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Review

APOBEC3G: an intracellular centurion

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The intrinsic antiretroviral factor APOBEC3G (A3G) is highly active against HIV-1 and other retroviruses. In different cell types, A3G is expressed in high-molecular-mass (HMM) RNA–protein complexes or low-molecular-mass (LMM) forms displaying different biological activities. In resting CD4 T cells, a LMM form of A3G potently restricts HIV-1 infection soon after virion entry. However, when T cells are activated, LMM A3G is recruited into HMM complexes that include Staufen-containing RNA granules. These complexes are probably nucleated by the induced expression of Alu/hY retroelement RNAs that accompany T-cell activation. HMM A3G sequesters these retroelement RNAs away from the nuclear long interspersed nuclear element-derived enzymes required for Alu/hY retrotransposition. Human immunodeficiency virus (HIV) exploits this 'window of opportunity' provided by the loss of LMM A3G in activated CD4 T cells to productively infect these cells. During HIV virion formation, newly synthesized LMM A3G is preferentially encapsidated but only under conditions where Vif is absent and thus not able to target A3G for proteasome-mediated degradation. Together, these findings highlight the discrete functions of the different forms of A3G. LMM A3G opposes the external threat posed by exogenous retroviruses, while HMM A3G complexes oppose the internal threat posed by the retrotransposition of select types of retroelements.

Keywords: APOBEC3G; HIV-1; Vif; Alu; retrotransposition; RNA granules

1. RETROVIRUSES AND CYTIDINE DEAMINASES

Biological interactions with retroviruses and related endogenous retroelements have played important roles in human evolution. These interactions are dynamic and reciprocal. Humans and retroviruses have evolved mechanisms to counteract the other's biological activities. Since the identification of human immunodeficiency virus (HIV) in 1983, this pathogenic retrovirus has become a prototype for studies of many aspects of retroviral biology, including virus–host interactions.

HIV-1 is a member of the primate lentivirus family of retroviruses and is the product of a cross-species (zoonotic) transmission event of related but distinct lentiviruses (simian immunodeficiency viruses or SIVs) that naturally infect non-human primates in sub-Saharan Africa. SIVcpz in chimpanzees (*Pan troglodytes*) is the precursor of pandemic HIV-1. Similarly, SIVsm from sooty mangabeys (*Cercocebus atys*) is the immediate source of HIV-2. Other lentiviruses, such as SIVagm from African green monkeys, have not been transmitted to humans. It is clear that species-specific barriers to infection oppose successful establishment of various zoonotic retroviral infections. These barriers, which are now becoming better understood, provide an important resource for better understanding the evolutionary conflict that continues to occur between pathogenic retroviruses and their cellular hosts.

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2. THE HIV-1 VIF PHENOTYPE

In contrast to simple retroviruses encoding only Gag, Pol and Env gene products, HIV-1 encodes six additional auxiliary proteins (Tat, Rev, Nef, Vif, Vpr and Vpu) that orchestrate the pathogenic interplay of HIV-1 with its human host. While the major functions of Vpr, Vpu, Tat, Rev and Nef were largely unravelled within a few years after the HIV-1 genome was sequenced, the mechanism underlying the function of Vif remained shrouded in mystery for almost two decades.

Vif corresponds to a basic 23 kDa phosphoprotein that is expressed late in the retroviral life cycle and is highly conserved among all of the primate lentiviruses with the notable exception of the equine infectious anaemia virus. The function of Vif is tightly linked to the nature of the virus-producing cell (Gabuzda *et al.* 1992). Several T-cell lines (e.g. Jurkat and SupT1) and non-haemopoietic cell lines (e.g. HeLa, 293T and COS) produce infectious HIV-1 virions in the absence of Vif (Δ*vif* HIV-1) and are termed 'permissive'. Conversely, Δ*vif* HIV-1 virions derived from non-permissive cells, including primary CD4 T cells and macrophages, the natural targets of HIV-1 infection, are non-infectious (Sova & Volsky 1993; von Schwedler *et al.* 1993). However, the molecular basis for these interesting cell-dependent differences remained unclear for many years.

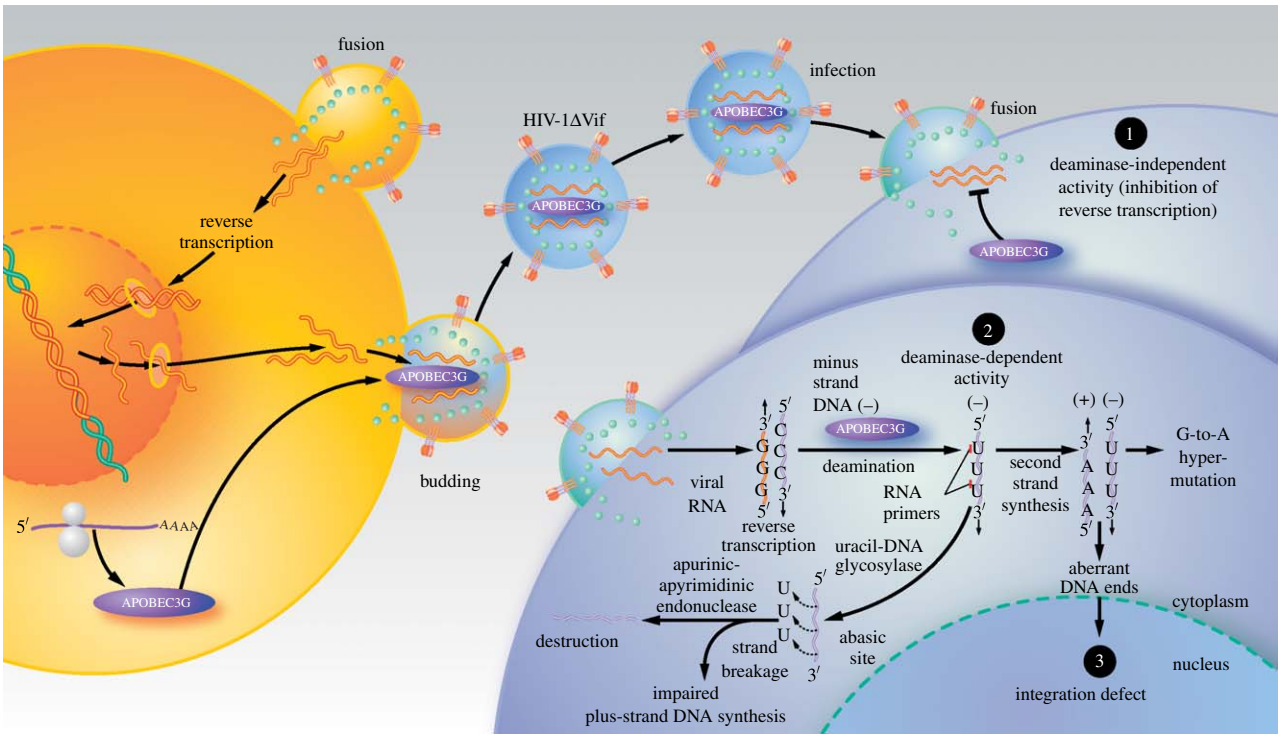

Figure 1. Sequential strategies for the restriction of HIV-1 by virion-incorporated A3G. In the absence of Vif, A3G in the cytoplasm of virus-producing cells is effectively incorporated into budding virions and carried forward into the next target cells, where it can act as a potent inhibitor of HIV-1 replication. These inhibitory effects involve both deaminase-independent and -dependent antiviral actions. A3G bound to HIV-1 RNA may physically impede reverse transcriptase movement on the viral RNA template, resulting in a deaminase-independent block in reverse transcription (1). However, this inhibition is frequently incomplete, and minus-strand viral DNA is generated. A3G mediates extensive deamination of dC residues in this newly synthesized single-stranded viral DNA. This action of A3G effectively halts HIV replication because of the resulting dG-to-dA changes in the viral plus strand or because the uracil-containing minus strand is destroyed by the actions of uracil DNA glycosylase and apurinic–apyrimidinic endonuclease (2). Additionally, diminished chromosomal integration of the double-stranded viral DNA may occur due to defects in $tRNA^{Lys3}$ primer cleavage, leading to the formation of viral DNA with aberrant ends (3).

3. INVESTIGATING VIF ACTION REVEALS APOBEC3G AS AN INNATE ANTIVIRAL FACTOR

The identification of human APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G or A3G) in non-permissive cells as a potent innate inhibitor of retroviruses emerged from studies investigating HIV-1 Vif action.

A key insight into how Vif allowed wild-type HIV-1 to readily spread in cultures of non-permissive cells was made when heterokaryons of permissive and non-permissive cells were produced and infected with Δ*vif* HIV-1 (Madani & Kabat 1998; Simon *et al.* 1998). Analysis of the progeny virions emanating from these heterokaryons revealed that they were non-infectious, which suggested that Vif somehow overcomes the inhibitory effect of one or more factors produced by non-permissive cells. Using subtractive hybridization techniques for analysis of two closely related cells differing in permissivity, Sheehy *et al.* (2002) succeeded in identifying A3G, a known cytidine deaminase, as the host antiviral factor produced by non-permissive cells and thwarted by Vif. Virions budding from non-permissive cells were found to contain A3G, providing an explanation for how viral replication might be altered during the next round of viral spread (figure 1).

4. APOBEC3G AND OTHER MEMBERS OF THE CYTIDINE DEAMINASE FAMILY

A3G (384 amino acids, 46 kDa) belongs to a much larger family of cytidine deaminases that share a conserved zinc-binding motif (Cys/His)-Xaa-Glu-$Xaa_{23–28}$-Pro-Cys-$Xaa_{2–4}$-Cys (Jarmuz *et al.* 2002). These enzymes mediate hydrolytic deamination at the C4 position of the C (or dC) base, converting C to U (or dC to dU). These changes are often referred to as RNA or DNA editing (Teng *et al.* 1993; Harris *et al.* 2002). In humans, the APOBEC enzyme family includes activation-induced deaminase (AID), APOBEC1, APOBEC2, APOBEC3A–H and APOBEC4 (Jarmuz *et al.* 2002; Conticello *et al.* 2005; Rogozin *et al.* 2005; OhAinle *et al.* 2006).

APOBEC1 is primarily expressed in gastrointestinal tissue (Teng *et al.* 1993). As the central component of an RNA editosome complex, it edits the apolipoprotein B mRNA transcript at cytosine^{6666} converting a glutamine at this position to an in-frame stop codon giving rise to a truncated version of the apoB protein (Teng *et al.* 1993; Yamanaka *et al.* 1995; Mehta *et al.* 2000). The longer apoB100 and the shorter apoB48 proteins differ in their effects on cholesterol metabolism.

AID is selectively expressed in germinal centre B cells (Muramatsu *et al.* 1999), where it catalyses deamination

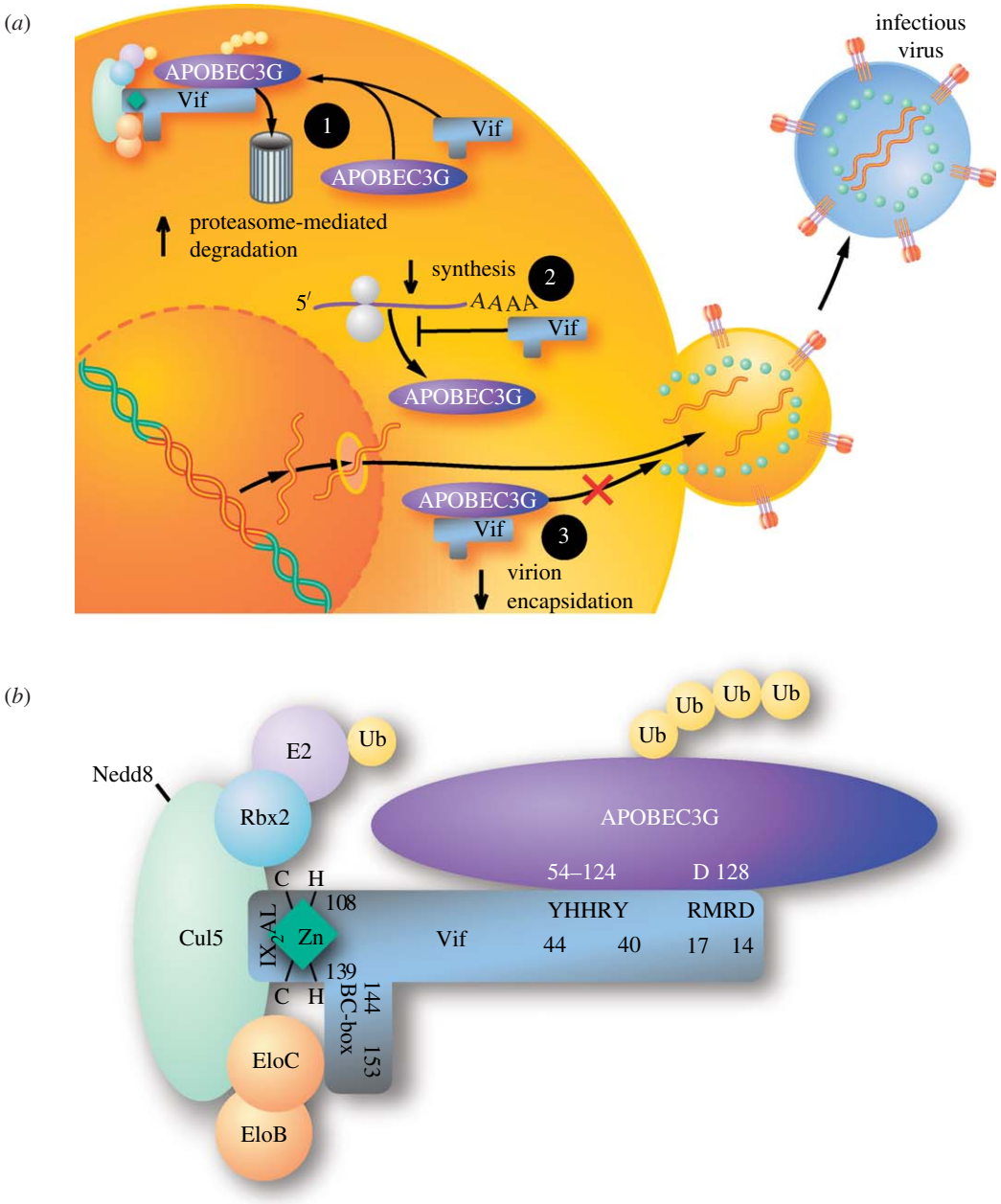

Figure 2. The interplay of Vif and APOBEC3G. (*a*) Neutralization of A3G in virus-producing cells by HIV-1 Vif. Vif defeats the antiviral activity of A3G principally by both binding to A3G and recruiting an E3 ligase complex that mediates polyubiquitylation (Ubn) of A3G and its accelerated degradation in 26S proteasomes (1). Vif also partially impairs the translation of A3G mRNA (2). These dual effects of Vif effectively deplete A3G in the virus-producing cells and, thus, A3G is not available for incorporation into virions budding from these cells. Other auxiliary functions of Vif have been proposed, including physical exclusion of A3G from virion encapsidation in the absence of degradation, perhaps due to sequestration of A3G away from the sites of viral assembly/budding (3). (*b*) Model of the Vif–cullin5–elongin BC complex. Vif employs multiple protein interaction domains to orchestrate A3G degradation. The N-terminal region of Vif binds to an N-terminal region of A3G (amino acids 54–124). The SLQ(Y/F)LA motif (amino acids 144–150) of Vif mediates binding to the elongin C (EloC) component of the E3 ligase complex. Finally, a novel zinc-binding motif, (HCCH, amino acids 108–139) within Vif, containing two conserved cysteines, mediates a second interaction with the cullin 5 (Cul5) component. The Cul5–Vif E3 ubiquitin ligase binds A3G and brings it into close proximity with the E2 ubiquitin-conjugating enzyme.

at the DNA level, and thus promotes immunoglobulin gene diversification via somatic hypermutation and class switch recombination (Muramatsu *et al.* 2000).

The highly related APOBEC3 genes (*APOBEC3A–H*) (7, 10, 12 and 125) are located in a single cluster on human chromosome 22 at q13.2. Four of the APOBEC3 proteins (A3B, A3DE, A3F and A3G) have duplicated cytidine deaminase domains (CDs) while A3A, A3C and A3H have only one CD (Jarmuz *et al.* 2002; Conticello *et al.* 2005; Dang *et al.* 2006; OhAinle *et al.* 2006). Phylogenetic analyses suggest that the primordial APOBEC3 contained only one CD, which might have evolved from AID or APOBEC2 during the onset of vertebrate speciation (Conticello *et al.* 2005).

APOBEC2, a single-domain cytidine deaminase, is principally expressed in the cardiac and skeletal muscle. Despite rather extensive study, its function in muscle biology remains unknown. APOBEC2 is the closest known paralog of APOBEC3 for which a high-resolution structure is available (Prochnow *et al.*

2007). However, this deaminase lacks enzymatic activity and its precise function in cells is also unknown.

When expressed in *Escherichia coli*, A3G, AID and even APOBEC1 catalyse the deamination of dC residues in single-stranded DNA (Harris *et al.* 2002; Beale *et al.* 2004), suggesting that single-stranded DNA is the favoured substrate for A3G. This finding provides an explanation for how viral replication might be blocked in the next target cell via the inherent deaminase activity of A3G acting on the nascent reverse transcribed cDNAs produced by the virus (figure 1).

5. VIF CIRCUMVENTS ANTIVIRAL ACTIVITY OF APOBEC3G

Human A3G expressed in non-permissive cells poses a significant threat to the replication and spread of HIV-1. Vif counters this threat by directly interacting with A3G and linking A3G to an efficient E3 ubiquitin ligase complex that mediates polyubiquitylation of both A3G and Vif. These events promote accelerated degradation of these proteins by the 26S proteasome (Conticello *et al.* 2003; Marin *et al.* 2003; Sheehy *et al.* 2003; Stopak *et al.* 2003). Vif also partially impairs the translation of A3G mRNA, although the mechanism remains undefined (Stopak *et al.* 2003). Nevertheless, the combined effects of accelerated degradation and diminished synthesis result in the nearly complete depletion of intracellular A3G in the producer cell during wild-type HIV-1 infection. These actions of Vif prevent virion encapsidation of A3G, thereby ensuring high infectivity of the progeny virions (Mariani *et al.* 2003; Stopak *et al.* 2003; figure 2*a*).

In the absence of Vif, A3G effectively incorporates into budding HIV-1 virions (Mariani *et al.* 2003; Stopak *et al.* 2003) by interacting with the nucleocapsid region of the Gag polyprotein (Alce & Popik 2004; Cen *et al.* 2004; Luo *et al.* 2004). This interaction is further strengthened by A3G's propensity to bind single-stranded nucleic acids, particularly viral RNA at the plasma membrane site of virion budding (Schafer *et al.* 2004; Svarovskaia *et al.* 2004; Zennou *et al.* 2004; Khan *et al.* 2005; Burnett & Spearman 2007; Khan *et al.* 2007; Soros *et al.* 2007). The incorporation of only seven molecules of A3G into Δ*vif* HIV virions produced by human peripheral blood mononuclear cells is sufficient to greatly impair HIV-1 replication (Xu *et al.* 2007). Thus, the A3G–Vif–E3 ligase axis is a compelling new target for the development of novel antiviral drugs that could re-enable the potent defensive properties of A3G.

6. FURTHER INSIGHTS INTO VIF ACTION

Deletional mutagenesis coupled with co-immunoprecipitation analyses reveals that the N-terminal region of Vif binds to the N-terminal region of A3G (amino acids 54–124; Conticello *et al.* 2003; Marin *et al.* 2003; Simon *et al.* 2005; Wichroski *et al.* 2005; figure 2). Further mechanistic insights have emerged from cross-species studies. For example, Vif from SIVagm effectively triggers the degradation of African green monkey A3G but fails to neutralize either human or chimpanzee A3G. Similarly, HIV-1 Vif cannot induce degradation of African green monkey or rhesus macaque A3G. These species-specific effects appear to be governed by a single amino acid (residue 128) in A3G (Mariani *et al.* 2003; Bogerd *et al.* 2004; Mangeat *et al.* 2004; Schrofelbauer *et al.* 2004; Xu *et al.* 2004) and amino acids 14–17 (DRMR) in Vif (Schrofelbauer *et al.* 2006). These species-specific limitations in Vif activity probably form an important barrier that minimizes the frequency of zoonotic transmission of many primate lentiviruses. The fact that the Vif gene products of SIVcpz and SIVsm, the precursors of HIV-1 and 2, respectively, degrade human A3G (Mariani *et al.* 2003; Gaddis *et al.* 2004; Xu *et al.* 2004) provides a compelling explanation for how these viruses successfully spawned the HIV-1 and 2 epidemics in humans.

The regions in Vif critical for interacting with the E3 ligase complex have also been mapped by mutagenesis (figure 2*b*). The SLQ(Y/F)LA motif (amino acids 144–150) of Vif interacts directly with elongin C (Marin *et al.* 2003; Yu *et al.* 2003, 2004*c*; Mehle *et al.* 2004*a*,*b*). A cryptic zinc-coordination motif, His-Xaa_5-Cys-Xaa_{17-18}-Cys-Xaa_{3-5}-His (HCCH, amino acids 108–139), connects Vif to cullin 5 (Luo *et al.* 2005; Mehle *et al.* 2006; Xiao *et al.* 2006). Through these interactions, Vif recruits a ubiquitin ligase (E3) complex comprising elongin C, elongin B, cullin 5, Nedd8 and Rbx1 (Yu *et al.* 2003, 2004*c*) that mediates the polyubiquitylation and triggers degradation of A3G (Conticello *et al.* 2003; Marin *et al.* 2003; Sheehy *et al.* 2003; Stopak *et al.* 2003). Mutation of the SLQ or the HCCH motif in Vif, or overexpression of cullin 5 mutants that fail to engage Nedd8 or Rbx1 renders Vif incapable of degrading A3G and leads to greater A3G antiviral activity (Mehle *et al.* 2004*a*,*b*, 2006; Yu *et al.* 2003, 2004*c*). Thus, the Vif:cullin 5 and Vif:elongin C interaction sites are additional potential targets for antiviral drug design.

Although Vif-induced degradation of A3G is important in overcoming the antiviral effects of A3G, non-degradative mechanisms of Vif action have also been proposed. Vif may physically exclude A3G from sites of viral assembly/budding or inhibit A3G encapsidation by competing for binding to viral components, such as the nucleocapsid or viral genomic RNA (Mariani *et al.* 2003; Kao *et al.* 2004; Opi *et al.* 2007; figure 2*a*).

7. REGULATION OF CYTIDINE DEAMINASES AND CANCER

How is the potentially promiscuous mutagenic activity of the cytidine deaminases controlled, particularly during cell division when this cytoplasmic enzyme could readily access nuclear DNA? In this regard, forced expression of APOBEC1 as a transgene in the livers of mice consistently leads to hepatic dysplasia and hepatocellular carcinoma, possibly due to promiscuous RNA editing of tumour suppressors or oncogenes (Yamanaka *et al.* 1995). High-level expression of AID is similarly linked with the development of various large B-cell and non-Hodgkin's lymphomas (Revy *et al.* 2000). The constitutive and ubiquitous transgenic expression of AID in mice similarly results in the development of various cancers, specifically T-cell lymphomas, micro-adenomas and lung adenocarcinomas (Okazaki *et al.* 2003). The associations of

APOBEC1 and AID with cancer emphasize how tight intracellular regulation of these cytidine deaminases is probably required to minimize the chances of mutations in genomic DNA leading to cellular transformation.

8. ASSEMBLY INTO LARGE RNA–PROTEIN COMPLEXES INHIBITS APOBEC3G ENZYMATIC ACTIVITY

How is the potentially mutagenic activity of the A3G enzymes negatively regulated? One possibility was suggested immediately by subcellular localization studies, which indicated that A3G is strongly retained in the cytoplasm (Mangeat *et al.* 2003; Muckenfuss *et al.* 2006; OhAinle *et al.* 2006; Wichroski *et al.* 2006; Gallois-Montbrun *et al.* 2007). While such sequestration would limit promiscuous editing of genomic DNA during many phases of the cell cycle, nuclear access could occur during mitosis when nuclear membranes break down. Another possibility involving negative regulation by complex assembly was suggested by functional clues from APOBEC1 and AID. The enzymatic activity of APOBEC1 is regulated by its assembly with an additional factor termed ACF (APOBEC1 complementing factor), leading to the formation of a multicomponent enzyme complex. AID has no measurable deaminase activity unless pretreated with RNase to remove inhibitory RNAs bound to AID. Indeed, endogenous A3G expressed in the cytoplasm of H9 T-cell lines and mitogen-activated CD4 T cells is assembled in 5–15 MDa high-molecular-mass (HMM) ribonucleoprotein (RNP) complexes, and the deaminase activity of A3G is greatly inhibited in these complexes. Interestingly, these HMM A3G complexes can be artificially converted to an enzymatically active low-molecular-mass (LMM) form by treatment with RNase A, suggesting that one or more cellular RNAs play an important role in the assembly of HMM A3G complexes (Chiu *et al.* 2005).

9. RNP COMPLEXES IN HMM A3G COMPLEX ASSEMBLY

The HMM A3G RNP complexes contain at least 95 different proteins, as determined by tandem affinity purification and mass spectrometry (Chiu *et al.* 2006; Kozak *et al.* 2006; Gallois-Montbrun *et al.* 2007). Numerous cellular RNA-binding proteins with diverse roles in RNA function, metabolism and fate determination are found in these HMM A3G complexes, and their participation in the complex occurs in an RNA-dependent manner (Chiu *et al.* 2006; Kozak *et al.* 2006; Gallois-Montbrun *et al.* 2007). Careful analysis suggests that these components fall into at least three previously defined multi-subunit RNP complexes in human cells: (i) Staufen-containing, polysome-associated RNA granules, (ii) Ro RNPs, and (iii) select components of prespliceosomes plus reservoirs for transcriptional regulators (Chiu *et al.* 2006). Of note, the protein cofactors in the latter class are quite multifunctional, and many participate in Staufen-containing RNA granules and function as cytoplasmic regulators of translation.

Staufen-containing RNA granules correspond to more than 10 MDa macromolecular RNP complexes comprising ribosomal subunits, scaffold proteins, translation machinery, RNA-binding proteins, helicases and various decay enzymes (Kanai *et al.* 2004; Villace *et al.* 2004; Anderson & Kedersha 2006; Kiebler & Bassell 2006). Ro RNPs are the major RNP autoantigens recognized by sera from patients with various connective tissue diseases. In human cells, Ro RNPs contain one of the four human small Y (hY) RNAs (hY1, hY3, hY4 and hY5) and two core proteins (60 kDa Ro and 50 kDa La; Fabini *et al.* 2000). Almost all of the proteins that participate in the formation of Staufen-containing RNA granules and Ro RNPs are readily detectable in the purified HMM A3G RNP complexes.

An association of A3G with exogenously expressed components of stress granules (SGs) and processing bodies (PBs) has been reported (Kozak *et al.* 2006; Wichroski *et al.* 2006; Gallois-Montbrun *et al.* 2007). The fact that Staufen RNA granules, SGs and PBs represent a continuum of granular-like structures in which cargos may be transferred back and forth could contribute to these results. These findings imply that the dynamic spatial organizations between RNA granules and related cytoplasmic complexes could affect the status of A3G complexes and control A3G's antiviral activity.

Intriguingly, sequencing of the most prominent RNA components in HMM A3G complexes identified human Alu and small hY (hY1–5) endogenous retroelement RNAs (Chiu *et al.* 2006). In the presence of A3G, Alu and hY RNAs are selectively recruited and enriched in Staufen RNA granules and Ro RNPs, respectively, suggesting a potential physiological function for these complexes (Chiu *et al.* 2006). Specifically, endogenous non-autonomous retroelements (i.e. Alu and hY RNAs) are probably the natural cellular targets of A3G.

10. INTRAVIRION APOBEC3G COMPLEXES: AN UNUSUAL VIRUS–HOST INTERACTION

The fact that cellular A3G principally resides in 5–15 MDa HMM RNP complexes in activated, virus-producing T cells prompted analysis to determine which form of A3G is actually incorporated into the budding virions. Pulse-chase radiolabelling and size-fractionation studies revealed that cellular A3G rapidly assembles (less than 30 min) into HMM complexes (Soros *et al.* 2007), and as a consequence only small quantities of newly synthesized LMM A3G occur in cells. However, pulse-chase studies further revealed that virion A3G is mainly recruited from the cellular pool of newly synthesized enzymes (Soros *et al.* 2007). Interaction of A3G with HIV genomic RNA in the virion core leads to the assembly of large intravirion A3G complexes and unexpectedly to inhibition of its enzymatic activity (Soros *et al.* 2007). This process is analogous to the inactivation of cellular A3G activity when it engages cellular RNAs in the HMM RNA–protein complexes (Chiu *et al.* 2005).

How is the enzyme ultimately activated? Intriguingly, the answer appears to involve reverse transcription and the action of HIV-1 RNase H. As the minus-strand cDNA is synthesized during HIV reverse transcription, the genomic RNA template is degraded by the RNase H

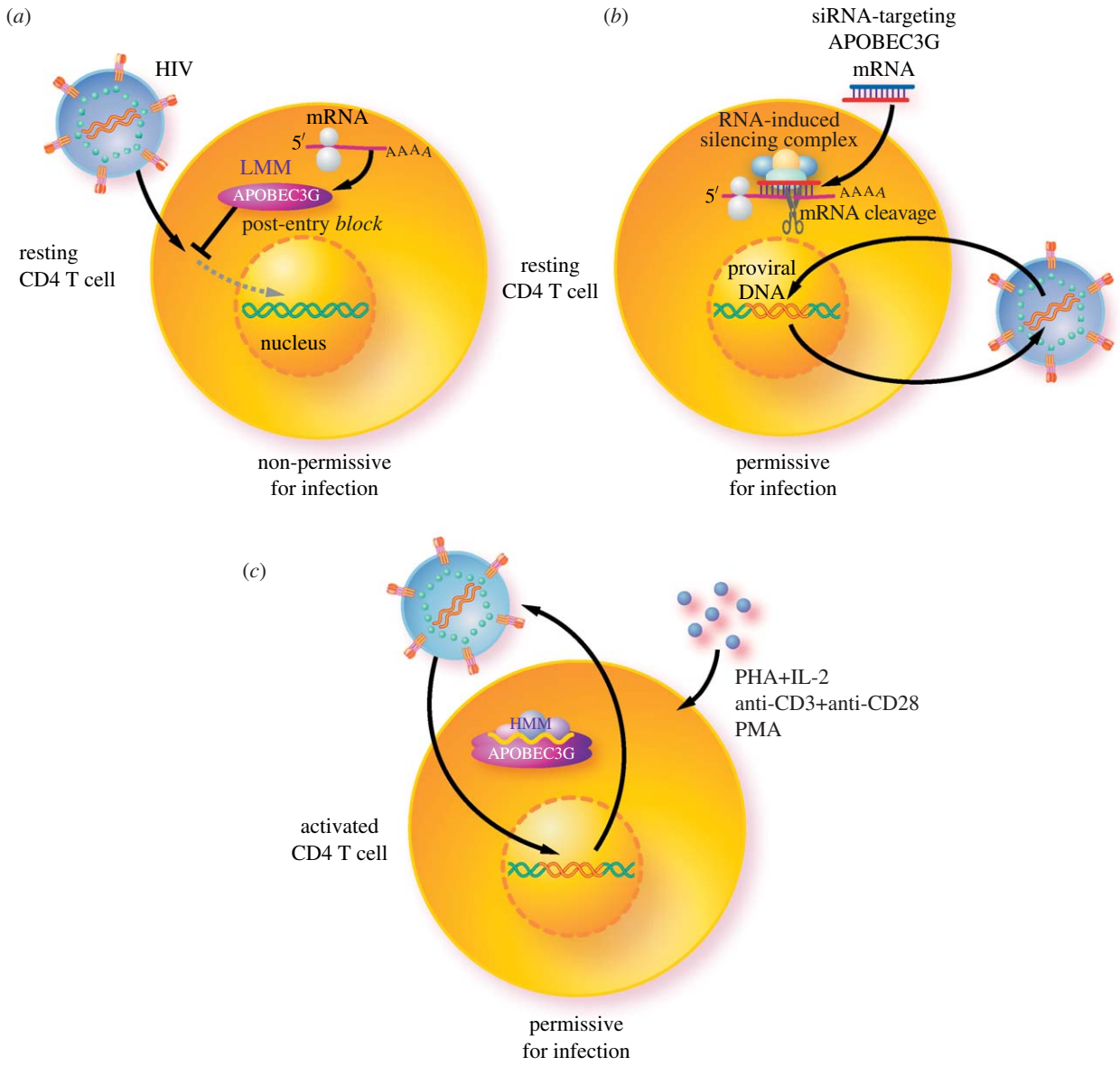

Figure 3. (*a*) LMM A3G restricts HIV-1 after entry into resting peripheral blood CD4 T cells. Human A3G exists exclusively in LMM forms in peripheral blood-derived resting CD4 T cells and functions as a post-entry restriction factor to block replication of incoming HIV-1 viral particles. (*b*) RNA interference-mediated depletion of LMM A3G in resting CD4 T cells is sufficient to render these cells permissive for HIV-1 infection. (*c*) This restricting activity is forfeited when A3G is recruited into the HMM A3G complex upon CD4 T-cell activation by various mitogens (anti-CD3/CD28 and phorbol myristate acetate (PMA)) and cytokines (IL-2, IL-7 and IL-15).

activity of the reverse transcriptase, generating the minus-strand DNA substrate of A3G and also removing the inhibitory RNA bound to A3G and thereby activating its deaminase activity (Soros *et al.* 2007). These findings highlight a most unusual virus–host interaction, in which initiation of the antiviral enzymatic activity of A3G is contingent on the prior action of an essential viral enzyme.

11. MECHANISMS OF APOBEC3G ANTIVIRAL AND ANTI-RETROELEMENT ACTION

The antiviral mechanism of A3G has been attributed to two fundamental properties: its ability to bind single-stranded RNA and its inherent deaminase activity on single-stranded DNA substrates. These two properties map to two A3G CDs. The N-terminal CD1 mediates RNA binding and virion encapsidation (Navarro *et al.* 2005; Newman *et al.* 2005; Iwatani *et al.* 2006). The C-terminal CD2 confers deaminase activity (Hache *et al.* 2005; Navarro *et al.* 2005; Newman *et al.* 2005; Iwatani *et al.* 2006) and sequence specificity for modification of the single-stranded DNA substrate (Harris *et al.* 2003; Zhang *et al.* 2003; Bishop *et al.* 2004; Liddament *et al.* 2004; Wiegand *et al.* 2004).

12. DEAMINASE-INDEPENDENT ANTIVIRAL ACTIVITY OF APOBEC3G

RNA binding appears to play a major role in rendering Δ*Vif* HIV-1 virions non-infectious. Specifically, A3G analogues containing inactivated C-terminal CD2 can still substantially reduce the infectivity of Δ*vif* HIV-1 (Newman *et al.* 2005; Bishop *et al.* 2006). The RNA-

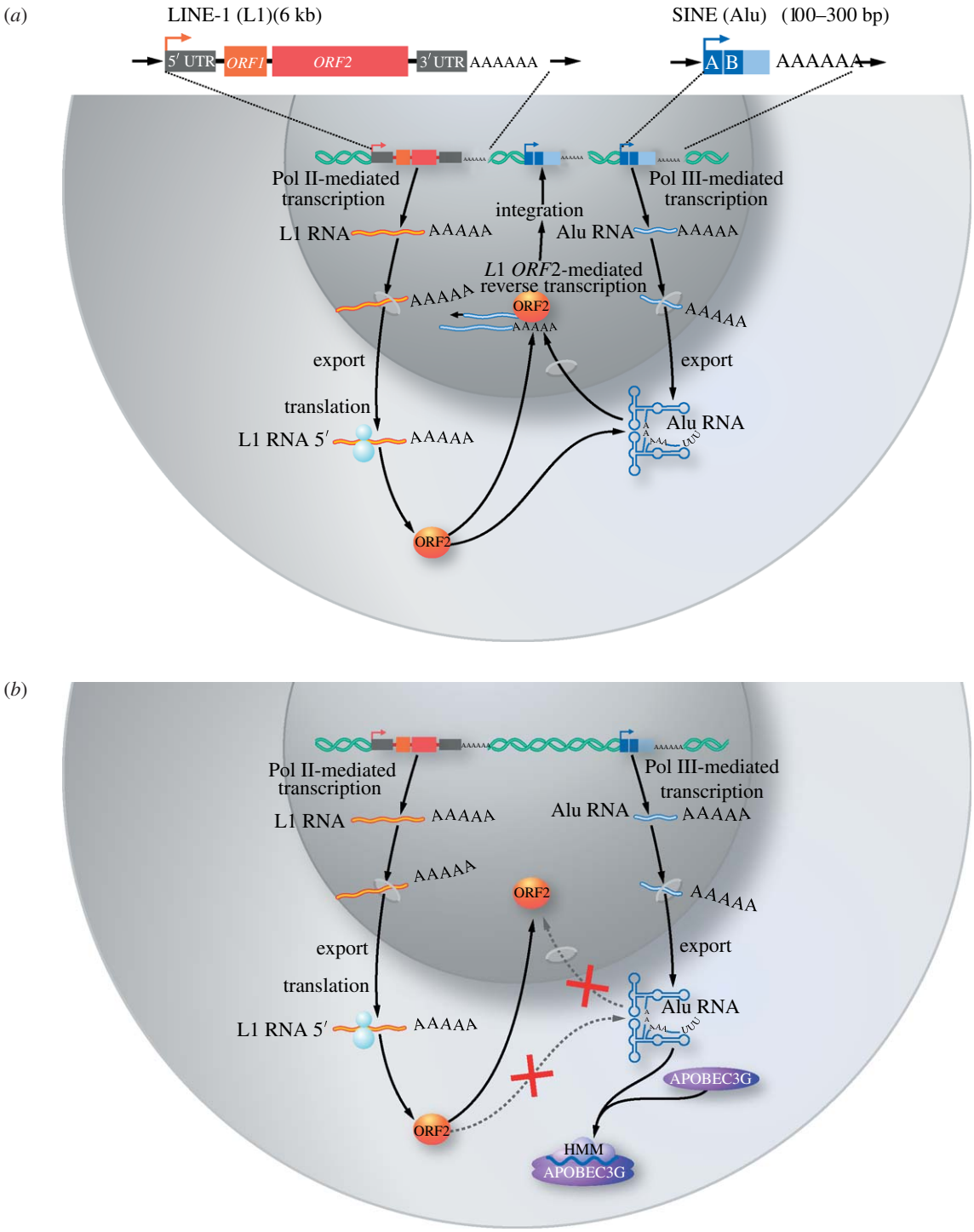

Figure 4. HMM A3G restricts Alu retrotransposition. (*a*) Alu retrotransposition mediated by long interspersed nuclear elements-1 (L1). Functional L1 elements are 6-kb-long autonomous retroelements that contain an internal RNA polymerase II (Pol II) promoter within their 5′-UTR, two open reading frames (ORF1 and ORF2), and a 3′-UTR. SINEs, including the most prominent and active member, Alu, are short RNA polymerase III (Pol III)-transcribed retroelements that contain an internal promoter but no protein coding capacity. Successful retrotransposition of Alu elements depends on their ability 'to steal' the reverse transcriptase/endonuclease enzymes encoded by L1 ORF2. (*b*) Non-enzymatic inhibitory mechanism for A3G to restrict Alu. Human A3G impairs the retrotransposition of Alu by sequestering Alu RNA transcripts in the cytoplasmic HMM complexes, especially Staufen-containing RNA granules, away from the nuclear L1 machinery, thereby interdicting the retrotransposition cycle.

binding activity of the N-terminal CD1 appears critically involved in this non-enzymatic form of inhibition, which probably involves binding to HIV-1 RNA in the virion core and physical impairment of reverse transcriptase activity (figure 1). Additionally, A3G in Δ*vif* HIV-1 virions reduces the ability of $tRNA^{Lys3}$ primers to initiate reverse transcription by 50 per cent or more, again providing a mechanism for a block at the level of reverse transcription (Guo *et al.* 2006). A3G might also cause defects in $tRNA^{Lys3}$ cleavage during plus-strand DNA transfer, leading to the formation of aberrant viral DNA ends that could interfere with subsequent chromosomal integration (Mbisa *et al.* 2007). Of note, the N-terminal linker region of A3G has been implicated as a docking site for the C-terminal domain of HIV-1 integrase. The association of A3G with components of the

preintegration complex, such as integrase, might negatively influence nuclear import of the complex, thereby further impairing viral DNA integration (Luo *et al.* 2007).

The role of non-enzymatic mechanisms of A3G antiviral function has been evaluated in other systems. For example, A3G also mediates deaminase-independent antiviral activity against human T-cell leukaemia virus type-1 (HTLV-1; Mahieux *et al.* 2005; Sasada *et al.* 2005) and hepatitis B virus (HBV; Rosler *et al.* 2004; Turelli *et al.* 2004*a*). Intriguingly, some retroviruses, such as HTLV-I, murine leukaemia virus (MLV) and Mason–Pfizer monkey virus, have evolved mechanisms to escape human A3G, mouse APOBEC3 (mA3) or rhesus A3G, respectively, by targeting the RNA-binding properties of these antiviral proteins (Kobayashi *et al.* 2004; Abudu *et al.* 2006; Derse *et al.* 2007; Doehle *et al.* 2005*b*, 2006).

13. DEAMINASE-DEPENDENT ANTIVIRAL ACTIVITY OF APOBEC3G

If RNA binding solely accounts for the antiviral activity of A3G, why would the deaminase function be conserved? Although we cannot exclude the existence of other physiological functions, we believe that the conserved deaminase property of A3G provides an even more potent 'second antiviral punch' (figure 1).

During reverse transcription, the C-terminal CD2 of virion-incorporated A3G catalyses extensive dC-to-dU deamination, preferentially at the 3′-dC of the 5′-CC dinucleotides (Harris *et al.* 2003; Mangeat *et al.* 2003; Zhang *et al.* 2003; Bishop *et al.* 2004; Liddament *et al.* 2004; Wiegand *et al.* 2004) in the newly synthesized minus-strand viral DNA (Yu *et al.* 2004*b*; Chelico *et al.* 2006; Suspene *et al.* 2006). Some of the uracils generated by A3G may be excised by uracil DNA glycosylase, leading to abasic sites that could cause DNA degradation in the presence of apurinic–apyrimidinic endonucleases (Schrofelbauer *et al.* 2005; Yang *et al.* 2007*a*). A few viral minus strands appear to survive this attack and serve as templates for plus-strand synthesis, where the dU promotes dA misincorporation. The resultant dG-to-dA mutations further negate HIV-1 replication by altering viral open reading frames and introducing inappropriate translation termination codons (Harris *et al.* 2003; Lecossier *et al.* 2003; Mangeat *et al.* 2003; Zhang *et al.* 2003; figure 3). Additionally, the accumulation of dUs in minus-strand DNA may lead to decreased plus-strand synthesis due to aberrant initiation (Klarmann *et al.* 2003; figure 1).

These enzyme-dependent effects of A3G produce antiviral responses against many retroviruses in addition to HIV-1. These include SIV and equine infectious anaemia virus, and even distantly related retroviruses, such as MLV and foamy viruses (Harris *et al.* 2003; Mangeat *et al.* 2003; Mariani *et al.* 2003; Lochelt *et al.* 2005; Russell *et al.* 2005; Delebecque *et al.* 2006). Similarly, G-to-A hypermutation, albeit at low levels, has also been detected in HBV (Rosler *et al.* 2004; Suspene *et al.* 2005; Bonvin *et al.* 2006) and HTLV-1 (Mahieux *et al.* 2005) genomes produced in cells expressing A3G.

These studies and those interrogating intravirion A3G imply sequential strategies for virion-incorporated A3G to restrict HIV-1 (figure 1). Initially, the enzymatically latent form of A3G bound to HIV-1 RNA may impair generation of minus-strand DNA by physically impeding reverse transcriptase on its viral RNA template. However, this physical block may be incomplete and minus-strand viral DNA may be generated. Subsequently, A3G deaminase activity is restored when RNase H degrades the viral RNA and leaves the single-stranded DNA template intact for plus-strand synthesis, allowing extensive deamination of the minus-strand DNA. The relative effectiveness of these two antiviral actions in different cellular environments could explain why HBV replication is inhibited by deaminase-dependent actions of A3G in HepG2 cells but by deaminase-independent action in Huh7 cells (Rosler *et al.* 2004; Turelli *et al.* 2004*a*; Suspene *et al.* 2005; Bonvin *et al.* 2006).

14. POST-ENTRY RESTRICTION OF EXOGENOUS VIRAL INFECTION BY LMM APOBEC3G

The finding of different forms of A3G has shed light on a long-standing mystery in HIV-1 biology: why resting CD4 T cells in lymphoid tissue are permissive to HIV-1 infection, while CD4 T cells circulating in the peripheral blood are not, even though A3G exists in both cell types. The answer is that A3G is expressed in two very different forms in these two populations of CD4 T cells. Circulating resting CD4 T cells have LMM A3G (Chiu *et al.* 2005) and are refractory to HIV-1 infection, at least in part, owing to an early post-entry restriction block at or immediately after the reverse transcription step (figure 3*a*). In sharp contrast, in lymphoid tissue-resident resting CD4 T cells, which display increased permissiveness to HIV-1 infection, A3G is predominantly found in HMM complexes (Kreisberg *et al.* 2006). RNA interference-mediated depletion of LMM A3G in resting CD4 T cells is sufficient to render these cells permissive for HIV-1 infection (Chiu *et al.* 2005; figure 3*b*). These results provide the first evidence that LMM A3G can function as a potent post-entry restriction factor that inhibits the replication of incoming HIV-1 virions in target cells independently of its prior incorporation into virions. However, this restricting activity is forfeited when A3G is recruited into the HMM A3G complex upon CD4 T-cell activation by various mitogens (anti-CD3/CD28 and phorbol myristate acetate) and cytokines (interleukins (IL)-2 and -15) (Chiu *et al.* 2005; Kreisberg *et al.* 2006; Stopak *et al.* 2007; figure 3*c*).

LMM A3G also governs the resistance of cells of monocyte lineage to infection with HIV-1 (Chiu *et al.* 2005; Pion *et al.* 2006; Ellery *et al.* 2007; Peng *et al.* 2007). Specifically, freshly isolated monocytes are refractory to infection and have LMM A3G (Chiu *et al.* 2005). Differentiation of these cells into macrophages promotes assembly of the HMM A3G complex (Chiu *et al.* 2005), a transition that correlates with a sharp increase in permissiveness to HIV-1 infection. HMM A3G complexes also are observed in the CD16+ subset of monocytes, which are more permissive to HIV infection than other monocytes

(Ellery *et al.* 2007). Maturation of dendritic cells is associated with a sharp increase in A3G expression and the additional expression of LMM A3G as well as less permissiveness to HIV infection (Pion *et al.* 2006; Stopak *et al.* 2007).

Interestingly, the post-entry antiviral action of cellular LMM A3G does not depend strictly on editing. Instead, it involves significant delays in the accumulation of HIV-1 late reverse transcription products (Chiu *et al.* 2005). As such, RNA binding by A3G is probably involved in the restriction of incoming HIV-1 by LMM A3G. However, G-to-A hypermutation has been noted in a minor subset of reverse transcripts from unstimulated peripheral blood mononuclear cells (Janini *et al.* 2001) and resting CD4 T cells (Chiu *et al.* 2005) infected with HIV-1. Although a recent study suggested that T cells contain an RNase-insensitive inhibitor of A3G deaminase activity (Thielen *et al.* 2007), LMM A3G possesses detectable enzymatic activity *in vivo*. Thus, A3G probably employs a dual strategy involving sequential non-enzymatic and enzymatic actions to achieve its potent post-entry antiviral effects.

15. HMM APOBEC3G OPPOSES ENDOGENOUS ALU RETROELEMENT RETROTRANSPOSITION

Identifying the RNA components of HMM A3G complexes (Chiu *et al.* 2006) has yielded important insights into their physiological functions and suggested that endogenous non-autonomous retroelements (i.e. Alu RNAs; Kazazian 2004) probably are the natural cellular targets of A3G.

Alu elements, the most abundant short interspersed nucleoside elements, are a highly successful group of retroelements in humans, accounting for approximately 10 per cent of the human genome. They are amplified through retrotransposition, an intracellular process that involves cytoplasmic RNA intermediates, reverse transcription in the nucleus, and integration of the newly formed retroelement DNA at novel chromosomal sites. Alu elements encode no protein but can retrotranspose by 'stealing' the reverse transcriptase and endonuclease enzymes encoded by a set of autonomous retroelements termed long interspersed nuclear elements-1 (L-1; Dewannieux *et al.* 2003; Kazazian 2004; figure 4*a*).

When tested in an *in vitro* assay measuring Alu retrotransposition in living cells (Dewannieux *et al.* 2003), A3G greatly inhibited L1-dependent retrotransposition of Alu elements (Chiu *et al.* 2006; Hulme *et al.* 2007). Notably, A3G does not affect the retrotransposition of L1 (Turelli *et al.* 2004*b*; Esnault *et al.* 2005; Bogerd *et al.* 2006; Chen *et al.* 2006; Muckenfuss *et al.* 2006; Stenglein & Harris 2006). Catalytically inactive mutants of A3G also effectively inhibit Alu retrotransposition, suggesting a deaminase-independent mechanism for A3G action (Chiu *et al.* 2006; Hulme *et al.* 2007). Since A3G is primarily cytoplasmic, and Alu RNA is recruited to Staufen RNA granules in an A3G-dependent manner, A3G probably interrupts retrotransposition by sequestering transcribed Alu RNAs in the cytoplasm, denying Alu RNAs access to the nuclear enzymatic machinery of L1 (Chiu *et al.* 2006; figure 4*b*). Unfortunately, the assembly of HMM A3G complexes to combat Alu retrotransposition opens the door for HIV-1 infection as the post-entry restricting activity of LMM A3G is forfeited.

The assembly of HMM A3G complexes appears to require entry into the $G1_b$ phase of the cell cycle (Chiu *et al.* 2005), a stage characterized by RNA synthesis. Activation of CD4 T cells with phytohaemagglutinin and IL-2 induces high-level expression of selected endogenous retroelement, including Alu and hY RNAs. Conversely, almost all of the protein cofactors that participate in the HMM A3G complexes are constitutively expressed in resting CD4 T cells but are not assembled into complexes (Chiu *et al.* 2006). These findings raise the distinct possibility that the induced expression of Alu RNAs is the driving force for HMM A3G complex assembly. These findings also suggest that A3G directly binds to Alu and hY retroelement RNAs.

In sharp contrast to human APOBEC3 family genes (*A3A–A3H*) on chromosome 22, only a single APOBEC3 gene (*mA3*) is present on the syntenic chromosome 15 in rodents, suggesting that the APOBEC3 locus expanded after the genetic radiation of mice and humans. Phylogenetic analysis of primate A3G proteins reveals a high rate of positive selection (non-synonymous mutations) that predates the emergence and diversification of primate lentiviruses (Sawyer *et al.* 2004; Zhang & Webb 2004), strengthening the notion that the APOBEC3 gene family expanded to curtail the genomic instability caused by endogenous retroelements. Consistent with this notion, the activity of retrotransposons is at least 100-fold higher in mouse cells than in human cells (Maksakova *et al.* 2006).

16. APOBEC3 DEAMINASES AS INHIBITORS OF ENDOGENOUS RETROELEMENTS

APOBEC3 proteins do, in fact, inhibit LTR retrotransposons. Human A3B, A3C, A3F, A3G and mA3 all effectively inhibit mouse IAP and MusD retroelements (Esnault *et al.* 2005; Bogerd *et al.* 2006; Chen *et al.* 2006), while A3F restricts a pseudo-ancestral human endogenous retrovirus (HERV-K) DNA sequence that was recently reconstructed based on the fossil record of ancient endogenous retroviruses (Lee & Bieniasz 2007). Reminiscent of the dual inhibitory effects of A3G in HIV-1 replication, APOBEC3 proteins exert dual restricting effects on these endogenous retroviruses, involving both a decrease in the number of transposed DNA copies and extensive editing of the transposed copies (Esnault *et al.* 2005, 2006). Interestingly, A3A inhibits IAP and MusD retrotransposition through a novel deamination-independent mechanism (Bogerd *et al.* 2006).

Human APOBEC3 proteins, including A3A, A3B, A3C and A3F, inhibit non-LTR retrotransposons, such as L1 retroelements (Bogerd *et al.* 2006; Chen *et al.* 2006; Muckenfuss *et al.* 2006; Stenglein & Harris 2006); A3A, A3B, and to a lesser extent, A3C also inhibit L1-mediated Alu retrotransposition (Bogerd *et al.* 2006). This finding could reflect the ability of these deaminases to enter the nucleus (Bogerd *et al.* 2006; Chen *et al.* 2006; Muckenfuss

et al. 2006), where L1-mediated reverse transcription occurs. Human A3B and A3F may also directly interact with the L1 enzymatic machinery through a highly homologous region in these two deaminases (Stenglein & Harris 2006). The relatively high-level expression of APOBEC3 proteins in human testis and ovary (A3G, A3F and A3C) (Jarmuz *et al.* 2002; OhAinle *et al.* 2006) and embryonic stem cells (A3B) (Bogerd *et al.* 2006), where extensive genome demethylation prevails and retroelements are thought to be transcribed (Kazazian 2004; Maksakova *et al.* 2006), points to a physiologically relevant role for these deaminases in the protection of these cells from the potentially deleterious effects of endogenous retrotransposition.

17. APOBEC3 DEAMINASES AS INNATE ANTIVIRAL RESTRICTION FACTORS

Considerable effort has been devoted to defining the function and spectrum of antiviral activities for each of the members of the extended human APOBEC3 family. Apart from A3G, several human APOBEC3 family members (e.g. A3F, A3B and A3DE) also inhibit HIV-1. A3F is coordinately expressed with A3G in primary cellular targets of HIV-1 (CD4 T cells and macrophages) (Bishop *et al.* 2004; Liddament *et al.* 2004; Wiegand *et al.* 2004). Similar to A3G, virion-incorporated A3F probably achieves its restrictive effect through a two-pronged sequential attack: initial impairment of viral reverse transcription followed by mutational inactivation of successfully formed reverse transcripts (Bishop *et al.* 2006; Holmes *et al.* 2007; Yang *et al.* 2007*b*). LMM forms of A3F also probably mediate post-entry restriction of HIV-1. The dG-to-dA hypermutations matching the recognized sequence preferences for both A3G (CC) and A3F (TC) are detectable in a minor subset of slowly formed reverse transcripts isolated from resting CD4 T cells infected with HIV-1 (Chiu *et al.* 2005). Furthermore, siRNA-mediated downregulation of A3F expression in dendritic cells increases the permissiveness of these cells to HIV-1 infection (Pion *et al.* 2006). Human A3B also has moderate activity against HIV-1 when packaged into virions and is quite resistant to Vif-induced degradation (Bishop *et al.* 2004; Doehle *et al.* 2005*a*). Human A3DE is the most recent APOBEC3 protein found to suppress HIV-1 infection. It deaminates HIV-1 minus-strand DNA preferentially at an AC dinucleotide motif that is distinct from that of other APOBEC3 members, but signatures of this type of mutation are evident in clinical HIV-1 isolates (Dang *et al.* 2006).

HIV is not the sole viral target of the APOBEC3 gene products. Indeed, human A3G actively suppresses the spread of HIV-1, SIV and MLV; A3F, A3B and A3DE inhibit the replication of HIV-1 and SIV, but not MLV (Bishop *et al.* 2004; Doehle *et al.* 2005*a*; Dang *et al.* 2006). Human A3C blocks SIV and HIV-1, although the anti-HIV effects are quite modest (Yu *et al.* 2004*a*). Within the nucleus, A3A blocks the replication of adeno-associated virus, which replicates as a single-stranded DNA in the nuclei of infected cells (Chen *et al.* 2006). In close parallel to the post-entry restricting function of A3G and A3F against HIV-1, the deaminase-independent actions of A3G, A3F, A3B and A3C sharply interfere with the HBV replication in cotransfected hepatoma cell lines. Mouse protein mA3 also appears to restrict mouse mammary tumour virus, as highlighted by the increased vulnerability of mA3 knockout mice to MMTV infection (Okeoma *et al.* 2007). This fascinating spectrum of antiviral activities for the APOBEC3 family of enzymes is certainly consistent with the impressive expansion and rapid evolution of the primate A3 proteins.

18. CONCLUSIONS

Studies of A3G biology highlight the multifunctional properties of A3G as an antiviral factor. Using its inherent deaminase activity or deaminase-independent RNA-binding anti-viral mechanisms, A3G acts at distinct steps along the retroviral life cycle. The ability of LMM A3G to counteract HIV-1 at the post-entry level and to inhibit Alu retrotransposition in its HMM form further highlights the economical use of this enzyme in the host to minimize the threat posed by both exogenous retroviruses and endogenous retroviral elements. The disproportionate activities, expression profiles, and even the intracellular localizations of the various APOBEC3 members (please refer to Chiu & Greene (2008) for a comprehensive review) further support the notion that members of this gene family may have evolved to function as important guardians of the integrity of the human genome that counter the adverse effects of both exogenous retroviruses and endogenous retroelements.

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