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Host Restriction Factors Blocking Retroviral Replication

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Abstract

Retroviruses are highly successful intracellular parasites, and as such they are found in nearly all branches of life. Some are relatively benign, but many are highly pathogenic and can cause either acute or chronic diseases. Therefore, there is tremendous selective pressure on the host to prevent retroviral replication, and for this reason cells have evolved a variety of restriction factors that act to inhibit or block the viruses. This review is a survey of the best-characterized restriction factors capable of inhibiting retroviral replication and aims to highlight the diversity of strategies used for this task.

Keywords

Trim5α; Trim28; Zap; Tetherin; APOBEC; intrinsic immunity

INTRODUCTION

Retroviruses are extremely successful pathogens affecting virtually all branches of life. These viruses are champions of persistence, maintained as proviral DNAs integrated into the genome of somatic cells and even entering into the germ line. Infection can result in cell death, or in oncogenic transformation by insertional mutagenesis. Thus, there is tremendous selective pressure to block or prevent retrovirus replication. In recent years, it has become apparent that mammalian cells have evolved a number of powerful mechanisms to limit or restrict virus replication, constituting novel aspects of intrinsic immunity. These mechanisms act at many diverse steps in the life cycle. The potential importance of these restriction factors is highlighted by the fact that many retroviruses, in turn, have evolved mechanisms to inactivate or overcome the blocks to infection. The picture now emerging is one of an ongoing battle between virus and host.

In this review, we summarize what is known about several key restriction factors, those providing the best understood and perhaps most powerful blocks to infection. Proceeding from early to late in the course of viral infection, we discuss the following: the APOBECs (apolipoprotein B mRNA-editing catalytic polypeptides), cytidine deaminases attacking the viral DNA as it is synthesized; Fv1, TRIM5α, and TRIMCyp, all attacking the viral capsid (CA) soon after entry into the cell; TRIM28, blocking viral transcription; ZAP (zinc-finger antiviral protein), directing the degradation of the viral RNAs; and tetherin, trapping the virions on the surface of the producer cell. For an overview of the retroviral life cycle and the restriction factors discussed herein see Figure 1.

DISCLOSURE STATEMENT
The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

APOBECs: CYTIDINE DEAMINASES FOR VIRAL DNAs

The existence of the APOBEC restriction system was uncovered through the study of the viral infectivity factor (Vif) of HIV-1. It was long known that the Vif protein, encoded by one of the so-called accessory genes of the virus, was dispensable for viral replication in certain "permissive" cell lines such as CEM-SS and SupT1, but essential for replication in "nonpermissive" cells such as primary$CD4^+$ T-cells, monocyte-derived macrophages, and certain T cell leukemia lines such as CEM (44, 48, 134, 151, 154). The effect of Vif deletion appeared to be dependent on the cell line in which the virus was produced. Vif-minus virions produced by nonpermissive cells were poorly infectious, whereas Vif-minus virions produced in permissive cells were able to infect both permissive and nonpermissive cell types (48, 134, 151). These observations coupled with heterokaryon fusion experiments between permissive and nonpermissive cells suggested the existence of a dominantly acting antiviral factor that Vif was able to neutralize (96, 147). The identity of this factor was uncovered in 2002 through a cDNA subtraction screen for transcripts specifically expressed in nonpermissive cells (144). Of the large number of such genes identified, only one, originally known as CEM15, could convert a permissive cell into a nonpermissive one (144). Furthermore, CEM15 was found to be highly expressed in nonpermissive cells and weakly expressed or absent in permissive cells. This gene was subsequently identified as a member of the small APOBEC gene family and was named APOBEC3G.

APOBEC proteins constitute a family of polynucleotide cytidine deaminases, named after apolipoprotein B mRNA-editing catalytic polypeptide 1 (APOBEC1), the first protein of this class identified (163). All proteins in this family are characterized as having a His-X-Glu-$X_{23–28}$-Pro-Cys-$X_{2–4}$-Cys catalytic motif (71) that, based on studies of yeast and bacterial homologs, coordinates a Zn^{2+} ion (73, 79). Catalysis by the enzyme results in the hydrolytic deamination at the C4 position of the cytosine base of either DNA or RNA, thereby converting cytidine (C) to uridine (U). APOBEC1 was cloned because of its ability to specifically deaminate cytidine 6666 of the APOB mRNA, generating a stop codon and leading to the production of a truncated APO B protein. Another prominent member of the APOBEC family, AID, is expressed in activated B cells and is essential for several processes involved in antibody gene diversification, including somatic hypermutation (102).

The cytidine deaminase activity of the restriction factor neutralized by Vif strongly suggested that the restriction it conveyed could be due to specific deamination of either viral RNA or DNA. This prediction was quickly borne out by several groups who showed that APOBEC3G restriction correlated with the presence of G-to-A mutations in the sense strand of the retroviral DNA formed after infection of nonpermissive cells (60, 97, 193). This suggested that APOBEC3G was deaminating cytosines in the minus or antisense DNA strand during the first steps of reverse transcription. Such deaminated DNA could be subject to removal of these uracil bases by the uracil N-glycosidase (UNG) and subsequent cleavage by DNA repair enzymes such as apurinic/apyrimidinic endonuclease-1 (APE1) (183). Should such a modified provirus escape degradation and become integrated, it would most likely be inactive due to the G-to-A mutagenesis (189). Such inactivation would be the result of multiple mutation events, including missense changes, mutation of start codons, the introduction of new splice sites, and premature termination codons (189). Selective pressure to evade deamination by APOBEC proteins may explain why HIV-1 genes have atypical codon frequencies (9).

Consistent with the model that APOBEC3G-mediated antiviral activity is generated in the cell in which the viral particle is produced, APOBEC3G was found to be incorporated into the HIV-1 particle (60, 97, 144). This incorporation was attributed to a specific interaction between APOBEC3G and the nucleocapsid (NC) portion of the virus group-specific antigen

(gag) protein (3, 28, 38, 137, 191). It has also been proposed that the NC-APOPEC3G interaction is dependent on RNA (137, 161, 191), and this assertion is plausible as both NC and APOBEC3G bind RNA (10, 86). The details of how APOBEC3G becomes encapsidated remain controversial, and more biophysical analysis is required for resolution of this question. It has been suggested that the reason mouse APOBEC3 is unable to restrict murine leukemia virus (MLV) is the absence of an APOBEC3-NC interaction, which results in no APOBEC3 being incorporated into MLV virions (37). However, some studies have shown that APOBEC3 is incorporated into MLV particles, and it has been suggested that instead MLV protease is able to inactivate APOBEC3 (1, 100).

To prevent APOBEC3G-mediated restriction, the Vif protein must be able to neutralize it during viral particle production. The Vif protein likely achieves this primarily by inducing proteasomal-mediated degradation of the APOBEC3G protein (31, 91, 101, 106, 145, 153). It induces degradation by bridging an interaction between APOBEC3G and a ubiquitin E3 ligase complex consisting of Elongin B, Elongin C, Cullin 5, and Ring Box-1 (190). This interaction leads to the polyubiquination of the APOBEC3G protein and its subsequent degradation by the proteosome (190). There is also some evidence that APOBEC3G is excluded fromHIV-1 virions directly through an interaction with Vif (75, 100). The interaction between APOBEC3G and Vif is species specific, and a single amino acid difference between African green monkey (AGM) and humanAPOBEC3G at residue 128 is sufficient to render the AGM APOBEC3G resistant to HIV-1 Vif-mediated degradation (17, 98, 139, 180). Conversely, the Vif protein from the AGM simian immunodeficiency virus (SIV_{AGM}) is able to degrade AGM APOBEC3G but not human (17, 139). The species specificity of Vif for its APOBEC targets is in part responsible for the host range of the virus that carries it (63). This interaction therefore represents a therapeutic target, and the identification of pharmacological agents able to disrupt this interaction could lead to novel anti-HIV-1 therapeutics.

APOBEC3G is not the only cytidine deaminase to have specific antiretroviral function; multiple studies have shown that APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3F, and APOBEC3D/E are all, to varying degrees, able to restrict HIV-1 in the absence of Vif (16, 24, 33, 88, 132). It is therefore important to ascertain which of these proteins is most likely to be influencing HIV-1 replication in vivo. There is as yet no definitive answer to this question, but several lines of evidence point to APOBEC3G and APOBEC3F as being the predominant restriction factors during HIV-1 infection. First, Vif can only substantially inhibit APOBEC3G and APOBEC3F (17, 36, 84, 100, 145, 153, 174), which implies that these are the enzymes likely to be most potent in their ability to restrict HIV-1. Second, APOBEC3G and APOBEC3F are expressed in a number of tissues appropriate for HIV replication, including $CD4^+$ T-cells and macrophages (88, 174), whereas many of the other APOBEC family members are not (16, 30, 37). Finally, different members of the APOBEC family have different substrate specificities. APOBEC3B, -3C, and -3F all have similar preferences for 5′-TC dinucleotides on the antisense strand, which manifest as 5′-GA to AA mutations in the sense strand of HIV-1, and patient-derived HIV-1 DNA sequences have been shown to carry these mutations frequently (23, 45, 171). The same data also show many 5′-GG to AG mutations, which are characteristic of APOBEC3G and which result from its preference for modifying CC dinucleotides on the antisense strand. These results make it probable that during a productive infection in humans, HIV-1 is encountering at least two of these enzymes: APOBEC3G and either APOBEC3B, -3C, or -3F.

Recent evidence suggests that the deaminase activity of the APOBEC enzymes is sometimes dispensable for their restrictive activity (116), which raises the question as to whether these proteins have an additional mode of retroviral restriction. Subsequent studies have shown that many of the APOBEC proteins mutated in their cytidine deaminase domains are still

able to reduce the accumulation of reverse transcription products during the early stages of viral replication (15, 67). More recent studies have shown that APOBEC3G is able to specifically inhibit all stages of reverse transcription in vitro (70). The precise relevance of these in vitro effects to the in vivo situation is uncertain as several groups have demonstrated that the restriction caused by APOBEC family members in the absence of deaminase activity is negligible when the mutant proteins are expressed in cells at levels similar to those found physiologically (109, 142).

The APOBEC enzymes are likely responsible for innate or “intrinsic” immunity toward retroviruses but also toward other cellular pathogens, as well as acting as a guard against endogenous retroelements. APOBEC3G is able to block hepatitis B virus (HBV) replication (168), although no significant nucleotide changes were detected in the viral cDNA, and the cytidine deaminase activity of APOBEC3G was completely dispensable for this restriction. It appears rather that APOBEC3G is able to restrict HBV by interfering with the packaging of pregenomic RNA into subviral particles (168). Additional studies have shown that APOBEC3F and 3B can also restrict HBV, and some of these studies have shown the presence of hypermutation in the HBV cDNA, presumably due to cytidine deaminase activity. Therefore, in a situation similar to HIV-1, the exact mechanism of the APOBECs restriction of HBV remains under debate (20, 120, 121, 133, 160). The APOBEC enzymes are also able to inhibit a variety of retrotransposons such as MusD and IAP (intracisternal A-Particle) and non-LTR (long terminal repeat) retrotransposons such as LINE-1, and Alu elements (18, 30, 40, 41, 69, 111). Though many of the APOBECs appear to have some ability to restrict these retroelements, APOBEC3A and APOBEC3B appear to be the most potent and therefore most likely to be the most physiologically relevant to this activity (18, 19).

EARLY POSTENTRY BLOCKS TO INFECTION: FV1, TRIM5α, AND TRIMCYP

Two related retroviral restriction factors that block the retroviral life cycle subsequent to cell entry but before nuclear entry are Fv1 and Trim5α proteins. The *Fv1* gene was first identified as a mouse locus that determined susceptibility to the Friend murine leukemia virus (89, 127, 128), and was subsequently shown to also convey resistance to other murine leukemia viruses (MLVs). Lilly and coworkers went on to describe two major naturally occurring *Fv1* alleles among inbred mouse strains: the $Fv1^b$ allele from Balb/c mice, which allowed replication of a subset of MLVs named B-tropic and blocked replication of MLVs named N-tropic, and the $Fv1^n$ allele from NIH Swiss mice, which allowed replication of N-tropic MLVs and blocked B-tropic MLVs. The alleles were shown to be dominant, and thus $Fv1^{n/b}$ heterozygous mice restrict both N- and B-tropic MLVs. A third set of MLVs termed NB-tropic are able to infect animals with both $Fv1^b$ or $Fv1^n$ alleles; the well-characterized Moloney-MLV (Mo-MLV) falls into this class. The presence of a restrictive *Fv1* allele during MLV infection leads to replication being blocked largely after reverse transcription, but before nuclear translocation of the preintegration complex (PIC) (74, 184). PICs isolated from an infected restrictive cell remain competent for integration when assayed in vitro, suggesting that the *Fv1*-mediated restriction prevents their translocation in the nucleus (129). The determinant of viral sensitivity to *Fv1* was shown to lie in the Gag protein (34, 50, 68), specifically at residue 110 of the capsid (CA) polypeptide (21, 22, 80). This fact remains the best evidence for an active role of the CA protein during the early events of the retroviral life cycle.

The *Fv1* gene was identified in 1996 by a positional cloning strategy (12) and was found to encode a Gag-like protein with sequence similarity to the ERV-L family of endogenous retroviruses (12). The expression of the appropriate *Fv1* gene is sufficient to induce restriction of a sensitive MLV strain (14), and this restriction is thought to function via a

direct interaction between the Fv1 protein and the CA protein of the incoming MLV virions. This interaction has never been directly observed, and perhaps the best evidence for its existence are experiments showing that Fv1-mediated restriction is saturable by infection with very high multiplicities of virus (8, 39). Importantly, this saturation can also be achieved by viral particles that are defective for replication, but only if they are of a tropism restricted by the Fv1 they are being used to saturate (8). Such saturation of Fv1-mediated restriction by an excess of restricted viral particles is termed “abrogation” and suggests that Fv1 is being specifically bound by incoming CA and titrated in these experiments.

These studies of Fv1-mediated restriction became of considerably greater interest to the general retroviral community when it was discovered that human cell lines exhibited a resistance to N-tropic MLV similar to that observed in cells expressing $Fv1^b$ (11, 164). Amazingly, the determinant for susceptibility to this restriction was also amino acid 110 of CA, though the factor in human cells appeared to block infection at a slightly earlier stage than Fv1, i.e., before reverse transcription. This restriction, named Ref1, was found to be saturable in a manner similar to Fv1 (165). Further interest in this restriction was generated by the discovery that many cell lines originally deemed to be nonpermissive for HIV-1 replication (65, 146) were in fact restricting HIV-1 in a saturable manner (66, 164). As for Ref1 and Fv1, susceptibility to the HIV-1 restriction was determined by sequences within the HIV-1 CA protein (32, 61, 122). The protein mediating both of these restrictions was determined to be TRIM5α (62, 76, 125, 157, 186).

The TRIM5α gene was identified by a cDNA library screen to identify the factor mediating HIV-1 resistance in rhesus macaque lung fibroblasts and had no homology to the *Fv1* gene (157). Rather, the TRIM5α gene product is a member of the *tri*partite *m*otif (TRIM) family of proteins characterized as having three domains usually at the N terminus of the protein: a RING domain, either one or two B-boxes, and a coiled-coil domain (118). The RING domain is a cysteine-rich zinc binding domain, which is commonly found in E3 ubiquitin ligases, and indeed there is evidence to suggest that TRIM5α is a ubiquitin ligase (35, 181). The B-box domains are thought to act as an interaction domain and thus determine RING box ubiquitin ligase substrate specificity (103, 104). The coiled-coil domain has been shown to be involved in homo- and heteromultimerization of the TRIM proteins (123, 130). TRIM5α forms trimers in solution and its coiled-coil domain is required for this self-assembly (72, 107, 124, 192). The C terminus of TRIM5α contains a B30.2 domain; this domain binds to CA molecules of incoming retroviruses, and therefore its sequence determines which retroviruses a specific TRIM5α will restrict (62, 113, 124, 125, 143, 158, 159, 187).

Subsequent to the cloning of rhesus macaque TRIM5α, TRIM5α genes have been cloned from a variety of species and tested for their ability to restrict different retroviruses (62, 124, 135, 138, 148, 157, 186, 188). It was found that species variation of TRIM5α sequences (specifically in the B30.2 domain) led to differences in their ability to restrict HIV-1 and other retroviruses. For instance, human TRIM5α potently restricts N-tropic MLV but not B-tropic MLV, HIV-1, or SIV_{mac}, whereas rhesus macaque TRIM5α potently restricts HIV-1 N-tropic MLV but not SIV_{mac} (62, 124, 135, 148, 157, 186). In this way, TRIM5α is a major determinant of retroviral species tropism (63).

The mechanism of TRIM5α-mediated restriction remains to be fully elucidated. Under normal circumstances, it blocks retroviral replication early in the life cycle, after viral entry but before reverse transcription (65, 146). Inhibition of the proteosome during infection allows reverse transcription to take place; however, the PICs are still not able to enter the nucleus and thus infectivity is not rescued by such treatment (5, 179). As TRIM5α is an E3 ubiquitin ligase it is tempting to speculate that the effect is due to a TRIM5α–specific

targeting of CA or another viral protein for degradation. However, it has been shown that disruption of E3 ligase activity by point mutation does not completely abolish the ability ofTRIM5αto induce restriction (35) and therefore TRIM5α certainly also induces restriction in a proteosome-independent fashion. TRIM5α has been observed using a capsid sedimentation assay to promote the rapid uncoating of incoming HIV-1 capsids that may be detrimental to PIC formation (158). Also, TRIM5α induces the degradation of CA in a proteosome-independent manner that may also contribute to its ability to restrict HIV-1 (29). Perhaps all of these mechanisms contribute to TRIM5α-mediated restriction.

Trimeric TRIM5α interacts with capsids via its B30.2 domain (143, 158), and a wealth of evidence intimates an interaction between capsid and the Fv1 protein (21, 22, 80). Both proteins have no sequence homology and yet seem to bind to the same very small area of the CA protein centered around amino acid 110. The fact that this interaction and a subsequent early block to retroviral restriction have evolved independently at least twice in mammals suggests that CA has a conserved and vital role in early events of retroviral replication that host cells can disrupt to induce restriction. Lending further credence to this hypothesis was the discovery of the TRIM5α-cyclophilin A (CypA) fusion protein in owl monkey cells named TRIMCyp (136). CypA is a peptidyl prolyl isomerase and had been previously identified as a protein that specifically interacts with HIV-1 CA (95). This interaction is important for HIV-1 replication and can be disrupted by either the G89Vmutation inHIV-1CAor by competition with the drug cyclosporine A (CSA), which is a competitive inhibitor of CypA. The TRIMCyp fusion in owl monkey cells appears to have formed by the retrotransposition of a CypA pseudogene into the 3′ section of the TRIM5 gene and creates an in-frame fusion ofTRIM5α and CypA in which CypA replaces the B30.2 domain. In this way, the TRIMCyp molecule uses the CypA protein to bind specifically to CA and thereby restrict HIV-1 replication (136). In agreement with this theory, it has been demonstrated that CSA treatment of owl monkey cells disrupts HIV-1 restriction and allows replication to proceed normally (166). Very recently there has been yet another twist in this story with the discovery that the TRIM5α CypA fusion protein has itself evolved independently on two separate occasions. The second occurrence of the TRIMCyp fusion protein is found in several Old World monkeys, including both pigtailed and rhesus macaques. This second allele arose by convergent evolution as the CypA insertion occurred in a slightly different location in the TRIM5 gene (25, 87, 117, 173, 176).

The inhibition of the early stages of the retroviral replication cycle using a protein that is able to recognize CA has clearly occurred independently several times over the course of evolution. The restriction induced by these proteins is extremely potent and therefore enhancement of this restriction remains a tantalizing prospect for antiretroviral therapies.

DESTRUCTION OF VIRAL RNAs BY A ZINC-FINGER ANTIVIRAL PROTEIN

The zinc-finger antiviral protein (ZAP) was isolated using a screen for dominant-acting antiretroviral genes (49). RAT2 cells (which are highly permissive for MLV replication) were stably transduced with a rat cDNA library, and cDNAs that induced resistance to MLV infection were then selected after repeated infection with an MLV vector expressing the herpes thymidine kinase (TK) gene (49). Subsequent treatment of these cells with trifluorothymidine killed all cells that had become TK^+ and thereby selected for cells which had remained TK-minus through resistance to infection. One of the resulting clones from this screen expressed the N-terminal portion of a novel protein ZAP, encompassing a cluster of four CCCH-type zinc fingers. CCCH zinc fingers are uncommon and are found only in a small group of RNA-binding proteins known as the tristetraprolin (TTP) tandem zinc finger (TZF) family (83). Members of the TTP protein family have been shown to bind specifically to AU-rich elements (ARE) in the 3′ untranslated regions of several cytokine mRNAs and to

lead to their degradation (26, 27, 82, 83). This finding immediately suggested that ZAP would most likely interfere with an RNA component of the retroviral life cycle. In agreement with this notion, ZAP-expressing cells were found to block the MLV life cycle by preventing postintegration accumulation of viral RNA in the cytoplasm (49). The block was posttranscriptional, with no effect on the amounts of viral RNA in the nucleus but causing a dramatic decrease in steady-state levels in the cytoplasm (49). Further studies demonstrated that ZAP specifically interacted with the 3′LTR of MLV and that mutations that abolished this interaction (in either the second or fourth CCCH zinc finger) concomitantly abolished the ability of ZAP to mediate viral restriction (56). The 3′LTR of MLV contains no obvious AREs, and ZAP is also not able to specifically mediate the destruction of ARE-containing mRNA transcripts (56), suggesting that the CCCH motifs in ZAP recognize a different viral-specific motif. The question of what RNA feature ZAP specifically was recognizing was made more pertinent by the revelation that ZAP is also able to induce the restriction of alphaviruses such as Sindbis virus (SIN) (13, 56, 194) and filoviruses such as Ebola virus (EBOV) (112). Studies into ZAP-mediated restriction of these viruses suggest that ZAP is targeting viral RNA for degradation. In the case of SIN, the region of the viral genome with which ZAP is able to interact was mapped and showed no significant homology to the 3′LTR of MLV(56). The studies of ZAP-mediated restriction of other viruses has also led to the discovery that ZAP is an interferon-stimulated gene whose upregulation is partially required for interferon-induced cellular restriction of SIN (194).

A possible mechanism by which ZAP leads to the degradation of cytoplasmic RNAs is suggested by a recent study showing that ZAP is able to specifically interact with components of the exosome (57). The exosome is an evolutionarily highly conserved 3′–5′ exoribonuclease complex found both in the nucleus and cytoplasm of eukaryotes (4, 42, 108). Guo and coworkers showed that ZAP coimmunoprecipitates with exosome components Rrp40p and Rrp46p and crucially that RNAi-mediated knockdown of these proteins attenuates ZAP-mediated restriction of MLV (57). This finding strongly suggests that exosome activity is required for ZAP activity. Therefore, the current model for ZAP-mediated restriction is that it bridges an interaction between viral RNA species and the exosome and thereby targets them for degradation. A very recent study has also shown that a longer splice variant of ZAP (termed ZAP(L)) exists in both humans and rats that is more potent in its ability to restrict MLV. ZAP(L) contains a poly(ADP-ribose) polymerase (PARP) domain (77), and comparison of the domain from many primates suggests that this region has evolved under positive selection throughout primate evolution (77). Such positive selection is often attributed to host-pathogen interactions, suggesting that some viral proteins may bind to the PARP domain. No such positive selection was observed in the CCCH domain that binds to viral RNA (77). These data regarding ZAP(L) suggest that ZAP may have additional, as yet uncharacterized, antiviral activities.

PRIMER BINDING SITE (PBS)-MEDIATED RESTRICTION

Teich and colleagues observed in 1977 that murine leukemia viruses (MLVs) were unable to replicate in embryonic carcinoma (EC) cells (162). These pioneering studies reported that when EC cells were infected with MLV, integration of the proviruses proceeded normally; however, no viral mRNA transcripts could be detected (162). This study also revealed that EC cells, if forced to differentiate into a nonpluripotent state, were rendered permissive for MLV replication (162). Subsequently, it was demonstrated that such differentiation only rendered EC cells permissive to new infection by MLV but did not result in reactivation of viruses integrated prior to differentiation (119). To reactivate these silenced viruses, it was necessary to both differentiate the infected EC cells and also to treat them with the DNA demethylating agent 5′azacytidine (5–azaC) (119). These results strongly suggested that the mechanism by which MLV was silenced occurred in two stages: The first stage involved

EC-specific factors, and the second involved DNA methylation that occurred subsequent to the initial silencing and persisted even in differentiated cells (119).

The nature of the EC-specific transcriptional silencing was delineated by several groups in a flurry of activity in the late 1980s. Both reduced transcription factor binding to the viral enhancers in the LTR (64, 90, 92, 152, 167) and the presence of repressive *trans*acting factors were involved (2, 6, 43, 46, 52, 92, 167). The genomic site of action of one of these *trans*-acting factors was determined by a screen to identify MLV escape mutations to EC cell-mediated restriction. This screen identified a single base pair mutation (named B2) that greatly reduced this restriction (6). The B2 mutation is located within the primer binding site (PBS) of MLV, and further study identified 17 bp of the 18 bp of the PBS as essential for EC cell-specific restriction of MLV (6, 43, 92). The PBS of the MLV genome is complementary to 18 nucleotides at the 3′ end of the host proline tRNA and is critical for virus replication. The $tRNA^{pro}$ is annealed to the PBS (PBS^{Pro}) in the RNA genome at the time of virus assembly, and upon infection is used as the primer for minus-strand DNA synthesis during reverse transcription (59). That the PBS is both a target for EC cell-specific restriction and is required for priming of reverse transcription suggests that this restriction could be occurring at the level of DNA or RNA. A DNA-binding *trans*-acting factor was postulated to exist based on a series of experiments characterizing the nature of PBS-mediated restriction in EC cells. It was observed that the PBS^{Pro} was able to silence LTR-driven transcription from reporter constructs in EC cells, independently of orientation and position, even when placed outside of the transcriptional unit (94, 126). It was also demonstrated that the MLV PBS functioned to silence transcription from promoters other than the MLV LTR and that the restriction activity was saturable by transfection of increasing amounts of DNA containing the PBS^{Pro} sequence (93). The presence of this transcriptional silencer was detected in EC cell nuclear extracts using a probe spanning the PBS^{Pro} sequence of MLV by exonuclease III protection assays (94). This exonuclease III protection footprint was reduced upon either differentiation of the EC cells or the use of a probe containing the B2 point mutation, suggesting the presence of a DNA-binding factor that correlates with this repression. Similar experiments performed using MLV PBS^{Pro} in electrophoretic mobility shift assays (EMSA) showed that DNA binding activity was enriched in nuclear extracts from EC cells when compared with differentiated cell lines (126).

Further study of the PBS-mediated restriction of MLV showed that it was not limited to EC cells but also occurred in ES cells (55) as well in several hemapoietic cells lines (58). PBS^{Pro}-mediated restriction was also found to occur in human hematopoietic cells, either transformed or primary cells from cord blood (58). Another PBS sequence, corresponding to $tRNA^{Lys1,2}$ ($PBS^{Lys1,2}$), utilized by retroviruses such as visna, spuma, and Mason-Pfizer monkey virus (105, 149, 150), also leads to restriction in EC cells (110, 182). A binding complex is detected by EMSA in restrictive cells, and in a manner similar to the B2 mutation in PBS^{Pro}, a single point mutation in the $PBS^{Lys1,2}$ sequence is able to relieve the restriction (6, 110, 182). These observations suggest that the $PBS^{Lys1,2}$ may recruit silencing machinery similar to the PBS^{Pro}. As a whole, these data suggest that PBS-mediated restriction occurs in many cell types from multiple species and targets multiple different retroviruses.

Recently, our laboratory identified TRIM28 as a factor required for PBS^{Pro}-mediated restriction of MLV in EC cells. TRIM28 was identified by biochemical purification of the PBS^{Pro} binding complex as observed by EMSA (178). TRIM28 (also known as Kap-1, or Tif1-beta) is a transcriptional corepressor that is recruited to its target genes by interactions with the Kr uppel associated box (KRAB) zinc finger DNA-binding proteins (47). This interaction is mediated by the KRAB box domain and leads to the complex acting as a

sequence-specific transcriptional repressor (169). TRIM28 recruits several factors involved in transcriptional silencing and heterochromatin formation, including the histone H3 K9 methyltransferase ESET, the NuRD histone deacetylase complex, and the heterochromatin-associated protein HP1 (85, 140, 141). TRIM28 is required for PBS^{Pro}-mediated restriction in EC and is recruited to sites of proviral integration in a PBS^{Pro}-dependent manner (178). TRIM28 appears to specifically recruit the HP1 protein to the provirus, and this recruitment is absolutely required for the silencing of MLV (177, 178).

TRIM28 is expressed in many nonpluripotent cell types that do not exhibit PBS^{Pro}-mediated restriction, and thus TRIM28 expression appears to be necessary but not sufficient to induce restriction. This observation, combined with the fact that TRIM28 has no known DNA binding activity, suggests that a DNA-binding factor capable of binding the PBS^{Pro} sequence is differentially expressed between EC and differentiated cells. A KRAB zinc finger DNA-binding protein makes an attractive candidate for this DNA-binding activity, as these proteins interact with TRIM28 and through this interaction induce transcriptional silencing (169).

PBS-mediated silencing of retroviruses in pluripotent cells is likely to have evolved to protect the embryo from infection, but also from the reactivation of endogenous retroviruses and retrotransposons that could cause damaging mutations in the germ line and early progenitor cells. There is a rationale for the evolution of a repression machinery that targets the PBS elements: The PBS is essential for the priming of virus reverse transcription, and furthermore during reverse transcription the PBS sequence on one strand of the viral DNA is synthesized by reverse transcription of the cellular tRNA itself. Thus, the viral PBS sequence is a uniquely effective target site for repression by the host, since any escape point mutations in the PBS sequence would quickly revert to the original sequence during viral replication. The identification of TRIM28 as a component of this restriction machinery will likely aid greatly in the dissection of the molecular mechanism of this type of restriction.

INHIBITION OF RETROVIRAL PARTICLE RELEASE BY TETHERIN

Tetherin (also variously known as BST-2/HM1.24/CD317) was recently identified as a restriction factor that prevents retroviral particle release from the surface of producer cells (115, 170). Much as the APOBEC enzymes were discovered through the study of Vif (144), so tetherin was discovered through characterization of theHIV-1 accessory protein Vpu (115, 170). Vpu was first identified as a small transmembrane phosphoprotein expressed by HIV-1 that enhanced release of virions from infected cells (78, 155, 156). It was later shown that Vpu could also lead to the degradation of the HIV-1 receptor CD4 (175). This degradation is mediated through an interaction between Vpu and the F-box protein βTrCP, which targets CD4 for ubiquitination and subsequent proteasomal degradation (99). These two functions of Vpu are distinct, as Vpu can promote release of HIV-1 and other retroviral virions in cell lines that do not express CD4 (54, 185). The enhancement of virion release from HIV-1 infected cells by Vpu was found to be cell-type dependent (51, 54, 134), and heterokaryon fusion experiments demonstrated this dependence was due to a dominant acting restriction factor (172). This restriction factor is only effective against Vpu-minus HIV-1, and as such wild-type HIV-1 replication is unaffected by it (172). This restriction factor was also found to be upregulated in many permissive cell lines upon stimulation of interferon alpha (IFNα), which rendered these cells resistant to infection with Vpu-minus HIV-1 without significantly affecting wild-type HIV-1 replication (114). Production of Vpu-minus HIV-1 virions in cells expressing this restriction factor led to a striking phenotype, namely large numbers of fully formed viral particles bound to the extracellular leaflet of the cell's outer membrane (114). These bound or "tethered" virions are fully infectious, and

protease treatment of cells with such tethered virions causes a substantial increase in infectious particle release (114).

Tetherin was identified by comparative microarray analysis of cells that either did or did not require Vpu for efficient virion production, coupled with similar analysis of IFNα stimulated genes (115). Tetherin was one of fewer than ten genes whose expression correlated with a dependence on Vpu for efficient partial release and was shown to specially inhibit Vpu-minus HIV-1 production when expressed in the permissive 293T cell line (115). Tetherin is a transmembrane protein with a highly unusual topology including a N-terminal cytoplasmic tail, a single membrane-spanning helix, an extracellular coiled-coil domain, and a C-terminal glycosylphosphatidylinositol (GPI) membrane anchor (81, 131).With the possible exception of a minor variant of the prion protein, there exists no other mammalian protein with similar topology (131). It is not yet known how tetherin adheres virions to the extracellular membrane of cells. Its unusual topology and ability to dimerize (53) suggest several possibilities. One hypothesis would be that tetherin bridges an interaction between the cellular membrane and the viral envelope with one of its two lipid-bilayer anchoring domains in each membrane (115). Alternatively, the tethering could be mediated by dimerization of two tetherin molecules, one in the viral envelope and the other in the cell membrane (115). Further biophysical and biochemical analysis is required to determine which of these possibilities is correct.

The mechanism by which Vpu disrupts tetherin-mediated restriction is also not fully elucidated. Van Damme and colleagues have shown that expression of Vpu leads to a proteasome-independent downregulation of tetherin from the extracellular membrane of a target cell (170). It has also been noted that overexpressed Vpu leads to decreased steady state levels of tetherin (7). Furthermore, like Vif, Vpu may be species specific in its ability to inactivate tetherin, as HIV-1 Vpu is unable to relieve what is presumed to be tetherinmediated restriction of HIV-1 particle production in IFNα–treated African green monkey cells (114).

The nature of tetherin-mediated restriction suggests that tetherin might restrict a wide range of enveloped viruses. As yet, there is only sparse evidence to support this notion, though it has been shown that IFNα treatment of cells leads to a specific block in Ebola virus–like particle release from cells, and that this block can be abrogated by the expression of Vpu (114). Suggestive findings come from experiments showing that the Kaposi sarcoma–associated herpesvirus (KSHV) K5 protein is able to decrease the cellular steady-state levels of tetherin (7). This implies that tetherin may also be detrimental to KSHV replication. Further studies into this rapidly expanding field will likely shed light on these interesting questions.

SUMMARY AND CONCLUSIONS

Much of the early work on retroviruses focused on elucidating the life cycle and determining how the virally encoded machinery drove its progression. Recently, there has been a growing realization that no viral processes take place in the absence of interactions with cellular host factors. Over the course of evolutionary time, the host and retrovirus have been locked in a constant struggle for survival. This has resulted in retroviruses evolving the capability of exploiting a multitude of cellular proteins to promote their life cycle. Reciprocally it has led to the host cell developing many restriction factors to inhibit the retroviral life cycle. As discussed in this review, the study of hostretroviral interactions has recently uncovered a number of these restriction factors and has been extremely illuminating to the field of intrinsic immunity as well as to molecular biology. The future study of these interactions will continue to provide insight into these endogenous cellular processes.

Finally, a greater understanding of the factors mediating intrinsic immunity may lead to the development of pharmacological agents that can boost their potency and thereby lead to treatments for viral disease.

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Glossary

Restriction factor	a host cell protein or factor whose expression restricts or inhibits the viral life cycle
APOBEC	apolipoprotein B mRNA-editing catalytic polypeptide
Capsid (CA)	the major structural constituent of the retroviral core, synthesized as a proteolytic cleavage product of the Gag precursor protein
ZAP	zinc-finger antiviral protein
Accessory gene	one of several small open reading frames found in complex retroviruses such as HIV-1. Accessory genes are so named as being dispensable for viral replication under some artificial conditions in tissue culture
Permissive	a cell line or organism that supports replication of the virus
Provirus	a DNA copy of the retroviral genome integrated into the host cell genome
Nucleocapsid (NC)	a proteolytic cleavage product of the Gag precursor protein, containing one or two zinc fingers, required for packaging of the RNA genome into the virion
Group-specific antigen (gag) gene	the retrovirus gene encoding the precursor protein that is cleaved to give rise to the major structural components of the virion particle
MLV	murine leukemia virus
LTR	long terminal repeat
PIC	preintegration complex
Primer binding site (PBS)	the region of a retroviral genome complementary to the 3′ end of a cellular tRNA sequence. During reverse transcription a cellular tRNA annealed to this sequence is used to prime the first strand of DNA synthesis

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SUMMARY POINTS

1. A large number of cellular proteins have evolved to interfere with the replication cycle of retroviruses. These proteins are known collectively as restriction factors.
2. Restriction factors have been shown to interfere with many stages of the retroviral life cycle.
3. Retroviral restriction factors are often able to restrict other nonretroviral viruses.
4. Several restriction factors have been identified through the study of HIV-1 accessory gene function. Accessory genes Vif and Vpu are both able to specifically inhibit cellular restriction factors.
5. Several other restriction factors have been identified by studying differences between permissive and nonpermissive cell lines.

FUTURE ISSUES

1. There are likely many other cellular restriction factors that remain unidentified; further characterization of the retroviral life cycle and cellular factors that inhibit it will, it is hoped, lead to their discovery.
2. As yet there has been no effective modification or stimulation of a cellular restriction factor to prevent a clinical disease. It is hoped that in the future such modification will be achieved either pharmacologically or genetically and that this will lead to effective clinical treatments for viral diseases.

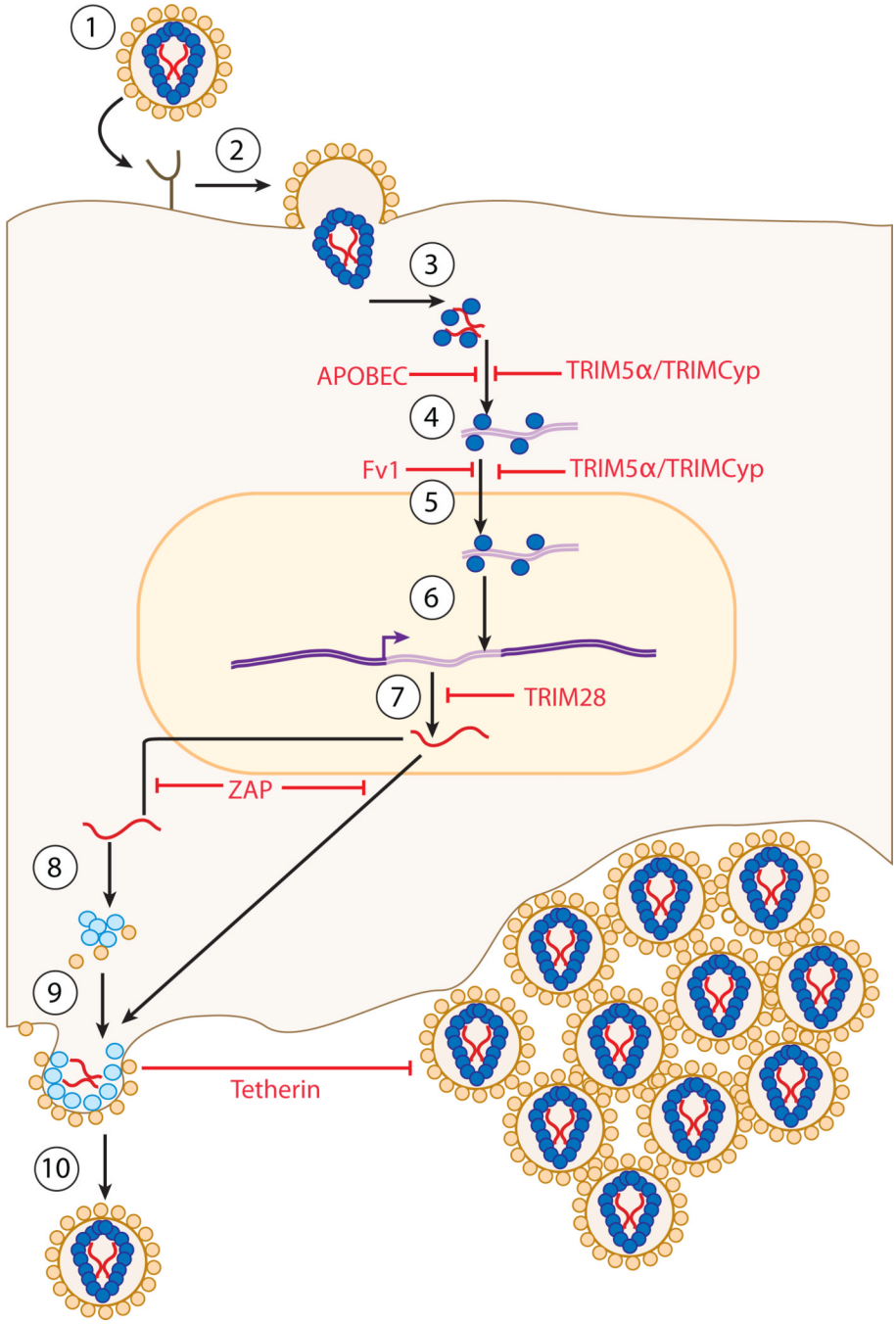

Figure 1.

Summary of the retroviral life cycle and points of action of restriction factors. Cartoon showing the simplified life cycle of a retrovirus. Stages of life cycle are numbered and are as follows: 1. Envelope-mediated binding of retrovirus to target cell receptor. 2. Fusion of retroviral envelope and target cell membrane, leading to core viral particle entering cell. 3. Uncoating of core viral particle. 4. Reverse transcription of viral ssRNA genome into dsDNA, and formation of preintegration complex (PIC). 5. Nuclear entry of PIC. 6. Integration of viral genome into target cell genome. 7. Production of new viral RNA genomes and mRNA molecules by transcription. 8. Nuclear export of viral RNA species followed by either translation into viral proteins, or packaging into forming viral particles at

a cellular membrane. 9. New translation of viral proteins and RNAs congregating at membrane and induction of membrane curvature as viral particles form. 10. Budding of fully formed viral particles from the membrane, and protease-mediated cleavage of viral proteins, leading to release of the fully mature virion. Dark blue circles denote protease cleaved viral proteins. Light blue circles denote viral proteins that have not been cleaved by protease. Small empty black circles denote viral envelope proteins. Wavy red lines denote viral RNAs; green wavy lines denote viral DNAs. Straight red lines denote point of interference in retroviral life cycle by restriction factors.