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Accessories to the Crime: Recent Advances in HIV Accessory Protein Biology

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Abstract

Recent advances in understanding the roles of the lentiviral accessory proteins have provided fascinating insight into the molecular biology of the virus and uncovered previously unappreciated innate immune mechanisms by which the host defends itself. HIV-1 and other lentiviruses have developed accessory proteins that counterattack the antiviral defenses in a sort of evolutionary battle. The virus is remarkably adept at co-opting cellular degradative pathways to destroy the protective proteins. This review focuses on recent advances in understanding three of the accessory proteins—virion infectivity factor (Vif), viral protein R (Vpr), and viral protein U (Vpu)—that target different restriction factors to ensure virus replication. These proteins may provide promising targets for the development of novel classes of antiretroviral drugs.

Introduction

The presence of additional open reading frames in lentiviruses compared with simpler genomes of the murine and avian oncoviruses is one of the fascinating features of HIV-1 and has been an important area of investigation since the genome of the virus was first sequenced 24 years ago. Indeed, much has been learned—not just about the virus, but also about the cell—in studies of these genes and their products.

The picture that has emerged is one of how deviously clever evolution has been. A newly appreciated theme from these studies is the extent to which the accessory proteins are directed at eluding host adaptive and innate immune mechanisms. A second theme is how effectively the virus makes use of the protein degradative pathways in the cell to remove proteins that interfere with its purposes. This review focuses on recent advances in understanding three of the accessory proteins—virion infectivity factor (Vif), viral protein R (Vpr) and the closely related viral protein X (Vpx), and viral protein U (Vpu)—each of which relieves an intracellular restriction to virus replication (Fig. 1).

Escaping APOBEC3 With Vif

To replicate in CD4⁺ T cells and macrophages, the natural targets of HIV-1, the virus needs a functional Vif [1,2]. HIV-1 that is engineered to lack Vif—termed *vif* HIV-1—fails to

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replicate in primary cells. The failure to replicate is caused by a dominant inhibitor in the cells, APOBEC3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G) [3] and, to a lesser extent, its closest relative, APOBEC3F. In the human genome, *APOBEC3* is a family of seven related genes (*APOBEC3A*, *APOBEC3B*, *APOBEC3C*, *APOBEC3D/E*, *APOBEC3F*, *APOBEC3G*, and *APOBEC3H*), that are tandemly arrayed on chromosome 22 [4] and play a specific role in the innate immune defense against viruses. APOBEC3G and APOBEC3F are enzymes with potent antiviral activity that are expressed in lymphocytes and monocytes. Whether all seven of the family members fight viruses is not clear, but the importance of the antiviral activities of APOBEC3F and APOBEC3G is demonstrated by Vif, an accessory protein dedicated to inducing their destruction.

Diverse roles for cytidine deaminases

A remarkable feature of the APOBEC family, which includes APOBEC1, APOBEC2, APOBEC3, and the related activation-induced deaminase, is that the members play key roles in very different physiologic systems but do so through a common catalytic activity: cytidine deamination. APOBEC1 is an RNA editor whose role is to post-transcriptionally “edit” the mRNA for the lipid-transport protein apolipoprotein A by changing a single cytosine to uracil to generate a premature translational stop codon [5]. The APOBEC3 proteins act on single-stranded DNA rather than RNA to generate multiple mutations fairly randomly by hitting CC dinucleotides. Activation-induced deaminase is involved in antibody production by hypermutating immunoglobulin genes and mediating isotype class switch recombination—processes that also proceed through cytidine deamination [5]. The role of APOBEC2 is still unknown.

Roles of Vif and APOBEC3G in HIV-1 replication

How does cytidine deamination prevent HIV-1 replication? The generally agreed upon scheme that emerged after the identification of the role of APOBEC3G in HIV-1 replication by Sheehy et al. [3] is as follows. In a cell infected with *vif* HIV-1, APOBEC3G is packaged into the virion as it assembles. The packaged APOBEC3G molecules deaminate cytosines to uracils in the minus strands of the viral reverse transcript as it is synthesized by reverse transcriptase. Because of the minus-strand C→U changes, the plus strands that are synthesized from the deaminated template are G→A hypermutated. A gradient of G→A changes is made in the 3′→5′ direction in the genome that results from the length of time the minus strand remains single stranded during reverse transcription and the enzyme’s tendency to processively slide along the DNA [6,7•]. As few as a handful of virion-packaged APOBEC3G molecules are sufficient to render the virus harmless [8]. The uracil-containing DNA is degraded by DNA repair enzymes or, if it escapes degradation and manages to integrate, is incapable of programming the production of replication-competent progeny.

When wild-type HIV-1 infects a cell, its RNA genome is copied into a linear, double-stranded DNA by reverse transcriptase that then becomes covalently linked to the target cell chromosomal DNA through the catalytic activity of the integrase protein. Once the provirus is formed, the structural and regulatory proteins of the virus are synthesized. The newly synthesized Vif molecules cluster near the plasma membrane, where they bind to APOBEC3G molecules before they can associate with the assembling virion. The Vif–

APOBEC3G complexes then associate with an E3 ubiquitin ligase, a multisubunit protein complex that acts as a macromolecular machine to conjugate the 76-amino acid ubiquitin onto cellular proteins to signal their degradation [9]. The E3 ubiquitin ligase is composed of the scaffold protein Cul5; the substrate recognition element elongin B/C; and Rbx1, a subunit that associates with the ubiquitin-conjugating enzyme. Vif contains amino acid-sequence motifs termed *BC box* and *Cul5 box* that bind to elongin and Cul5 in the E3 ubiquitin ligase, respectively [10,11]. Once tagged with ubiquitin, APOBEC3G is shunted to the proteasome for degradation.

RNA binding: a tough strategy to elude

As virions bud from the cell surface, they capture small quantities of many different cellular proteins. However, APOBEC3 is packaged at higher efficiency than can be accounted for by a nonspecific mechanism. Which feature of the APOBEC3 proteins causes them to be packaged? APOBEC3G is a protein consisting of two cytidine deaminase domains: CDD1 and CDD2. Each domain coordinates a zinc ion through an amino acid motif that forms an RNA-grasping structure. Of the two domains, only CDD2 has cytidine deaminase activity [12,13]. CDD1 lacks catalytic activity but retains the ability to bind nucleic acid. APOBEC3 binds to the viral genomic RNA via CDD1 and goes along for the ride as the virion assembles at the plasma membrane. In addition to the viral genomic RNA, virions package small cellular RNAs such as the 7SL RNA that brings additional APOBEC3 molecules into the virion [12]. How APOBEC3G derives specificity for binding to viral RNA rather than the myriad cellular RNA is not clear, but it seems to preferentially bind RNA that is coated with the viral nucleocapsid protein [13,14].

Using the viral RNA to access the virion is a strategy the virus cannot readily circumvent. The genomic RNA molecules are at the heart of the virus. There is no obvious way to alter or hide them. To solve this dilemma, the virus had to develop an accessory protein. Paradoxically, the simpler animal retroviruses (eg, murine leukemia virus) do not encode Vif but replicate well in vivo. How these viruses survive is not clear. In fact, they have not completely solved the APOBEC3 problem. Genetically engineered APOBEC3 knockout mice are more susceptible to infection with murine retroviruses [15,16], suggesting that APOBEC3 restricts the replication of the simpler retroviruses to some extent.

Evolution of the *APOBEC3* genes

What selective forces drove the evolution of the *APOBEC3* family? The *APOBEC3* genes arose more than 30 million years ago, predating the lentiviruses, which evolved approximately 1 million years ago. The selective force that drove evolution of the *APOBEC3* genes may have been the endogenous virus-like genetic elements that litter mammalian genomes [17]. These elements are divided into those that have long terminal repeats (LTRs), such as the human endogenous retroviruses, and the non-LTR elements, such as SINE and LINE. Mobilization of these elements in the genome can be deleterious if they jump into vital genes. Some of the APOBEC3 proteins, particularly APOBEC3A, are potent inhibitors of LTR and non-LTR elements [18,19]. In support of a role for APOBEC3 in suppressing retroelements, the endogenous elements in the mouse genome contain frequent G→A mutations in GG dinucleotides, the preferred target of murine APOBEC3

[20], indicating that they had been targeted over the course of evolution. In more recent evolutionary history, the *APOBEC3* genes have been shown to be under selective pressure that has driven their expansion and diversity [21]. The mouse genome has a single *APOBEC3*, whereas primates have seven, reflecting the rapid expansion of the gene family in response to pressure from retroviruses.

Development of a new class of antiretrovirals

The potency with which APOBEC3G inhibits HIV-1 suggests it may be possible to manipulate the protein for therapeutic advantage. Small molecules that target Vif to prevent its association with APOBEC3G may defeat the virus. Alternatively, disruption of the association of Vif with the Cul5-based E3 ubiquitin ligase or inhibiting its activity also may be effective, although the latter is complicated by the need to maintain the physiologic function of the complex. Therapeutic manipulation of APOBEC3G expression in T cells and monocytes also may slow the virus.

Vpr and Vpx: Keys to New Restriction Factors

Vpr is a small nuclear protein that is expressed late in the virus replication cycle. Because of the relatively modest effect on virus replication in vitro, its role has been difficult to nail down. *vpr* HIV-1 replicates efficiently in cell lines and in activated CD4⁺ T cells in culture but replicates less efficiently in macrophages and monocytoic dendritic cells (DCs). Interestingly, in HIV-2 and most simian immunodeficiency virus (SIV) isolates, Vpr has been duplicated to generate a neighboring open reading frame that encodes the related accessory protein Vpx [22]. Why some viruses require two similar proteins is a mystery, but given their amino acid sequence homology, it is reasonable to think that they have similar but not identical functions. Despite its modest phenotype in vitro, Vpr plays an important role in vivo. HIV-1 primary isolates almost always have an open reading frame for Vpr, and given the speed with which the virus replicates, nonessential genes are rapidly lost. In the rhesus macaque model, animals infected with *vpr* or *vpx* SIV from rhesus macaques progress to AIDS more slowly and maintain low virus loads. In some animals, the mutations revert, causing the virus to become more pathogenic, demonstrating the selective pressure on these genes [23].

A virion-packaged accessory protein

Vpr and Vpx are components of the virion, a feature that distinguishes them from the other lentiviral accessory and regulatory proteins. Although virions contain small amounts of Nef and Vif, Vpr and Vpx can be packaged at levels commensurate with capsid. Vpr and Vpx are packaged into the virion by binding to the carboxyterminal domain of the Gag polyprotein p6 as the virus assembles [24]. The presence of these accessory proteins in the virion indicates that they are present during the early steps of infection and suggests a role before integration. Vpr remains associated with the viral preintegration complex as it transits into the nucleus. This association and its propensity to localize to the nucleus led investigators to hypothesize that Vpr escorts the preintegration complex into the nucleus of a nondividing cell. However, recent evidence shows that *vpr* HIV-1 can efficiently infect nondividing cells [25].

The hallmark of Vpr is cell cycle arrest. Cells that express Vpr by transfection or infection with wild-type virus arrest in the G₂ phase, eventually dying by apoptosis. Whether this is the intended role of Vpr is not clear. It is not obvious why the virus would find it advantageous to arrest the cell cycle or to kill the producer cell. G₂ arrest results in somewhat more efficient production of viral proteins and RNA, but this effect is fairly modest [26]. Although G₂ arrest may not be the primary objective of Vpr, the phenomenon has provided a handle with which to address the problem.

Another E3 ubiquitin ligase-associated accessory protein

Understanding how Vpr arrests the cell cycle has provided important clues to its role in infection. Vpr causes the cell to respond as if its DNA had been damaged by UV light or γ rays. The DNA damage-sensing kinase, ataxia telangiectasia-mutated and Rad3-related protein (ATR), is activated and then phosphorylates various cell cycle-regulatory proteins, including H2A-X, Chk1, and the p53-binding protein 1 [27]. Vpr itself does not induce DNA damage but must affect a cellular pathway that activates the DNA damage-response pathway.

To identify how Vpr intersects with cellular pathways, several groups used biochemical fishing approaches to pull down Vpr-associated proteins from cells. Vpr was found to associate with two proteins, one of which had been identified years earlier but had been nearly forgotten—appropriately named *Vpr-binding protein* [28,29]—and a second protein, the damage DNA-specific binding protein 1 (DDB1) [30–32]. This provided an important clue. Vpr-binding protein (renamed *DDB1 cullin-associated factor 1* [DCAF1]) and DDB1 are components of an E3 ubiquitin ligase similar to the one that binds to Vif. In this case, the complex consists of DCAF1, DDB1, Cul4a, and Rbx1. In the complex, DDB1 has a triple-propeller bladed structure that binds to the Cul4a scaffold protein and a series of DCAF proteins. The DCAFs serve as adaptor proteins that target cellular proteins for ubiquitination [33]. Vpr is thought to bind directly to DCAF1 in the complex. In doing so, it stimulates the ubiquitin ligase activity of the complex. Vpx also associates with the complex, although with reduced affinity. Knockdown of DCAF1 with short interfering RNA prevents Vpr from inducing G₂ arrest, demonstrating that the association of Vpr with the ubiquitin ligase induces G₂ arrest [32].

Taken together, these findings lead to the hypothesis that Vpr and Vpx, like Vif, may cause the degradation of one or more cellular restriction factors that block virus replication in macrophages and DCs. The association of Vpr with the ubiquitin ligase may interfere with its role in cell cycle regulation, inadvertently arresting the cell cycle. Thus, G₂ arrest may not be the primary role of Vpr but rather an unintended consequence. Although this interaction is unhealthy for the cell, it does not matter much from the virus's perspective. Its strategy is just to produce a sufficient number of new virions before its lifestyle wreaks havoc on cellular physiology.

Evidence that Vpx neutralizes the effect of a restriction factor that operates in macrophages and DCs

In macrophages and DCs, *vpx* virus is blocked after entry but before reverse transcription. The block at reverse transcription argues against a role for Vpx in nuclear import, which occurs after the initiation of reverse transcription. The defect can be rescued by introducing Vpx into the cell a few hours before infection. To do this, cells are incubated with Vpx-containing virus-like particles (VLPs) [34,35,36]. VLPs are essentially virus shells generated in the laboratory that can fuse to the plasma membrane of a target cell and dump their contents into its cytoplasm. This ability of Vpx to work in trans can be explained by two models. It either provides a function that is required for the virus to proceed through reverse transcription or it blocks the activity of an inhibitor in the cell. Support for the second model was provided by cell–cell fusion experiments in which macrophages were fused to a permissive embryonic kidney cell line. The resulting heterokaryons were resistant to infection by *vpx* virus [37]. This finding suggested that macrophages contain a dominant inhibitor that diffused through the heterokaryons to block infection.

The structural and functional similarity of Vpr and Vpx suggests that they may have similar but not identical roles. For example, Vpr and Vpx may target two related restriction factors that are expressed in different cell types or regulated by different cytokines. The identification of these restriction elements will be required to resolve the many questions that remain about these elusive accessory proteins.

Letting Go: The Role of Vpu in Virus Release

Vpu is a membrane-associated phosphoprotein that is localized to the endoplasmic reticulum and expressed late in the virus replication cycle [38]. A Vpu gene is present in the genomes of HIV-1 and some SIV isolates (SIV_{gsn} and SIV_{cpz}) but absent from HIV-2 and most SIVs. Vpu is not essential for virus replication in vitro, but its conservation in primary virus isolates suggests its importance in vivo [38,39]. Although SIV lacks the Vpu open reading frame, SIV/HIV (SHIV) chimeric virus that encodes HIV-1 Vpu was used to test the role of Vpu in vivo. In animals infected with *vpu*-SHIV, as compared with wild-type SHIV, virus loads were low, and CD4⁺ T cells were depleted at a reduced rate [40].

Downregulation of cell-surface proteins

Studies of *vpu* HIV-1 replication in cultured cells revealed dual roles for Vpu. Vpu reduced the amount of CD4 and major histocompatibility protein complex (MHC) on the surface of infected cells and increased the amount of virus shed into the culture supernatant [38,41,42].

CD4 degradation—The reduction of cell-surface CD4 is caused by rerouting of CD4 during its biosynthesis to an endosomal location, where it is degraded [38]. To do this, Vpu interacts with an E3 ubiquitin ligase complex that consists of S-phase kinase-associated protein 1, Cullin1, and β -transducing repeat-containing protein. The complex catalyzes the ubiquitination of CD4, triggering its proteasomal degradation. CD4 degradation is useful to the virus because it serves to free gp160 from CD4, which traps it in the endoplasmic reticulum. By degrading CD4, gp160 is free to continue its transit to the plasma membrane,

where it is incorporated into the lipid bilayer of the budding virion [38]. In addition, removal of CD4 from the cell surface prevents the virus from futilely reinfecting the producer cell. Studies in the macaque model have shown the importance of CD4 degradation. A SHIV that contained a subtype C Vpu, which is less efficient at inducing CD4 degradation, was less pathogenic than a subtype B, Vpu-bearing SHIV in rhesus macaques [40].

MHC I and MHC II modulation—In addition to inducing CD4 degradation, Vpu reduces the amount of MHC I and MHC II on the infected cell surface [41,42]. This could help the virus to evade MHC-restricted T-cell responses. The reduction in MHC II results from the interaction of Vpu with the MHC II invariant chain, preventing its transit to the cell surface.

Counteracting the restriction to virus release

Recent studies aimed at understanding the role of Vpu in augmenting virus release have provided important insight into a novel retroviral restriction factor. Electron microscopic analysis of cells infected with *vpu* HIV-1 shows a striking tethering of the virions to the cell surface, as if the virions tried to finish budding but got stuck [43]. Chains of virions were seen, as they were apparently glued to one another. *vpu* HIV-1 was released poorly from primary T cells, macrophages, and HeLa cells but released efficiently from African green monkey–derived COS-7 cells [38]. In addition, Vpu acts on a range of retroviruses, including murine leukemia virus [39]. Cell fusion studies in which Vpu-independent and Vpu-dependent cells were fused showed that the block to virus release was dominant [38]. As was the case for Vif, the finding suggested that Vpu releases a block imposed by a dominant restriction factor. Protease treatment of the cells caused the trapped virions to be released, suggesting that the tethering was caused by a protein on the cell surface [44].

Tetherin/bone marrow stromal cell antigen 2: a novel restriction factor

A clue to the identity of the restriction factor was provided by the finding that the Vpu phenotype could be induced by interferon- α [45]. Armed with the knowledge that the restriction factor was likely to be a cell-surface protein and was inducible by IFN- α , Neil et al. [46] used microarrays to identify cellular proteins that fit those criteria. Only a handful of candidate genes fit. The list was narrowed by testing each individually for its ability to block the release of *vpu* HIV-1 without affecting wild-type virus. The gene that fit these requirements was *CD317/BST2* (bone marrow stromal cell antigen 2), which the investigators appropriately christened *tetherin* [46].

A second group independently identified BST2/tetherin through a very different route. Kaposi's sarcoma– associated herpes virus produces a protein called K5 that has ubiquitin ligase activity and was known to downregulate BST2/tetherin. In addition, Vpu was found to reduce the amount of cell-surface BST2/tetherin [47]. Van Damme et al. [48••] reasoned that BST2/tetherin may be the restriction factor neutralized by Vpu and used similar methods to show this to be true. Both groups found that RNA interference knockdown of BST2/tetherin reversed the block to release of *vpu* HIV-1. Moreover, transfection of Vpu-independent cells with a tetherin expression vector restored the block to virus release, causing the virions to accumulate at the plasma membrane [46,48••].

How does BST2/tetherin tether nascent virions? The protein has the unusual feature of being anchored in the plasma membrane at both ends. At its amino-terminus, it has a hydrophobic transmembrane spanning peptide, and at its carboxy-terminus, it is anchored by a linked lipid. One end may insert into the lipid bilayer of the virus, with the other in the cell's plasma membrane, tethering the virus to the cell. Alternatively, intermolecular interactions between tetherin/BST2 proteins in the virus and cell membrane would have the same effect [46]. The cell-bound virus later may be taken up through the endocytic pathway and subsequently degraded [46,49].

How does Vpu counteract BST2/tetherin? One possibility is that Vpu targets BST2/tetherin through an E3 ubiquitin ligase-proteasomal degradation pathway similar to that used to degrade CD4. In one study, Vpu decreased the abundance of tetherin/BST2 in the cell [47]; however, this effect was not detected by another group [46], and treatment of cells with a proteasome inhibitor did not reverse the Vpu dependence [48••]. Alternatively, Vpu may affect the endosomal trafficking of BST2/tetherin, leading to its downregulation from the cell surface and preventing it from capturing budding virions. In support of this scenario, tetherin/BST2 was found to colocalize in the cell with Vpu in an intracellular compartment.

A second protein targeted by Vpu

In another search for the target of Vpu, Varthakavi et al. [50•] used a yeast-two hybrid screen to identify cellular Vpu-binding proteins. The screen identified the protein calcium-modulating cyclophilin ligand (CAML), a membrane protein required for T-cell differentiation. Like BST2/tetherin, CAML is expressed in Vpu-dependent cells and blocked the release of *vpu* HIV-1 but not of wild-type HIV-1. Furthermore, knockdown of CAML in Vpu-dependent cells relieved the requirement for Vpu. Vpu and CAML colocalize in the cell, suggesting that Vpu may prevent CAML from recycling back to the cell surface.

Many other questions remain to be addressed. How do two different proteins both act as restriction factors that are targeted by a single viral accessory protein, yet on their own, both are necessary and sufficient to restrict the virus? Knockdown of one of these proteins on its own should not have been sufficient to relieve the block to the release of *vpu* HIV-1. How does Vpu interfere with tetherin/BST2? Do the proteins physically associate, or does Vpu act indirectly to influence the intracellular trafficking of BST2/tetherin? Which other viruses are restricted by BST2/tetherin? Why does SIV not need Vpu? Can Vpu be targeted for the development of a novel class of virus-release inhibitors?

Conclusions

In the evolutionary battle between virus and host, the virus has a major advantage. The human genome evolves at a snail-like pace over millions of years while the virus, with its tiny genome, compressed generation time, and propensity toward replication errors, nimbly eludes the sophisticated defenses of the host adaptive and innate immune systems, developing genes that neutralize host defenses and structural genes that encode highly plastic structural proteins. In the end, however, the host will win out, not by evolving new immune defense mechanisms, but by virtue of the genes that have endowed us with the

ability to understand our surroundings and to develop the means to thwart even the most sophisticated viral accessory genes and molecular escape mechanisms—we hope.

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Figure 1.

Virion infectivity factor (Vif), viral protein R (Vpr), viral protein X (Vpx), and viral protein U (Vpu) counteract cellular restriction factors. Vif, Vpr, Vpx, and Vpu associate with E3 ubiquitin (Ub) ligases to induce the proteasomal degradation of restriction factors (the E3 Ub ligase for Vpu is not shown). Vpr and Vpx overcome a postentry block to reverse transcription by targeting a yet-unidentified restriction factor indicated by *question marks*. Vif induces the degradation of apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) to prevent its packaging into the virion and the subsequent C→U deamination of the reverse transcribed viral DNA. Vpu antagonizes tetherin and calcium-modulating cyclophilin ligand (not shown), which hold the virus onto the cell surface. DCAF1—DDB1 cullin-associated factor 1; DDB1—damage DNA-specific binding protein 1; Elo—elongin; Env—envelope; LTR—long terminal repeat.