevant in applications such as gene therapy or as new drug targets to enhance or modulate their endogenous antiviral activity. In the case of TRIM5α, it is conceivable, for example, to develop pharmacologic means to mask the basic residue 332 in the SPRY domain and therefore switch its restriction specificity towards HIV-1. Ongoing studies of the TRIM5 antiviral activity in many excellent laboratories will undoubtedly shed more light on this unique restriction mechanism in the future.

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