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Review Article

Intracellular Defenses Against HIV, Viral Evasion and Novel Therapeutic Approaches

*Robert A Lever, Andrew M L Lever**

Human immunodeficiency virus (HIV), the causative agent of AIDS, is a retrovirus. It is estimated that, while in the cell, it interacts with almost 10% of cellular proteins. Several of these have evolved to protect the cell from infection with retroviruses and are known as "restriction factors". Restriction factors tell us much about how the virus functions and open up new paradigms for exploring novel antiviral therapeutics. This article gives an update on the three best studied restriction factors, their putative mechanisms of action and how the virus has overcome their effects, together with an indication of novel therapeutic approaches based on this knowledge.

Key Words: AIDS, HIV, restriction factor, retrovirus

Although human immunodeficiency virus (HIV) has had a devastating effect on the human race since it was first identified in the last few decades of the last century, it is not the first pathogen to do this, and it follows a recognizable pattern in that infections that cross from other species (zoonoses) commonly cause much more severe disease in their new host. In addition, HIV is not the first pathogenic retrovirus to infect humans. One other, HTLV-1, is almost as widespread[1] but, because of its very long association with the human species, both have adapted to each other and now it is of low pathogenicity, except in a small percentage of individuals in whom it causes disease.

The adaptive immune system is often thought of as our major defense against such invaders, but in fact, only vertebrates have this type of immunity, and invertebrates seem to resist infections well in general. The innate immune system, a much underrated defense network, has a large and diverse armamentarium against those infections to which we have been exposed for many years. As relics of our past encounters with pathogens, and presumably for other contemporary purposes, a number of intracellular proteins exist that are there to block virus infection. These are known as "restriction factors" and they comprise a growing family of gradually better understood cellular guardians. Restriction factors are often highly conserved, intimating at their essential nature, and they offer us a new insight into ways in which we might target invading pathogens by

Department of Medicine, Addenbrooke's Hospital, Cambridge UK.

***Correspondence to:** Professor Andrew Lever, University of Cambridge, Department of Medicine, Addenbrooke's Hospital, Hills Road, Cambridge UK CB0OQQ.

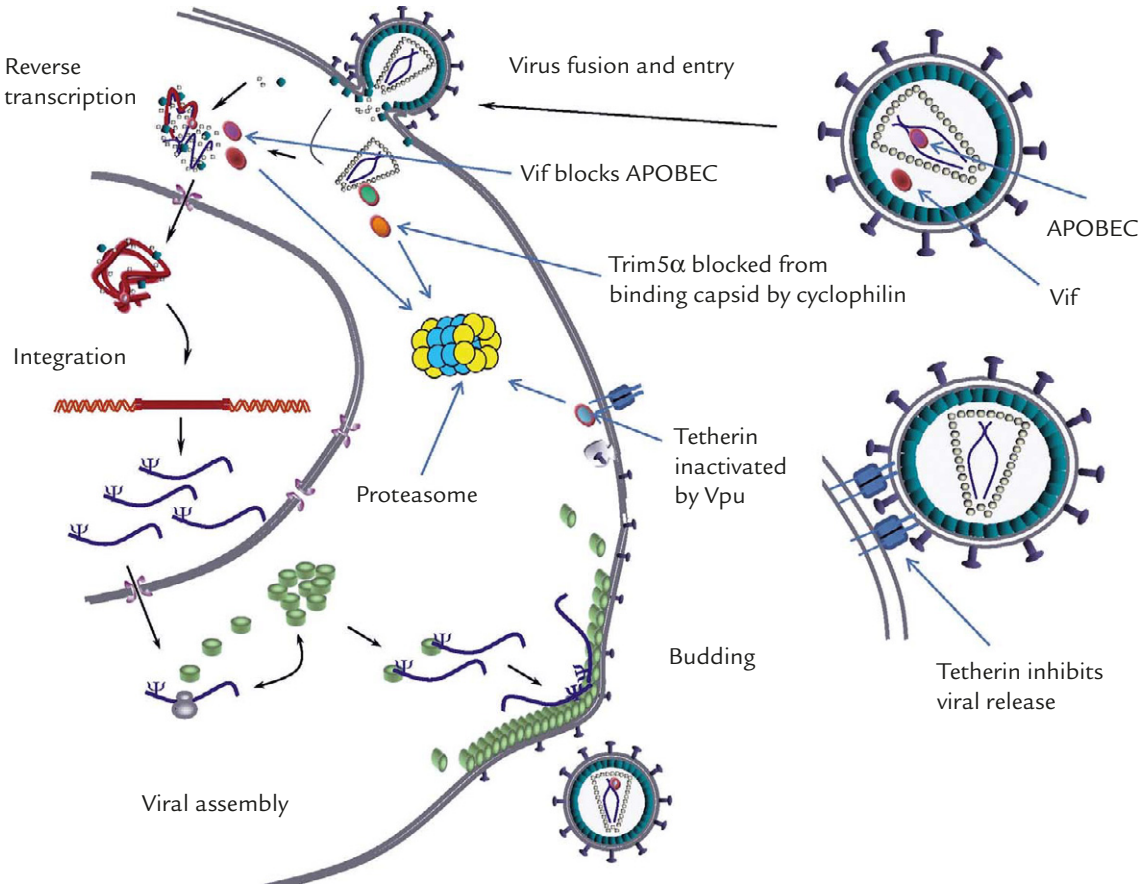

Figure. Sites of restriction factor action.

manipulating processes that have evolved naturally over many millions of years. This review focuses on three protein restriction factors that are important in the case of HIV infection. In all cases the defensive factor has been countered by strategies of HIV. There may be a symbiosis in which the virus uses the effect of the factor for its own purposes, or HIV has evolved an escape mechanism by generating its own interfering protein that nullifies the effect of the restriction factor. Another strategy of HIV is to hijack a second cellular protein for its own protection. The sites of action of the restriction factors and the counter measures of the virus are shown in the Figure.

TRIM5α

Simian immunodeficiency viruses such as SIVsm, SIVagm and SIVmac (the last of which has the highest sequence homology with HIV-1) are all able to infect immunological cells from old world monkeys (OWM) yet these latter resist *in vitro* infection with HIV-1.[2–6] Even getting the virus in by a "Trojan horse" approach of putting it in a new coat ("pseudotyping" it with VSV-G), which guarantees entry, does not overcome this so called "monkey block" telling us that the effect is occurring at a stage after viral entry. The restriction can be overcome experimentally by introducing an excess of virus cores into the cell, suggesting that an inhibitory factor is present that can be "saturated out". This factor was initially named Lv1 (lentiviral susceptibility factor 1).[7–10] In seeking to identify this factor, a mouse gene vector population containing a cDNA library of the genome of Rhesus macaques was introduced into (human) HeLa cells in culture, which were then challenged with VSV-G pseudotyped HIV and SIV vectors, and any cells which were resistant to infection by

HIV were isolated and grown out.[11] The resulting resistant clones were found to express a protein called simian TRIM5α. Knockdown of this protein by short interfering RNA abolished the blocking effect. The effect of TRIM5α was dependent on the viral capsid. Corroborating evidence soon followed from unexpected findings when Owl Monkey cells were challenged with HIV.[12,13] The Owl Monkey is a New World monkey, and as such would not be expected to constitutively restrict HIV-1, yet it showed a block of the same character as OWM cells. Analysis of the Owl Monkey TRIM5α sequence showed that it had fused with another cellular gene, cyclophilin A (CypA), which had previously been identified as a positive (infection enhancing) co-factor in early HIV-1 infection through its interaction with the p24 CA (capsid) protein of the virus, an effect that can be inhibited by the immunosuppressant drug and CypA inhibitor, cyclosporine A.[12,14] This fusion protein was named TRIMCyp, and it was hypothesized that the CypA sequence targeted the TRIM to the entering viral core, an idea supported by the elimination of restriction activity in cells treated with cyclosporine A.

TRIM is an abbreviation for Tripartite Motif, which refers to a group of three recognizably similar amino acid sequences (domains) found in a large family of proteins. These comprise an N-terminal RING (Really Interesting New Gene) domain, a B-box and a coiled coil. The protein family is large, with at least 68 members performing multiple functions within cells. Apart from antiviral activity, they have also been implicated in development and oncogenesis.[15–18] Present in cytoplasmic bodies, TRIM5α is universally and constitutively expressed while being under the control of the interferon sensitive IRF3 promoter, and is therefore upregulated in response to type I interferon.[19–21] The RING domain contains a zinc binding motif generally found in proteins that have E3 ubiquitin ligase activity.[22] This suggested that TRIM5 may undergo auto-ubiquitination and might also ubiquitinate other proteins.[23,24] The B-box is needed for the retroviral restriction activity, and point mutations of essential residues have been shown to eliminate the HIV-1 block.[25–27] Despite this, its function is obscure and it may be important for formation of higher order structures, intracellular localization and/or protein turnover.[28] Oligomerization represents the major function of the coiled-coil domain, where large numbers of α helices mediate protein-protein interactions thought to be important in the production of multimers. The major functional form of TRIM5α was believed to be a trimer,[29,30] but an elegant report in 2008 demonstrated that cross linkage of TRIM5α dimers bestowed anomalously slow electrophoretic mobility on the complex, making them appear trimeric.[31] The coiled coil may also contribute to species specificity.[32] The B30.2/SPRY C-terminal domain is responsible for viral capsid recognition and binding. This region contains non-structured interconnecting "variable" loops (v1–v4), which are critical for this function. Alterations in the sequence and structure of this domain are the most important factor in determination of species specificity of the TRIM5α protein.[33–38] The critical sequence contains PRY and SPRY domains.[27,34,39] The viral specificity of different TRIM5α proteins is concentrated in these regions.[33] One particular single amino acid substitution is of particular interest — a change from arginine to any non-positively charged amino acid at position 332 in the human TRIM5α elevates the potency of restriction against HIV-1 to that of the OWM TRIM5 molecules.

The exact mechanism by which TRIM5α binding effects retroviral restriction has yet to be elucidated. There are arguments for pathways both dependent (addition of ubiquitin and subsequent degradation)[40] and independent of the proteasome.[31,41] The RING domain possesses E3 ubiquitin ligase activity, and a recent paper set out to establish the effect of this activity in HIV restriction.[42] Previously, Rhesus macaque (Rh) TRIM5α activity had been shown to be reduced by addition of a proteasomal inhibitor.[43,44] The authors pursued this further and ascertained that the proteasomal dependence of restriction of distinct retroviruses is virus specific. For example proteasomal inhibition attenuated SIVmac block,

but had no effect on HIV-1 restriction by an African Green Monkey TRIM5α. It seems likely that, rather than polyubiquitinating the capsid protein itself, TRIM5α binds to the CA subunit and targets itself for degradation, a proposal supported by the potent ability of TRIM5α to auto-ubiquitinate.[24] The proteasomal independent pathway[45] may involve direct disassembly of viral cores, as has previously been demonstrated in cytoplasmic assays.[46] There is wide sequence divergence between TRIM5α in different primate species. However, there is remarkable sequence conservation in the human B30.2 domain within the human population, and specifically a complete absence of polymorphisms within the N-terminal region comprising the variable loops (V1–3).[47] This lack of diversity is unusual. It is speculated that the human sequence reflects evidence of a previous retroviral pandemic leading to a major survival benefit of the current version. Ironically the uniformity of the human TRIM5α gene could have contributed to the virtual universal susceptibility of humans to HIV and the consequent scale of the current HIV pandemic.[48] Several SNPs have been identified elsewhere in the gene, one in particular, the H43Y mutation, which shows alteration in activity *in vitro* attenuating TRIM5α activity. However, the clinical phenotype is inconsistent with protective,[49] neutral,[50] and negative effects described.[51]

Gene therapy experiments exploring potential applications for TRIM5α based HIV-1 treatment have used lentiviral vectors to express RhTRIM5α in a hematopoietic stem cell line that subsequently differentiated into HIV-1 resistant macrophages.[52] Combination vectors such as those containing a TAR decoy, CCR5 shRNA and a chimeric TRIM5α have also been shown to be effective in HIV-1 restriction in primary macrophages *in vitro* and probably represent a fruitful avenue for further gene therapy work.[53] An alternative has been to fuse cyclophilin A to the wild type human TRIM5α allele,[54] exploiting the fact that the basic defect in human TRIM5α mediated HIV-1 restriction is failure of B30.2 recognition. The investigators have effectively mimicked *in vitro* the process that *Aotus* sp and certain *Macaca* Old World Monkeys achieved by retrotransposition *in vivo*. Of note, one particular construct of this fusion protein has been shown to be more effective than the wild type Rhesus TRIM5α itself, and highly resistant to the evolution of viral resistance, both excellent signs for a potential gene therapy agent. Cyclophilin based strategies have also been explored in cats in attempts to restrict Feline Immunodeficiency Virus (FIV). This much underexploited animal model of HIV infection is well suited to such therapeutic trials.[55]

The APOBEC3 family and the vif gene

HIV-1 is a "complex" retrovirus, and as such codes for a variety of accessory and regulatory genes, in addition to the genes *gag*, *pol* and *env* that encode the structural proteins.[56] One of the accessory gene products, Vif, is a 23kDa highly basic protein that has long been known to be essential for *in vivo* viral replication, but is inconsistently required for infection of certain CD4+ T cell lines.[57–61] Exploitation of these *in vitro* differences between "permissive" cell lines [susceptible to infection with a Vif deleted (Δvif) virus] and "non-permissive" cell lines (non-susceptible) led to the gradual elucidation of the *in vivo* function of the Vif protein, and ultimately the discovery of a new class of restriction factors.[62] The first step was identifying whether there was an essential positive factor in permissive cells emulated by Vif in non-permissive cells, or whether the actions of Vif were to overcome the inhibitory effects of a gene which was only expressed in non-permissive cells. Hybrid permissive/non-permissive cell lines were created and infection with Δvif HIV-1 yielded defective virions, suggesting that Vif was acting to sabotage a cellular factor present only in non-permissive cells. Identification of this restriction factor followed 4 years later in an elegant series of experiments where the investigators first used subtractive hybridization to screen the transcriptome of non-permissive CEM cells versus a permissive version of the same cell line from which a candidate protein was identified. They then demonstrated reconstitution of the non-permissive phenotype by artificially expressing the protein in

permissive CEM-SS cells. This protein, APOBEC3G, is now recognized as a member of a family of cytidine deaminases, which operate to defend against exogenous retroviruses, endogenous retroelements and a variety of other viruses including the hepatitis B virus.[63,64] APOBEC3 proteins (so called due to their homology to the mRNA editing enzyme APOBEC1) are unique to mammals and are involved in DNA editing and mutation.[65] They have arisen via gene duplication on chromosome 22 and comprise seven genes in humans (APOBEC3A, 3B, 3C, etc through to APOBEC3H).[65,66] APOBEC3G (hA3G) and APOBEC3F (hA3F) are expressed in primary T cell lines, monocytes and macrophages rendering these cells resistant to vif deficient virus *ex vivo*.[67] Permissive cells produce Vif deficient virus that is infectious to non-permissive cells in single cycle assays, but Vif deficient virus produced from non-permissive cells is unable to replicate even in permissive cells.[68,69] This observation, in combination with the identification of hA3G in mature virions from permissive cell lines, intimated that the major site of hA3G action was prior to integration of the viral (proviral) DNA into the host cell chromosomes. It has since been shown that in Δvif mutants, hA3G bind both the viral nucleocapsid (NC) protein and viral genomic RNA and acts during the process of viral reverse transcription. During negative strand synthesis, hA3G is released by the action of RNase H at which point the single negative strand of viral cDNA is exposed.[70] hA3G then acts to deaminate cytidine to uridine on this strand.[71–77] Destruction of the heavily mutated DNA by cellular factors may then occur resulting in elimination of the proviral genome mid-reverse transcription.[78–80] If, however, the DNA is not destroyed, the deaminated cytosines on the DNA act as templates for adenine incorporation into the opposite (positive) strand of the immature proviral sequence. The subsequent G→A mutations result in missense or nonsense manipulation of the code, generally producing non-functional viral proteins post-integration, inhibiting further viral replication. Deamination of cytidines probably represents the most important activity of hA3 physiologically; however, several studies have reported a deaminase independent effect on viral and retroelement restriction,[81–86] and hA3 has been suggested to have a deaminase-independent effect on HIV-1 replication.[87–92] Criticisms at some of these studies have centered around the fact that they have relied on transient overexpression of hA3 genes (specifically hA3G and hA3F) at non physiological levels; however, since antiviral genes such as hA3G are likely to be upregulated in response to interferon *in vivo*,[93] it may not be a totally artificial scenario. *In vivo*, however, the virus only ever encounters deaminase competent A3G and A3F, and that activity may be so dominant that the alternative effects are minor or artifactual.

In general only hA3 proteins with two zinc binding domains can restrict HIV-1 to any significant degree and of these, only two, APOBEC3G and APOBEC3F, have noteworthy action (although several others have modest effects on HIV-1).[94,95] The presence of two zinc binding domains confers on these proteins the ability to homo-oligomerize in a context dependent manner.[96–98] Low molecular weight multimers are surmised to assemble in response to the presence of RNA and appear to be active in restriction, whereas higher molecular weight multimers complexed with RNA are not functional.[99] Vif operates to antagonize hA3G function in several ways, but primarily acts in a proteasome dependent fashion via the ubiquitination of hA3G.[100–104] Vif links hA3G to the ELONGINB/C-CULLIN5-RBX1 E3 ubiquitin ligase resulting in polyubiquitination and 26S proteasomal degradation.[105] In addition to this, Vif reduces translation of the hA3G mRNA *in vitro* by two independent mechanisms, the more important of which relies on Vif binding to the 5′ UTR of hA3G mRNA.[106] The co-evolution of Vif and A3G/A3F may represent a balance, as the mutagenic effect of APOBEC3 seems to have, in moderation, an advantageous effect in accelerating viral genomic mutation, and therefore immune escape and development of drug resistance.[107–112] Evidence for this partnership includes the persistence of new G→A mutations in appropriate contexts present in HIV-1 clinical isolates and the

preponderance of these changes in drug resistance genes. The most obvious therapeutic target in this process is the disruption of Vif mediated antagonism of hA3G and hA3F function. Clearly such manipulation could be achieved pharmaceutically by interruption of the binding of the two proteins by blocking the interacting surface on either the human or the viral protein. There is a small possibility that, by enhancing APOBEC3 function, a correspondingly higher mutation rate would predispose to an even more rapid production of highly drug and immune resistant viruses than is currently achieved by wild type virus. However, given the close relationship between Vif and APOBEC3 evolution, it seems likely that the current APOBEC induced mutation rate is optimal for HIV-1 survival. Another therapeutic approach would be the development of inhibitors targeted at binding the cellular hA3G. This approach has a number of advantages. (1) Altering hA3 action places the selection pressure on the stable cellular protein rather than the highly mutable Vif. (2) In contrast to the regions of Vif that bind hA3, the sequence binding Vif on hA3G is continuous, facilitating the design of specific shielding molecules.[113–118] Potential dangers of this strategy are related to the concept that hA3 proteins may also mutate cellular DNA, and this effect may be exaggerated to a harmful extent by enhancing their action.[119] Finally the homozygous absence of hA3B in certain human populations suggests that there may be a selective disadvantage to increased hA3 activity potentially through damaging hypermutation of DNA.[120] It is of note, however, that while there is no clinical phenotype associated with hA3B-/-, polymorphism studies have associated this genotype with increased risk of infection, faster progression to AIDS and an increase in baseline viraemia.[121]

Several small molecule inhibitors have been identified whose aim is to potentiate hA3G action via Vif inhibition. The first, RN-18, was shown to have efficacy through a screen of possible Vif antagonists.[122] It acts at the point of Vif induced recruitment of the E3 ubiquitin ligase complex prior to proteasomal degradation of hA3G by misdirecting polyubiquitination from hA3G onto the Vif molecule itself. This results in Vif destruction by the 26S proteasome, lower constitutive Vif effects and liberates the activity of hA3G allowing its incorporation into budding virions. More recently two further potential therapeutic inhibitors have been isolated from the collection at the Chinese Academy of Medical Sciences. IMB26 and IMB35 are analogous compounds found to disrupt Vif mediated degradation of hA3G. A negative effect on HIV-1 infectivity was shown with these compounds *in vitro*, which varied widely between T-cell lines and was positively related to APOBEC3G mRNA expression in the compound treated cells.[123] The authors were able to illustrate a direct interaction between IMB26/35 and hA3G and showed that, in contrast to RN-18, they acted to block Vif binding, rather than having a negative effect on Vif levels. Of note they also observed no increase in hA3G activity in the presence of the compound and an LD_{50} *in vivo* well outside any therapeutic range.[123] Clinically a number of studies have associated higher hA3G expression with elevated CD4+ counts and reduced viral set point,[124] however some reports cast doubt on these findings.[125–128] Exposed uninfected individuals appear to express higher levels of APOBEC3G compared with those that are infected.[129]

BST-2/tetherin

The viral accessory protein Vpu was first shown to be important in viral release in 1990, when its deletion resulted in viral accumulation in endosomes and on the cell surface.[130] Such viral retention was later shown to be cell type specific, and resembled the reaction seen in response to type I interferon.[131–134] The cell specificity of Vpu dependence was initially examined using fusions between cells of two cell lines exhibiting differential Vpu requirements. The results suggested the presence of a dominant cellular restriction factor antagonized by Vpu, a conclusion supported by the observation that exogenous Vpu could result

in release of an unrelated virus from the effects of this as yet unidentified cellular mechanism.[134,135] The retention imposed by this cellular factor was later shown to be relieved by exposure of the cells to subtilisin, a proteolytic enzyme, suggesting a cellular membrane bound protein was exerting the observed effect. The key discovery as to the identity of this protein was made almost simultaneously by two separate groups in 2008. Neil et al used a microarray comparison to see differences in the expression profile of Vpu dependent (HeLa) and Vpu independent (COS-7) cell lines that might account for their differential restriction. They identified the previously described BST-2, which they renamed tetherin in recognition of its action.[136] Van Damme et al made a similar discovery by following up some of their previous work on the K5 protein of the Kaposi's Sarcoma Herpes Virus (KSHV), which they had previously described as downregulating BST-2.[137,138] Tetherin is a 30–36 kDa type II membrane glycoprotein with an unusual structure.[139] Its N-terminal is an intracytoplasmic tail linked to a membrane-spanning alpha helical domain leading to a coiled-coil ectodomain and ending in a glycosylphosphatidylinositol (GPI) anchor at the C-terminus of the protein. The intracellular domain of tetherin contains a variety of important motifs; specifically a YxY domain mediating clathrin-linked endocytosis,[140] a KxxK motif targeted by the KSHV K5 protein,[141] and in non-human primates, a DDIWK sequence targeted by Nef both of the latter leading to degradation.[142] The coiled-coil consists of two alpha helices containing three cysteines mediating disulphide bonding and two asparagines representing putative glycosylation sites in the ectodomain, although the importance of both of these conserved residues is disputed.[143,144] Interestingly this coiled-coil structure is only metastable, being held together by disulphide bonds with conserved destabilizing amino acids resulting in the potential to form a rod like structure.[145] At the C-terminal, the GPI tail anchors the distal end of the protein in the membrane, allowing its localization to cholesterol rich lipid rafts,[139,146] a preferential site of HIV-1 (and Ebola virus) budding.[147] Apart from its strange arrangement, it is also atypical in that it is endocytosed in a clathrin dependent manner,[140] a feature highly unusual in a GPI linked protein.

Tetherin is under the transcriptional control of type I interferons which are released as part of the inflammatory response to HIV-1 via the stimulation of Toll Like Receptors (TLR) 7 and 9 in plasmacytoid dendritic cells.[148–151] The tetherin promoter contains an interferon response element (IRE) and three STAT3 response sites, allowing it to be induced by interleukin 6 and other stimulators of the JAK-STAT cascade.[152] The mechanism of action of tetherin is not well described, and contains several areas of contention. In the absence of Vpu activity, tetherin causes the retention of virions on the cell surface and in endosomal vesicles. This capture effect can be released either by physical shearing or by subtilisin treatment, resulting in the production of mature, functional virions.[143,153] The direct tethering of virions by tetherin has been supported by tetherin co-localizing with Gag.[136,154] Since several virus types are restricted by tetherin, any model involving specific interaction with a viral protein is highly unlikely, and a plausible idea is one in which one membrane anchor of tetherin is positioned in the cell membrane and the other in the viral envelope forming a physical bridge between them and preventing release.

Tetherin has been shown to dimerise,[144] but how this occurs and its functional significance are both unknown. Vpu is a 16 kDa Type I transmembrane protein whose functions include not only antagonism of tetherin, but also downregulation of CD4. It is unusual in that it is not present in most primate lentiviruses (including HIV-2), despite the highly conserved function of tetherin. Other accessory viral gene products such as Nef and Env substitute its function in many simian lentiviruses.[142,155,156] The tetherin transmembrane (TM) domain confers sensitivity and species specificity to Vpu as shown by experimental domain swapping to produce Rhesus-human fusion proteins.[157,158] It is also suggested by the high rate of non-synonymous mutations in this region.

In addition, a Vpu TM-mutant, still able to downregulate CD4, failed to negate the effects of tetherin.[159] Downmodulation of cell surface tetherin expression could be related to either destruction or reduction in total cell tetherin, or could be an outcome of retention of tetherin in intracellular compartments. Immunoblot analyses suggest the overall effect includes a direct downregulation of total cell tetherin, an observation supported by the effect of various lysosomal and ubiquitin inhibitors on Vpu action.[160–164] Vpu deficient viruses can still replicate, although they show more of a propensity to form syncytia with little free virion formation. This observation in itself implicates Vpu as a viral fitness factor possibly important in the transmission of HIV-1 via bodily fluids.[155] As yet, apart from involving the interferon system, there are no specific novel antiviral approaches to HIV involving the tetherin system.

Conclusion

This review has illustrated a small area of the intimate interaction that occurs between a viral pathogen such as HIV and the cells of its host. It emphasizes that we have much to learn about antiviral approaches from the innate immune system. Since to date there has been virtually complete failure to achieve protection from HIV infection by conventional approaches using the adaptive immune system, which has been so successfully exploited for other viral pathogens, it merits further investigation as to how we can use these ancient viral defense mechanisms to enhance our attempts at therapy and prevention of HIV/AIDS.

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