HIV Restriction Factors and Mechanisms of Evasion

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Retroviruses have long been a fertile model for discovering host–pathogen interactions and their associated biological principles and processes. These advances have not only informed fundamental concepts of viral replication and pathogenesis but have also provided novel insights into host cell biology. This is illustrated by the recent descriptions of host-encoded restriction factors that can serve as effective inhibitors of retroviral replication. Here, we review our understanding of the three restriction factors that have been widely shown to be potent inhibitors of HIV-1: namely, APOBEC3G, TRIM5α, and tetherin. In each case, we discuss how these unrelated proteins were identified, the mechanisms by which they inhibit replication, the means used by HIV-1 to evade their action, and their potential contributions to viral pathogenesis as well as inter- and intraspecies transmission.

HIV-1, in common with all viruses, requires the concerted contributions of numerous positively acting cellular factors and pathways to achieve efficient replication (Bushman et al. 2009). Conversely, mammalian cells also express a number of diverse, dominantly acting proteins that are widely expressed and function in a cell-autonomous manner to suppress virus replication. These have been termed restriction factors and/or intrinsic resistance factors, and they provide an initial (or early) line of defense against infection as a component of, or even preceding, innate antiviral responses. This work discusses the most extensively described examples of such factors, focusing on their impact on HIV-1. These are the apolipoprotein B messenger RNA (mRNA)-editing enzyme catalytic polypeptide-like 3 (APOBEC3) family of proteins (in particular, APOBEC3G), tetherin/ bone marrow stromal cell antigen 2 (BST2)/ CD317 (hereafter called tetherin), and tripartite-motif-containing 5α (TRIM5α).

A fundamental concept to the biology of restriction factors is that HIV-1 generally evades their potent inhibitory activities in human cells, thereby allowing virus replication to proceed efficiently. In contrast, the ability of HIV-1 to replicate in nonhuman cells is often severely compromised by restriction factors, thus marking these proteins as important determinants of viral host range and cross-species transmission. The mechanisms for evasion from restriction

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factors are virus encoded and frequently involve HIV-1's regulatory/accessory proteins, namely, Vif, Nef, Vpu, and Vpr. Indeed, the need to escape intrinsic resistance appears to have been an important driving force behind the acquisition of these viral genes. Aside from restriction factor evasion, the Vpu and Nef proteins regulate the expression and localization of a number of host proteins important during HIV-1 replication. Prominent among these interactions, Vpu and Nef both inhibit the cell-surface expression of the primary entry receptor CD4, as well as major histocompatibility class I complexes (MHC class I), whereas Nef also helps promote T-cell activation and HIV-1 particle infectivity (reviewed by Kirchhoff 2010).

HISTORY: DISCOVERY OF HIV-1 RESTRICTION FACTORS

The intellectual framework for considering restriction factors was established through studies of ecotropic murine leukemia virus (MLV). Specifically, mice encode a gene, $Fv1$, with two principal allelic forms, $Fv1^n$ and $Fv1^b$. $Fv1^n$ cells are up to 1000-fold more susceptible to infection by N-tropic strains of MLV than B-tropic strains, and $Fv1^n$ mice are correspondingly highly receptive to N-MLV induced disease. The opposite is true for $Fv1^b$ cells and animals, which are susceptible to B- rather than N-MLV. Heterozygous $Fv1^{n/b}$ cells are resistant to both N and B viruses, illustrating the general principle that a restricting phenotype is dominant over susceptibility. The sequence of the $Fv1$ gene most closely resembles that of an endogenous retrovirus gag gene (Best et al. 1996), Fv1 blocks infection by a poorly understood mechanism that operates after reverse transcription but before integration and likely requires direct recognition of infecting viral capsids, as N/B-tropism is determined by sequence differences in the capsid (CA) portion of the viral Gag protein.

Beginning in the 1990s, sporadic evidence emerged that hinted at the existence of additional restriction factors, including factors affecting HIV-1. For instance, (1) virus infectivity or the capacity of viral accessory genes to function could be profoundly affected by the animal species of the cells under experimental examination (Simon et al. 1998b; Hofmann et al. 1999); and (2) the requirements for individual accessory genes during virus replication could vary enormously between human cell lines (Gabuzda et al. 1992; Varthakavi et al. 2003). Drawing on the Fv1 analogy, but recognizing the lack of similarity among the phenotypic manifestations of these replication barriers, the concept that primate cells express a range of restriction factors that target HIV-1 and other lentiviruses gradually gained acceptance.

One experimental approach that added weight to these arguments, and parallels the resistance of $Fv1^{n/b}$ cells to N- and B-MLV infection, is illustrated in Figure 1. Here, cells that are restrictive or susceptible for a viral function of step or replication (also called nonpermissive and permissive cells, respectively) are fused in vitro to form heterokaryons that consequently express the contents of both cells. The capacity of these cell hybrids to support the viral activity in question is then assessed. A restricting phenotype points to the presence of a dominant restriction factor that is absent from susceptible cells, whereas a susceptible phenotype suggests that a positively acting cofactor has been lost from the nonpermissive cells. Cell fusion studies of this genre established that distinct restriction factor activities were apparently countered by HIV-1 Vif and Vpu, or evaded by sequence changes in CA (Madani and Kabat 1998; Simon et al. 1998a; Cowan et al. 2002; Varthakavi et al. 2003).
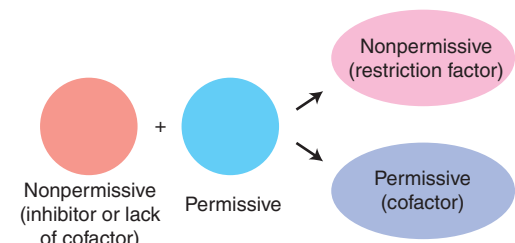
Figure 1. Cell fusion studies can illustrate restriction factor activity.

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Two experimental strategies have been used to discover genes encoding restriction factors. First, comparative transcriptomics (e.g., gene arrays or copy DNA [cDNA] subtraction) has been used to identify genes that are preferentially expressed in restrictive cells relative to susceptible cells. Lists of candidates may be pruned further when additional characteristics, such as interferon responsiveness, are known. Candidate cDNAs are then validated functionally by asking whether ectopic expression converts susceptible cells into restrictive cells. A second more direct screening approach has exploited the expression of cDNA libraries derived from restrictive cells in susceptible cells, followed by the selection of cells that have acquired viral resistance and the isolation of the cDNA conferring resistance. The former approach was used to identify APOBEC3G and tetherin (Sheehy et al. 2002; Neil et al. 2008), which initially engage their viral substrates at postintegration stages of replication, whereas the latter scheme is well suited for finding factors that act at preintegration steps, and led to the identification of TRIM5α (Stremlau et al. 2004). In all cases, a cardinal feature that defines a restriction factor is the capacity to display potent antiviral function as single genes (i.e., without the requirement for specific cellular cofactors). This and other shared features of the known restriction factors are listed in Table 1.

Table 1. Cardinal and shared features of HIV-1 restriction factors

Germline-encoded, expressed constitutively, and interferon (IFN)-inducible.

Dominantly acting, cell-autonomous mechanisms of action.

Largely inactive against contemporary "wild-type" viruses in cells of natural hosts.

Mediate potent species-specific suppression: control of cross-species transmission?

Some (APOBEC3 and tetherin) are regulated by HIV/SIV accessory proteins.

Display hallmarks of positive genetic selection (high d_N/d_S ratio), reflecting host–pathogen coevolution.

Function and/or regulation involves the cellular ubiquitin/proteasome system.

KEY ADVANCES IN THE STUDY OF RESTRICTION FACTORS

Beyond the recognition of their existence, major advances in restriction factor biology include the identification of the proteins responsible for restriction activity, the elucidation of mechanisms of action, the recognition of specific viral countermeasures and means of evasion, and the emerging paradigm that restriction factors and their antagonists continually coevolve. For each of the factors highlighted in this work, remarkable and unanticipated biology has been uncovered.

APOBEC3 PROTEINS AND Vif

Identification

The interplay between human APOBEC3 proteins and HIV-1 was discovered through efforts to understand the function of the ∼23 kDa viral protein Vif (an acronymn for virion infectivity factor) (reviewed by Malim 2009). Vif is required for HIV-1 replication in primary cell types, particularly CD4+ T cells, as well as some cell lines, yet is dispensable in other lines. Cell fusion experiments attributed causation to a restriction factor, and a cDNA subtraction-based screen revealed the human gene *APOBEC3G* as being sufficient to repress the replication of *vif*-deficient HIV-1 (Sheehy et al. 2002).
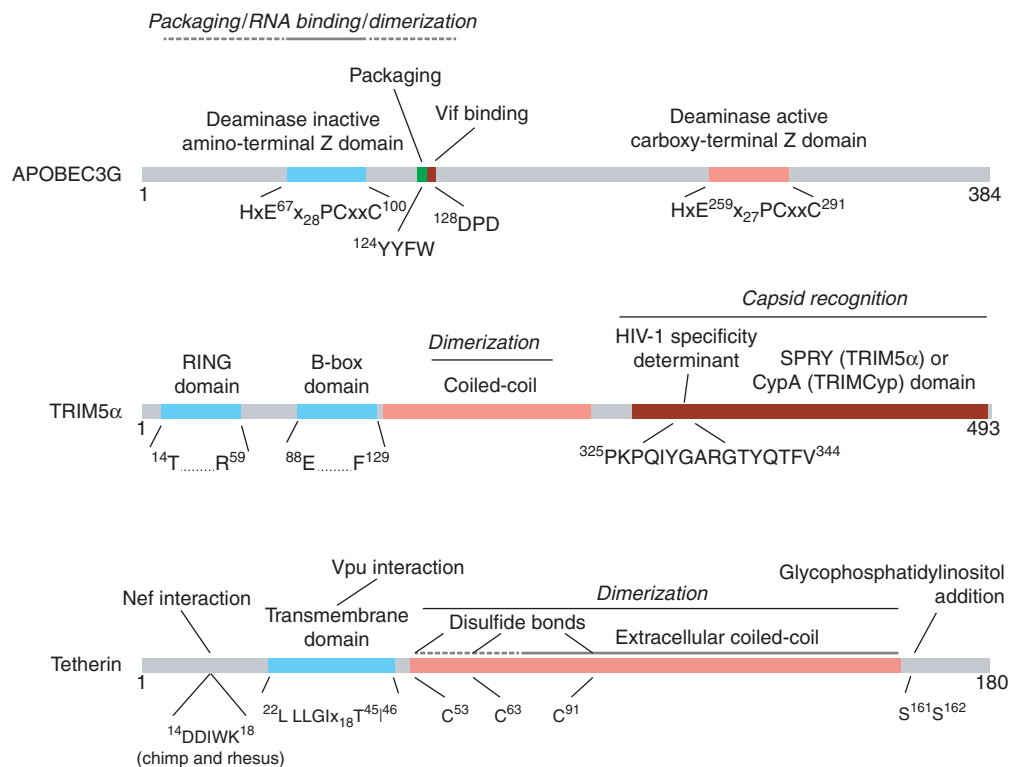
APOBEC3 Proteins

APOBEC3G (A3G) is a member of a family of vertebrate proteins (humans encode 11) with polynucleotide (RNA or DNA) cytidine deaminase activity. This reaction results in the postsynthetic editing of cytidine residues to uridines, thereby altering the nucleotide sequence and, with DNA substrates, introducing an unnatural base. APOBEC3 proteins are expressed widely in human tissues and cell types, and particularly in hematopoietic cells (Koning et al. 2009; Refsland et al. 2010). All APOBEC proteins contain one or two copies of a characteristic zinc-coordinating deaminase domain (the Z domain) (LaRue et al. 2009) that comprises a platform of five β strands, flanking α helices

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and connecting loops, a constellation of three histidine or cysteine residues that coordinate an essential Zn^{2+} ion, and a catalytic glutamic acid residue (Chen et al. 2008). A3G has two such domains: the carboxy-terminal domain mediates deamination, whereas the amino-terminal Z domain does not have catalytic activity (for unknown reasons), mediates incorporation into HIV-1 particles (see below), and is recognized by Vif (see below) (Fig. 2) (reviewed by Malim 2009; Albin and Harris 2010). A3G also forms dimers, in an RNA-dependent manner, and this attribute is thought to be important for packaging and antiviral function (Huthoff et al. 2009). However, in the absence of structures for full length A3G, there is ongoing debate not only regarding the relative arrangement of the Z domains within a single A3G molecule, but also into the nature, determinants, and significance of dimerization.

Viral Hypermutation

In the absence of Vif, A3G is packaged into assembling HIV-1 virions through the combined action of RNA binding and interactions between the amino-terminal Z domain of A3G and the nucleocapsid (NC) region of Gag (Bogerd and Cullen 2008). A3G is transferred to target cells by ensuing virus infection where, through its association with the viral reverse transcriptase complex (RTC), it deaminates cytidine residues in nascent single-stranded (mostly) negative-strand cDNA (Harris et al. 2003; Mangeat et al. 2003; Zhang et al. 2003; Yu et al. 2004). Up to 10% of cytidines may be edited, resulting in guanosine-to-adenosine hypermutation of viral plus strand sequence and the debilitating loss of genetic integrity (this can be considered as error catastrophe) (Fig. 3). Not all cytidines are equivalent targets

Figure 2. Domain organization of the human APOBEC3G, TRIM5α, and tetherin proteins. Critical domains and motifs are highlighted in color, their functions and attributes are indicated above, and important sequence motifs are shown below. The number of amino acids in each protein is also indicated.

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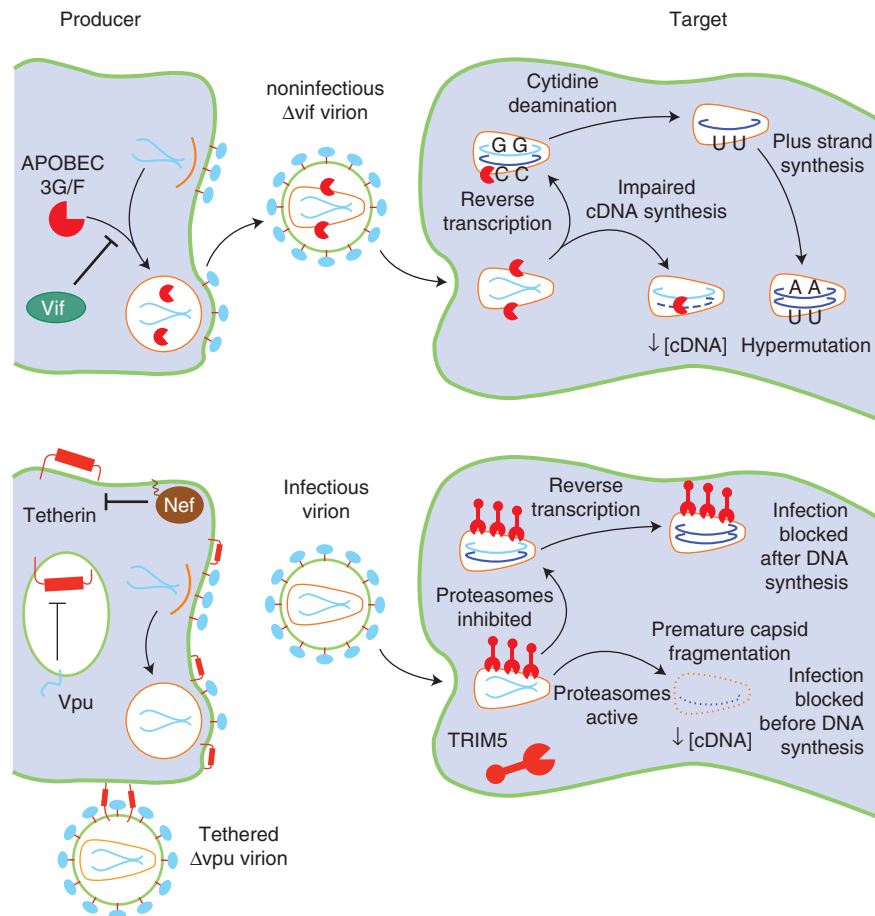
Figure 3. Mechanisms of restriction factor function and evasion. HIV-1 RNA is shown in light blue, HIV-1 cDNA in dark blue, and restriction factors in red. The sites of Vif, Vpu, and Nef antagonism are indicated. Refer to text for further details.

for A3G and there is a marked local sequence preference for 5′-CC**C**A (the deaminated cytidine is underlined) (Harris et al. 2003; Yu et al. 2004). Studies in vitro indicate that partiality for the 3′ cytidine is attributable to the 3′-to-5′ processivity of the enzyme on its DNA substrate (Chelico et al. 2006).

Mutation is not the only consequence of A3G action, as the levels of cDNA that accumulate during new HIV-1 infection are also diminished. It was attractive to believe that recognition of uridine-containing DNA by host DNA repair enzymes could initiate DNA degradation; however, this notion has been discounted because inhibition of uracil DNA

glycosidases fails to reverse A3G's effect on DNA levels (Langlois and Neuberger 2008). Rather, it appears that A3G impedes the translocation of reverse transcriptase along the viral RNA template, although the mechanistic underpinning of this effect awaits full elucidation (Fig. 3) (Iwatani et al. 2007; Bishop et al. 2008).

Vif Inhibits A3G Function

The antiviral activity of A3G is antagonized by HIV-1 Vif: Indeed, Vif is so efficient that physiologic levels of A3G have no overt effect on wild-type (Vif expressing) HIV-1 infection or replication in cultured cells. Vif's principal

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activity is to bind to A3G and recruit it to a cellular ubiquitin ligase complex that comprises the cullin5 scaffold protein, elongins B and C, Rbx2, and an as yet unidentified E2 conjugating enzyme (Yu et al. 2003). This results in A3G polyubiquitylation and proteasomal degradation, and therefore averts the encapsidation of A3G into nascent viral particles (Marin et al. 2003; Sheehy et al. 2003; Stopak et al. 2003; Yu et al. 2003). Recently, it has also been proposed that the central polypurine tract (cPPT) of HIV-1 helps mitigate the mutagenic effects of A3G by limiting substrate availability through reducing the length of time that minus-strand cDNA remains single stranded (Hu et al. 2010).

Extensive investigations have delineated various interactions that contribute to the assembly of the A3G-Vif-ligase complex. These are portrayed in Figure 4, although their temporal relationships with each other during complex formation are unknown (reviewed by Malim 2009; Albin and Harris 2010). Critical interactions include the binding of Vif's suppressor of cytokine signaling (SOCS) box to the elonginB/C heterodimer (Bergeron et al. 2010), the interaction between the Zn^{2+} coordinating motif of Vif and cullin5, and the recognition of the A3G substrate by discontinuous elements within the amino-terminal region of HIV-1 Vif. This last interaction is of particular interest as it modulates the species-specific regulation of A3G by HIV and SIV Vif proteins; e.g., the African green monkey (AGM) A3G protein contains a lysine at the position corresponding to the aspartic acid at position 128 in human A3G, and this permits recognition and regulation by SIV_{AGM}, but not HIV-1, Vif (reviewed by Malim 2009; Albin and Harris 2010). Indeed, it has been argued that the capacity of the Vif proteins of ancestral viruses to counteract the APOBEC3 proteins of new hosts has played an important role in past zoonotic transmissions of SIVs into humans (Gaddis et al. 2004).

APOBEC3F and APOBEC3H

Of the remaining 10 human APOBEC proteins, many have been assigned HIV-1 inhibitory function in the context of overexpression studies in vitro. However, at more relevant levels of expression, the current weight of evidence indicates that only APOBEC3F (A3F) and one allelic form of APOBEC3H (A3H), haplotype II, significantly suppress HIV-1 (reviewed by Albin and Harris 2010). This can be viewed as making biological sense because these two proteins (but not other APOBEC3 proteins) are regulated by Vif, perhaps implying that Vif-mediated neutralization is only important for APOBEC3 proteins that naturally encounter HIV-1 during in vivo infections. The mechanisms of antiviral action of A3F/H mirror those of A3G, although their intrinsic potencies appear to be lower (Holmes et al. 2007; Miyagi et al. 2010), and the preferred target site for deamination is 5′-TC rather than 5′-CC. Their levels of expression are also lower (Koning et al. 2009; Refsland et al. 2010), supporting the view that A3G is the most significant family member for inhibiting HIV-1 infection.
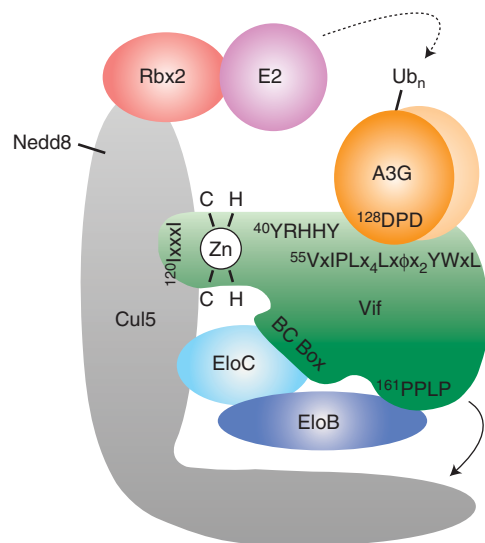
Figure 4. Components and intermolecular contacts in the cullin5-elonginBC-Vif ubiquitin ligase complex. HIV-1 Vif serves as a receptor protein that interacts with cullin5, elonginB (through a proline-rich motif), elonginC (through a BC box), and A3G. Refer to text for further details.

Regulation of A3G

The most obvious form of regulation is Vif-initiated degradation during virus infection (above). There is a consensus that APOBEC3 proteins are transcriptionally induced by type 1 IFNs, particularly in myeloid cells (Koning et al. 2009; Refsland et al. 2010), a feature that characterizes many cellular proteins involved in early/innate control of viral infection. Being a DNA mutator, it is also important to consider how host cell (chromosomal) DNA might be spared. There are a number of possibilities: (1) It is not, but mutations are repaired before fixation; (2) it is partially repaired/protected and some level of mutagenesis takes place; (3) A3G is localized to the cytoplasm; and (4) A3G is sequestered in ribonucleoprotein particles (RNPs) that suppress deaminase activity (Chiu et al. 2006; Kozak et al. 2006; Gallois-Montbrun et al. 2007). Interestingly, A3G-RNPs (and A3F-RNPs) further accumulate in mRNA processing bodies (P-bodies) (Wichroski et al. 2006; Gallois-Montbrun et al. 2007), although the relevance of this to A3G function or antiviral activity is uncertain.

APOBEC3 Proteins and Natural HIV-1 Infections

A3G/F/H appear to encounter HIV-1 during in vivo infection. In addition to the sensitivity of these proteins to Vif, guanosine-to-adenosine hypermutated HIV-1 sequences with the expected local nucleotide preferences are readily recovered from infected persons. This shows that APOBEC3 proteins can escape complete inhibition by Vif, and this may be due to variation in Vif function, allelic variation in *APOBEC3* genes, excessive APOBEC3 protein expression or activity, or simply stochastic events. It has also been proposed that infrequent APOBEC3-induced mutations (as opposed to hypermutation) can (1) contribute to sequence diversification and evolution, perhaps in ways that are beneficial to the virus in terms of immune escape or drug resistance (Wood et al. 2009; Kim et al. 2010; Sadler et al. 2010); or (2) generate nonsense (or missense) mutations that result in the expression of truncated

or misfolded viral proteins, and the enhanced presentation of viral epitopes to cytotoxic T cells (Casartelli et al. 2010).

Can variation in the APOBEC3 landscape influence the course of HIV-1 infection in humans? Many publications have started to address this point, although it remains challenging to distinguish between causation and consequence. Even though there are inconsistencies among some findings, there is a discernible trend that increased levels of A3G/F expression tend to correlate with clinical benefit (reviewed by Albin and Harris 2010).

TRIM5α AND TRIMCYP

Identification

The existence of an antiretroviral protein that targets HIV-1, SIV, and other retroviral capsids was predicted by descriptions of restricted infection in a number of mammalian cell lines (Bieniasz 2003). The characteristics of these resistance phenotypes were highly reminiscent of those displayed by Fv1: Restriction was saturable, dominant in heterokaryons, independent of the route of entry, and could be encountered or avoided by manipulating retroviral CA sequence. However, resistance was apparent in nonmurine species, including humans, which lack the *Fv1* gene. A screen for rhesus macaque genes that could restrict HIV-1 infection when expressed in human cells resulted in the identification of TRIM5α (Stremlau et al. 2004), and this protein has subsequently been shown to be responsible for the majority of similar postentry restriction phenomena in a number of mammalian species (Hatziioannou et al. 2004; Keckesova et al. 2004; Perron et al. 2004; Yap et al. 2004; Johnson and Sawyer 2009).

TRIM5α and TRIMCyp Proteins

TRIM5α is an ∼500 amino acid cytoplasmic protein that acts following the entry of retroviral capsids and their contents into the cytoplasm of target cells. Its action is generally accompanied by a failure to synthesize viral cDNA (Stremlau et al. 2004). TRIM5 is one of a family of ∼70 so-called "tripartite motif" (TRIM)-containing

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proteins. Family members have a broadly similar domain organization (Nisole et al. 2005). The TRIM domain is composed of amino-terminal RING and B-box type 2 domains linked to a central coiled-coil domain (Fig. 2). In the case of TRIM5, the coiled coil drives the formation of dimers. The nature of the carboxy-terminal domain can vary widely among proteins of the TRIM family (Nisole et al. 2005). In the case of TRIM5α, and several other TRIM proteins, this carboxy-terminal domain is called the B30.2 or PRYSPRY domain.

The range of retroviruses that are inhibited by a particular TRIM5α varies dramatically, depending on the species of origin. For instance, the human TRIM5α protein is an effective inhibitor of N-MLV, as well as equine infectious anemia virus (EIAV) (Hatziioannou et al. 2004; Keckesova et al. 2004; Perron et al. 2004; Yu et al. 2004); however, it is virtually inactive against HIV-1. Conversely, TRIM5α proteins from Old World monkey species generally inhibit HIV-1 infection (Stremlau et al. 2004). In general, TRIM5α proteins are poor inhibitors of retroviruses that are found naturally in the same host species, but are quite often active against retroviruses that are found in other species. As such, TRIM5α can impose a quite formidable barrier to cross-species transmission of primate lentiviruses (Hatziioannou et al. 2006).

The carboxy-terminal SPRY domain contains most of the determinants that govern substrate selection for a given TRIM5α protein, and has undergone rapid evolution, as evidenced by high numbers of nonsynonymous differences in interspecies sequence comparisons compared to the genome average (Sawyer et al. 2005; Song et al. 2005; Johnson and Sawyer 2009). Three peptide segments (V1–V3) within the SPRY domain that are hypervariable in both length and sequence likely encode surface exposed loops, by analogy with SPRY domains of known structure. At least one of these variable loops (V1) can be shown experimentally to be a key determinant of antiretroviral specificity. Indeed, it is possible to make a small number of changes, even a single amino acid substitution, in V1 segments of primate TRIM5α proteins and alter

their ability to recognize HIV-1 and SIV strains (Perez-Caballero et al. 2005; Sawyer et al. 2005; Stremlau et al. 2005; Yap et al. 2005). This genetic evidence, coupled with studies showing that the PRYSPRY governs the ability of TRIM5α proteins to bind to HIV-1 capsid-like assemblies in vitro (Stremlau et al. 2006a), indicates that this TRIM5α domain has evolved to specifically recognize particular capsids in the cytoplasm of target cells.

A number of lentivirus capsids bind to the abundant host cell chaperone protein, cyclophilin A (CypA), via a peptide loop that is exposed on the surface of the assembled capsid (see Sundquist and Kraeusslich 2011). The precise role for this interaction is not completely clear, but both the sequence of this exposed loop and the CypA protein itself can affect the sensitivity of HIV-1 to TRIM5α (Berthoux et al. 2005; Keckesova et al. 2006; Stremlau et al. 2006b). In owl monkeys (Sayah et al. 2004), and independently in some macaques (Liao et al. 2007; Brennan et al. 2008; Newman et al. 2008; Virgen et al. 2008; Wilson et al. 2008), retrotransposition events have placed CypA cDNAs into the TRIM5 locus, so that the resulting chimeric gene is expressed as a TRIM5-CypA fusion protein (TRIMCyp) with a CypA protein domain replacing the PRYSPRY domain. Predictably, TRIMCyp proteins are, in general, potent inhibitors of lentiviruses whose capsids bind CypA. However, evolved modification of the capsid binding specificity of the CypA domains in TRIMPCyp proteins can occur through mutation in the CypA encoding sequence, acquired during or after its retrotransposition into the TRIM5 locus. For example, the TRIMCyp proteins found in macaques and owl monkeys have been shown to differ greatly in their ability to inhibit HIV-1 infection (Virgen et al. 2008; Price et al. 2009).

Mechanisms of Infection Inhibition by TRIM5α and TRIMCyp Proteins

The mechanisms by which TRIM5 proteins act to block retroviral infection are not completely understood (Fig. 3). This is at least partly because the processes that occur during the

postentry phase of the retroviral life cycle that are perturbed by TRIM5 proteins are not easily analyzed with currently available biochemical and biophysical techniques. Nevertheless, it is clear that TRIM5α and TRIMCyp bind directly to HIV-1 capsids (Stremlau et al. 2006a) and, at least in the case of TRIMCyp, recognition of the incoming capsid must occur within 15–30 min of viral entry for inhibition to be effective (Perez-Caballero et al. 2005). Some studies have also revealed that incoming retroviral capsids lose their particulate nature on entry into the target cell cytoplasm if they encounter a TRIM5α or TRIMCyp protein (Stremlau et al. 2006a). These findings suggest a model in which TRIM5α and TRIMCyp accelerate capsid fragmentation soon after viral entry, thereby disrupting RTC architecture and blocking reverse transcription (Fig. 3).

Two zinc-binding domains (RING and B-box type 2) at the amino terminus of TRIM5α protein are important for the full antiviral activity of the protein. The B-box domain, although not required for TRIM5α dimerization, appears to constitute a second self-associating domain (Li and Sodroski 2008). Thus, the B-box contributes to the formation of higher-order multimers and the propensity of TRIM5α to assemble into preaggresomal structures, termed "cytoplasmic bodies," which are visible by fluorescent microscopy. Although cytoplasmic body formation may not be essential for antiviral activity, higher-order TRIM5α multimerization does appear to increase the efficiency with which TRIM5α interacts with the capsid lattice, and thus promotes antiviral potency (Diaz-Griffero et al. 2009). Indeed, recent cryoelectron microscopy analyses of purified TRIM5α show a propensity to assemble into hexagonal lattices that can interact in an ordered, polyvalent manner with preformed hexagonal lattices of HIV-1 CA (Ganser-Pornillos et al. 2011).

Although the RING domain of TRIM5α proteins possesses E3 ubiquitin ligase activity, ubiquitin or proteasomes appear not to be essential for antiviral function. Indeed, TRIM5α and TRIMCyp display potent anti-HIV-1 activity in the presence of proteasome inhibitors, or in a cell line containing an inactive ubiquitin activating (E1) enzyme (Perez-Caballero et al. 2005). However, proteasome inhibition prevents TRIM5-promoted capsid disassembly and restores reverse transcription, without enabling infection (Wu et al. 2006; Diaz-Griffero et al. 2007). Thus, it appears that neither proteasome activity nor accelerated capsid fragmentation or inhibition of reverse transcription is absolutely required for TRIM5α to exert antiretroviral activity (Fig. 3). In fact, aside from capsid binding and multimerization, no activity that has been associated with TRIM5α has been definitively shown to be required for antiretroviral activity. One possible explanation for this is that TRIM5α is capable of inhibiting infection in two or more redundant ways. Alternatively, it is conceivable that perturbing the ubiquitin/proteasome system simply slows whatever process (e.g., capsid fragmentation) that is responsible for inhibiting infection, such that it is not completed until after reverse transcription.

TETHERIN

Identification

The identification of tetherin was based on the finding that the Vpu accessory protein was required for efficient virion release from some cell lines but completely dispensable in others. The requirement for Vpu was found to be dominant in heterokaryons (Varthakavi et al. 2003) and inducible in cells from which it was ordinarily absent by treatment with IFN-α (Neil et al. 2007). Other studies indicated that the absence of Vpu rendered HIV-1 sensitive to a protein-based adhesive or tethering mechanism that trapped nascent virions on the surface of infected cells (Neil et al. 2006). These accumulated findings suggested the existence of IFN-induced protein tethers and provided the basis for a microarray/candidate gene-based discovery of tetherin, a membrane protein whose expression was necessary and sufficient to impose a requirement Vpu for the efficient release of HIV-1 particles (Neil et al. 2008; Van Damme et al. 2008).

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The Tetherin Protein and Mechanism of Virion Retention

In the absence of Vpu, and the presence of tetherin, HIV-1 particles are assembled normally, their lipid envelopes undergo ESCRT-protein-mediated fission from the plasma membrane, and they adopt a mature morphology. However, tetherin causes virions to remain trapped at the surface of the infected cell from which they are derived and to accumulate thereafter in endosomes following internalization (Neil et al. 2006). Tetherin is an unusual type II single-pass transmembrane protein in that it has both a transmembrane anchor close to its amino terminus and a glycophosphatidylinositol (GPI) lipid anchor at its carboxyl terminus (Fig. 2) (Kupzig et al. 2003). The entire extracellular portion of tetherin forms a single long α helix, much of which adopts a canonical dimeric coiled-coil structure (Fig. 5) (Hinz et al. 2010; Schubert et al. 2010; Yang et al. 2010). Additionally, each of three extracellular cysteines in each monomer forms disulfide bonds with a corresponding cysteine in another tetherin molecule (Figs. 2 and 5).

Several lines of evidence suggest that a simple and direct tethering mechanism underlies the ability of tetherin to cause retention of nascent virions (Fig. 5). Although tetherin's overall configuration (Fig. 2) is required for virion retention, it is surprisingly tolerant of mutations, including substitution of entire tetherin domains with protein domains that have similar predicted structures but lack sequence homology (Perez-Caballero et al. 2009). Indeed, a completely artificial tetherin-like protein, assembled from structurally similar but unrelated protein domains, can effectively mimic its activity. This fact, and the finding that tetherin can block viruses from various families whose structural proteins have no sequence or structural homology to each other, makes it unlikely that specific recognition of viral proteins is required for function (Jouvenet et al. 2009; Kaletsky et al. 2009; Mansouri et al. 2009).

Both the amino-terminal transmembrane domain and the carboxy-terminal GPI modification are essential for tethering function, and
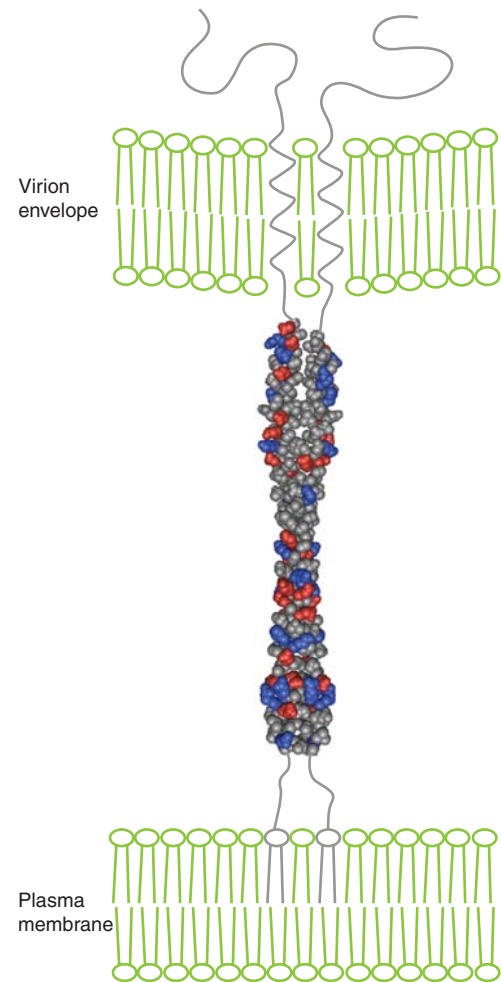
Figure 5. A structural model of how tetherin causes virion retention. In the scenario depicted here, tetherin transmembrane domains infiltrate the virion envelope, whereas the glycophosphatidylinositol anchors remain in the plasma membrane. The tetherin coiled-coil domain thereby physically links cell and virion membranes. Refer to Perez-Caballero et al. (2009) and Fitzpatrick et al. (2010) for alternative models.

tetherin mutants lacking either are efficiently incorporated into the lipid envelope of HIV-1 particles via the remaining membrane anchor (Perez-Caballero et al. 2009). The intact tetherin protein can also be found in virions in some circumstances. A protected dimeric amino-terminal tetherin fragment can be found in the

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envelope of tethered virions that can be recovered from the surface of cells by protease, indicating that at least some tethered virions have dimeric tetherin amino termini inserted into their lipid envelope (Perez-Caballero et al. 2009). Thus, either or both tetherin membrane anchors seem to be inserted into the lipid envelope of budding virions as they emerge from infected cells (Fig. 3). Concordantly, both fluorescent and electron microscopic analyses reveal that tetherin colocalizes with virions on the cell surface (Neil et al. 2008; Jouvenet et al. 2009; Perez-Caballero et al. 2009; Fitzpatrick et al. 2010; Hammonds et al. 2010).

Models for tetherin action include the possible scenario that one pair of tetherin membrane anchors (e.g., the TM domains) infiltrates the lipid envelope of the assembling virion, whereas the other pair (e.g., the GPI anchors) remains in the cell membrane (Fig. 5). In this configuration, the coiled coil could promote the spatial separation of two pairs of membrane anchors, increasing the probability that one of the two pairs is incorporated into the virion envelope. Alternatively, it is possible that non-covalent interactions might mediate the adhesion of tetherin dimers that are incorporated into the virions and those that remain in the host cell membrane. Tetherin expression causes the accumulation of virions that appear to be tethered to each other as well as to the cell surface. Importantly, this is only possible if both types of TM anchor can be incorporated into virion envelopes, and can occur following the budding of a virion at the same site on the plasma membrane as that already occupied by a tethered virion.

Antagonism of Tetherin by HIV and SIV Vpu, Nef, and Env Proteins

Because the relatively invariant, host-derived lipid envelope, rather than a viral protein, is the target for tetherin action, it would seem difficult for a virus to evade tetherin by avoiding interaction with it. Faced with this problem, HIVs and SIVs have independently evolved new biological activities in the form of *trans*-acting tetherin antagonists.

The HIV-1 Vpu protein is used by HIV-1 strains as an antagonist of tetherin (Neil et al. 2008; Van Damme et al. 2008). It is ~14 kDa and is composed of a single transmembrane helix and a small cytoplasmic domain. There is some uncertainty as to how antagonism is achieved, as several different mechanisms have been reported. There is, however, general agreement that Vpu colocalizes with and can be coimmunoprecipitated with tetherin and reduces the level of tetherin at the cell surface (Van Damme et al. 2008), either by retarding its progress through the secretory pathway or by causing its internalization (Mitchell et al. 2009; Dube et al. 2010). Vpu can also reduce the overall steady-state level of tetherin in cells, at least under conditions of transient overexpression (Bartee et al. 2006). The latter effect is reversed by proteasome inhibitors (Douglas et al. 2009; Miyagi et al. 2009), but because proteasome inhibition can deplete ubiquitin and thereby affect the trafficking of some cargoes through the endosomal system, it is not yet clear whether the proteasome or the endolysosomal system is directly responsible for tetherin degradation. Some studies find that tetherin down-regulation from the cell surface and/or degradation is modest or nonexistent in cell types where Vpu appears fully functional. Thus, a clear overall picture of precisely how Vpu antagonizes tetherin is currently lacking.

Most SIVs do not encode Vpu proteins, and instead use another accessory gene product, the ~27 kDa Nef protein, to antagonize tetherin (Jia et al. 2009; Zhang et al. 2009). Nef is an amino-terminally myristoylated peripheral membrane protein that can interact with a number of cellular partners, including clathrin adapter protein (AP) complexes, several kinases, and dynamin-2. It is not currently known whether any of these Nef binding proteins play a role in tetherin antagonism or how antagonism is achieved. In some instances, primate lentivirus Env proteins can also act as tetherin antagonists, and in these cases it appears the Env proteins engage tetherin and cause it to be sequestered within intracellular compartments (Gupta et al. 2009b; Le Tortorec and Neil 2009).

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Evolution of Tetherin and Viral Antagonists

The HIV-1 Vpu protein is an efficient antagonist of human tetherin but is ineffective against tetherins from other animals such as monkeys or rodents. This species-specific action of Vpu has been used to derive genetic and biochemical evidence that Vpu and tetherin interact via their transmembrane helices (Fig. 2) (Gupta et al. 2009a; McNatt et al. 2009). Similarly, although SIV Nef proteins effectively counteract monkey tetherin proteins that are found in their natural host species, they are often inactive against other tetherins. In this case, sequence variation in the tetherin cytoplasmic tail defines its sensitivity to Nef, and a five codon deletion in the cytoplasmic tail of human tetherin that renders it Nef resistant defines a particularly important target of Nef action on tetherin (Fig. 2) (Jia et al. 2009; Zhang et al. 2009).

As outlined in Sharp and Hahn (2011), primate lentiviruses have been transmitted from species to species on numerous occasions. Most recently, humans have been the recipients of these zoonoses, acquiring SIVcpz from chimpanzees and SIVsm from sooty mangabeys, resulting in the viruses we now call HIV-1 and HIV-2. Analyses of Vpu and Nef proteins from many SIVs have revealed that these proteins effectively exchanged the role of tetherin antagonist as they were passed from species to species and encountered tetherin proteins with varying TM and cytoplasmic tail sequences (Sauter et al. 2009; Lim et al. 2010). Most notably, SIVcpz uses the Nef protein as a tetherin antagonist, whereas its immediate descendent, HIV-1, uses Vpu. Similarly, SIVsm (which lacks Vpu) uses Nef to counteract tetherin, whereas its descendent, HIV-2, uses its Env protein in this role. In both cases, the acquisition of tetherin antagonist activity by HIV-1 Vpu and HIV-2 Env proteins likely occurred because human tetherin lacks a key five residue determinant of Nef sensitivity in its cytoplasmic tail (Fig. 3) (Sauter et al. 2009; Lim et al. 2010). These gain-of-function events that have occurred as a consequence of transmission to a new species illustrate the functional plasticity of HIV/SIV accessory proteins.

NEW RESEARCH AREAS

Although much has been discovered about restriction factors, there is significant scope for future discovery and exploitation. There are strong suspicions that there are more, perhaps many more, restriction factors to be uncovered. For example, the basis for the inhibitory effects of type 1 IFNs on the early steps of HIV-1 infection are largely unexplained, and may involve the action of unidentified restriction factors (Goujon and Malim 2010). Additionally, the functions of the HIV-1 accessory genes that have not been elucidated in their entirety and, based on the precedents described herein, are quite likely to include interactions with, and regulation of, restriction factors. In addition to the general approaches described above that led to the discovery of APOBEC3, TRIM5, and tetherin, as well as the MLV inhibitor ZAP (Gao et al. 2002), we predict techniques that can identify factors that interact with regulators of viral infectivity, or can monitor alterations in protein abundance and form, such as SILAC (stable isotope labeling with amino acids in cell culture), will have utility for finding new restriction factors.

Indeed, two groups very recently reported using proteomic methods to identify SAMHD1 (sterile α motif domain-, HD domain-containing protein 1) as the myeloid-specific, degradable cellular target of HIV-2/SIVsm Vpx proteins (Hrecka et al. 2011; Laguette et al. 2011). This protein is of considerable interest as its restriction of HIV and SIV infections (Goujon et al. 2008) correlates with limited viral cDNA accumulation, and mutations in the *SAMHD1* gene in humans result in a disease called Aicardi-Goutières syndrome (AGS) that is characterized by excessive IFN production and inflammation, and therefore, mimics congenital virus infection (Lee-Kirsch 2010). The mechanism of SAMHD1-mediated restriction remains to be defined, but the involvement of HD domains in nucleotide metabolism is suggestive of direct interaction with viral nucleic acids.

Therapeutic exploitation of restriction factor biology has yet to receive widespread attention. Indeed, the pharmacologic mobilization

of restriction factors, possibly by blocking interactions between a viral antagonist and a host restriction factor, would appear to be an attractive approach for the development of novel antivirals. For instance, inhibitors of Vif function that have viral inhibitory activity in cell culture have recently been described (Nathans et al. 2008; Cen et al. 2010) and provide a paradigm for possible exploitation of such targets.

Host cell factors mentioned: APOBEC3G, TRIM5α, and tetherin.

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