Opinion

APOBEC3G: a double agent in defense

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APOBEC3G (A3G) is an effective cellular host defense factor under experimental conditions in which a functional form of the HIV-encoded protein Vif cannot be expressed. Wild-type Vif targets A3G for proteasomal degradation and when this happens, any host defense advantage A3G might provide is severely diminished or lost. Recent evidence cast doubt on the potency of A3G in host defense and suggested that it could, under some circumstances, promote the emergence of more virulent HIV strains. In this article, I suggest that it is time to recognize that A3G has the potential to act as a double agent. Future research should focus on understanding how cellular and viral regulatory mechanisms enable the antiviral function of A3G, and on the development of novel research reagents to explore these pathways.

The multifaceted characteristics of the double agent

APOBEC3G (A3G) is a member of a family of cytidine deaminases named after apolipoprotein B editing catalytic subunit 1 (APOBEC1) [1,2], which was the first enzyme discovered with the capacity for site-specific cytidine to uridine deamination (editing) of apolipoprotein B mRNA. Almost 9 years ago, Sheehy *et al*. published a paper showing that the reason why the HIV protein known as viral infectivity factor (Vif) was required for the virus to infect nonpermissive cells was that these cells expressed A3G. Vif enabled the virus to penetrate host defenses by inducing the destruction of A3G [3]. At that time, the story was simple and exciting: Vif induced the degradation of A3G [4,5], thus preventing A3G from being incorporated into nascent viral particles and thereby neutralizing the ability of A3G to hypermutate the single-stranded DNA (ssDNA) of HIV during reverse transcription after viral entry [6–8] (Figure 1, Box 1).

In the absence of functional Vif, A3G catalyzes dC to dU mutations, primarily in the minus strand reverse transcript, and this templates dG to dA transitions in the protein coding plus strand during viral replication (Box 1). Some mutated virions can then integrate into the host cell chromosome, leading to the expression of viral proteins with missense substitutions or nonsense codons [9,10] (Figure 1). These findings are consistent with sequence analysis of HIV isolated from infected patients who had numerous dG to dA polymorphisms; in some instances, inactivating substitutions in the Vif sequence were identified [9]. The flanking nucleotide sequences of these single nucleotide polymorphisms were the same as those preferred at editing sites for A3G and its homolog A3F [9]. In a recent study, HIV genomes recovered from infected humanized mice expressing human A3G also contained dG to dA mutations with an A3G flanking sequence preference [11]. In most experimental systems, the absence of Vif enables sufficient dC to dU mutations to produce a reduction in proviral DNA, caused by the creation of abasic sites by uracil-DNA glycosylase, which is followed by DNA degradation [12,13]. In summary, A3G-mediated mutations, which occur in addition to the mutations stemming from low-fidelity reverse transcription and recombination of viral genomes, were initially proposed to be solely detrimental to the virus, because of their location in the HIV genome or their abundance.

Glossary

APOBEC: A family of proteins containing a zinc-dependent deaminase motif, named after the first enzyme in the family discovered, apolipoprotein B editing catalytic subunit 1. The family consists of activation-induced deaminase (AID), A1, A2, A3A-A3H and A4. Although A3G and A3F have a high level of identity and similar structural organization, they have different nearest neighbor preferences in single-stranded DNA for deamination and are different in their interaction with Vif, with A3G being more susceptible to Vif-dependent degradation. A3G protein is natively expressed at higher levels than A3F.
HMM and LMM: High molecular mass and low molecular mass complexes (respectively) are operationally defined by biochemical sizing methods of A3G in cell extracts. They range in size from MDa to several hundred kDa for HMM, and a few hundred kDa to single subunits of A3G (46 kDa) for LMM. A3G HMM is heterogeneous in protein composition because A3G binds nonselectively to cellular RNAs, which in turn are associated with variety of cellular proteins. A3G LMM is considered to have few or no RNAs associated with A3G subunits.
Permissive and nonpermissive cells: An operational term used to describe the ability of a cell to undergo a productive infection by a particular retrovirus (permissive) or not (nonpermissive).
Proviral DNA: The double-stranded DNA copy of the retroviral RNA genome.
SOCS (suppressor of cytokine signaling) box protein: A member of a family of proteins containing the SOCS amino acid motif. SOCS box-containing proteins are known to mediate an interaction between protein substrates targeted for degradation and the respective components of the ubiquitylation machinery. They do so by binding to protein substrates, elongin B/C proteins, cullin 5 and RBX2, which together interact with an E3 ubiquitin-protein ligase complex. There is a large diversity of SOCS box-containing proteins that serve as receptors for docking different substrates into the ubiquitylation machinery. Vif mimics these receptors when it binds A3G, thereby inducing its destruction.
Vif (viral infectivity factor): a 21 kDa HIV-encoded protein containing multiple domains for diverse protein–protein and protein–RNA interactions.
Viral capsid: The retroviral RNA genome encased in an oligomeric viral protein shell minus the components that make up the viral envelope of the mature viral particle or virion.
Viral replication: An inclusive term referring to the entire viral life cycle, which is not limited to reverse transcription.
ZDD (zinc dependent-deaminase): A sequence that is part of the cytidine deaminase protein fold consisting of five anti-parallel β-sheets supported by two α-helices that position three cysteine/histidine residues for the coordination of a zinc atom, a water molecule and a glutamic acid residue for proton shuttling in a hydrolytic deamination reaction, leading to the conversion of cytidine/deoxycytidine to uridine/deoxyuridine with NH_3 as the leaving group.

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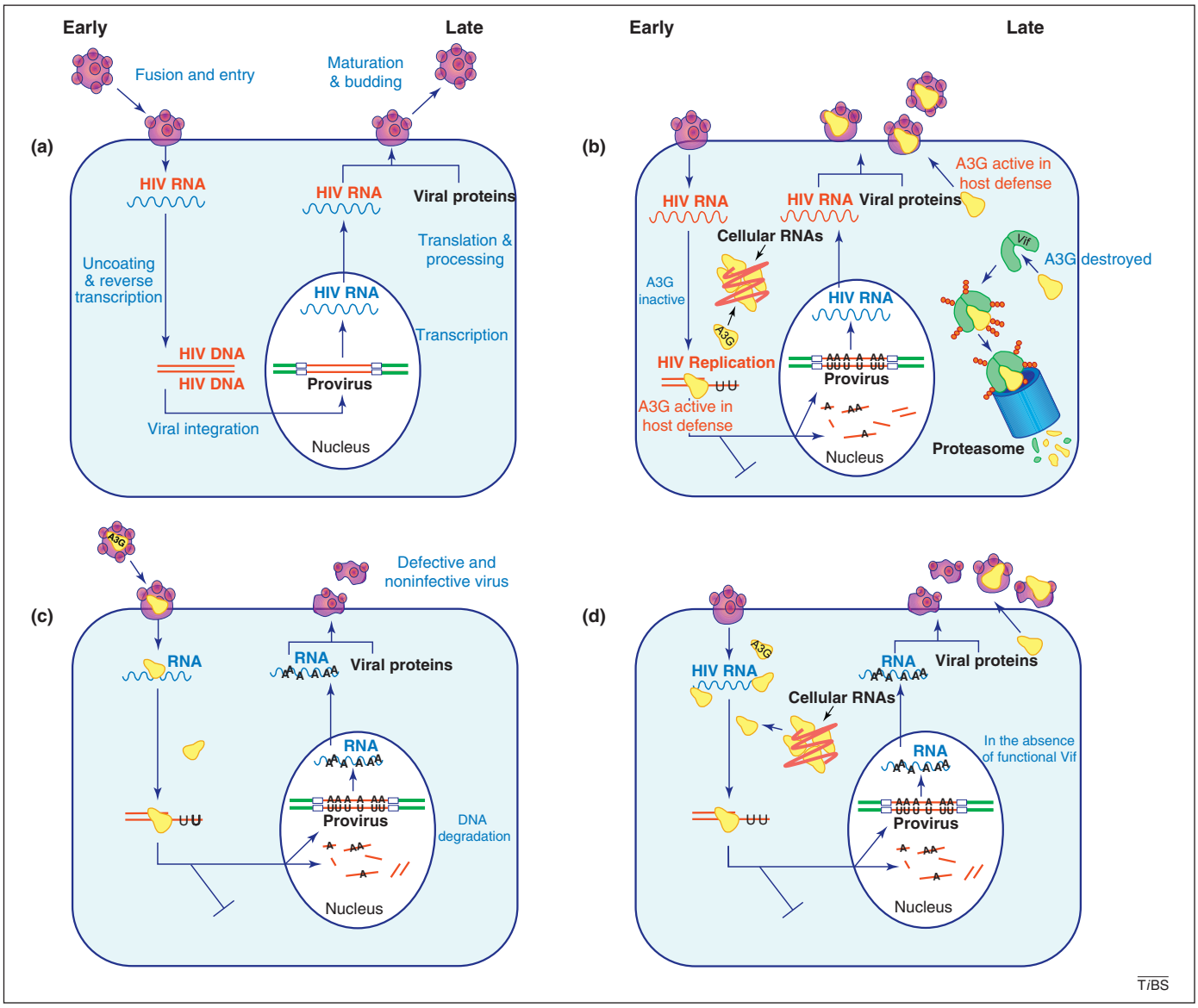

Figure 1. APOBEC3G during the early and late stages of HIV replication. **(a)** Viral functions crucial to A3G activity during the early and late stages of viral replication. The early stage events are as follows. **(i)** Entry: virions fuse with the surface of cells through receptor-mediated endocytosis, and following uncoating of the virion, two viral RNA genomes are reverse transcribed. **(ii)** Reverse transcription: the viral RNA genomes are reverse transcribed to proviral dsDNAs. **(iii)** Integration: the proviral DNAs interact with the cellular nuclear import machinery, and viral integrase mediates insertion of the HIV proviral genomes into the host cell chromosomes. The late stage events are as follows. **(iv)** Transcription: viral promoters drive transcription of the viral genome, which is then exported to the cytoplasm as viral RNA genomes and as unprocessed and processed mRNA templates for viral protein translation. **(v)** Protein expression and processing. **(vi)** Viral particle maturation: viral proteins assemble with viral genomes, and proteins undergo further processing in the formation and maturation of nascent viral particles. **(vii)** Budding, nascent virions are released from the cell by fusing with the host cell plasma membrane. **(b)** Active and inactive complexes of A3G mediated through deaminase-dependent and deaminase-independent interactions. During the early stage of viral replication, T cells become activated, and A3G binds to cellular RNAs and forms multimeric complexes (Box 2) that are considered to have low or no ability to react with viral replication complexes or to become packaged with virions during late infection. By contrast, LMM complexes of A3G are active in host defense once they have been assembled into virions. During the late stage of viral replication, integrated proviral DNA is transcribed and Vif is expressed. Vif forms homomeric and heteromeric protein complexes with A3G, elongin B/C, cullin 5, RBX2 and E3 ubiquitin ligase (Box 1). These complexes are required for polyubiquitination and destruction of A3G in the 26S proteasome. Both HMM and LMM A3G are targeted by Vif. Alternatively, if LMM A3G escapes Vif-dependent degradation, it can be incorporated into virions. **(c)** Current hypothesis: A3G must enter cells with the virion before it can exert its antiviral activity. A3G entering cells with virions and bound to the HIV RNA genome will exert deaminase-dependent and deaminase-independent antiviral activities during reverse transcription (Box 1). The outcome of the A3G antiviral activity is that viral genomes: **(i)** are not reverse transcribed effectively, **(ii)** are not integrated, or **(iii)** that sufficient dC to dU mutations arise to induce degradation of the proviral DNA or to code for nonfunctional viral proteins and defective or noninfectious virions. Vif-dependent degradation of A3G reduces or eliminates these antiviral mechanisms. **(d)** Opinion: activating cellular A3g host defense might protect cells from an infection. A3G pre-existing in cells might be able to pre-emptively inhibit the replication of incoming virus through both deaminase-dependent and deaminase-independent mechanisms if these activities become sufficiently robust to exceed a mutagenic threshold at which HIV becomes noninfective. However, the current hypothesis predicts that A3G in cells is not capable of accessing viral replication complexes unless it has been incorporated within virions by binding to viral RNA (Box 2). If A3G could be liberated from RNA and HMM complexes by activating cellular regulatory pathways or by therapeutic intervention, A3G might be able to pre-emptively access virions during the uncoating and reverse transcription processes, and thus inhibit viral replication.

Soon after the discovery of A3G deaminase-dependent antiviral activity, experiments evaluating the functional requirement of residues in the catalytic domain of A3G through site-directed mutagenesis and deletion analyses revealed that A3G has deaminase-independent antiviral activities [14,15]. Interactions of A3G with viral and host cell proteins and RNAs were shown to inhibit HIV reverse transcription and promote A3G assembly within viral

Box 1. Deaminase-dependent and deaminase-independent antiviral mechanisms

In the deaminase dependent mechanism, A3G catalyzes zinc-dependent hydrolytic deamination of deoxycytidine to form deoxyuridine in HIV DNA [6,7]. These mutations arise primarily on the HIV minus strand as a result of A3G having a requirement for a ssDNA substrate [8,43]. The frequency and distribution of mutations determined by the 3′ to 5′ processivity of A3G activity on ssDNA [44] and limited temporally by the transient availability of ssDNA arising from RNase H-mediated removal of the RNA genome template following reverse transcription [34] and before the formation of double-stranded DNA (dsDNA) by second strand synthesis. A3G deaminase activity is thought to be the consequence of protein–protein and protein–ssDNA interactions that drive assembly of larger A3G–ssDNA aggregates presumed to be essential for deaminase activity [8,43,45].

Vif is a mimic of cellular SOCS box proteins that function as receptors for protein substrates targeted for ubiquitylation through the elongin B/C–Cullin5–ring box protein 1(RBX2)–E3 ligase complex and degradation via the 26S proteasome [46,47]. Vif–A3G binding requires residues within the N terminus of A3G [29,48–50] and residues in the N terminus [31–33,51–53] and C terminus [4,54,55] of Vif.

In the deaminase-independent mechanism, A3G is predicted to contain an N-terminal and a C-terminal zinc-dependent deaminase or ZDD fold [1]. Efforts to delineate the contribution of each ZDD to antiviral activity showed that deaminase activity resides exclusively within the C-terminal ZDD [15,56–58]. These studies were controversial because they also suggested that deaminase activity might not be required for antiviral activity in experimental systems [14,58,59]. A3G has an intrinsic ability to bind RNA and ssDNA nonspecifically [43,57,60,61]. This characteristic is undoubtedly essential to the deaminase-independent antiviral activities that have been described in more recent literature, by which A3G binds nucleic acids to inhibit $tRNA_{lys3}$ priming of first strand synthesis [62], strand transfer activity [63], reverse transcript elongation [64] and inhibition of proviral dsDNA integration [65,66].

There is agreement that protein–protein and protein–RNA interactions with the N-terminal half of A3G are required for encapsidation. However, there is disagreement over whether the interactions of A3G with Gag [48,67,68] and/or viral RNA and/or cellular RNAs [60,61,69–72] are sufficient to place A3G, along with the viral genome, inside the virion core in such a way that it will be ideally positioned to inhibit reverse transcription after viral entry.

particles (Box 1, Figure 1). Proponents of the deaminase-dependent mechanism suggested that these deaminase-independent interactions were only apparent because of the supraphysiological levels of A3G expressed in transfected cell systems [16–18]. It is, however, an inescapable fact that A3G subunits have an intrinsic ability to bind proteins, ssDNA and RNA and therefore, deaminase-independent interactions could occur at all levels of expression. It is also important not to overlook the fact that deaminase-independent interactions determine A3G assembly with virions, an essential factor according to the deaminase-dependent hypothesis for A3G antiviral activity. What remains unclear is whether the deaminase-independent interactions of A3G that have been proposed to interfere with reverse transcription (Box 1) are sufficient to inhibit viral replication within the biological range of A3G expression.

Pursuing the possibility that A3G is a host defense factor, high-throughput screening of chemical libraries identified small molecules with antiviral activity that were selected based on their ability to inhibit Vif-dependent A3G degradation [19,20]. Similarly, a cell-transducing peptide mimic of the Vif dimerization domain was optimized [21] from phage display screening of peptides that disrupted Vif multimers and thereby inhibited viral replication in nonpermissive cells. Both the small molecules and peptides had the anticipated outcome of producing virions with a higher content of A3G and lower infectivity [19–21].

In my opinion, A3G has the potential of being a double agent enzyme, serving as an antiviral factor, or as a facilitator of viral genome diversification that can lead to the emergence of drug resistance. Sequence analysis data suggest that varying levels of A3G deaminase-dependent (and therefore deaminase-independent) activity can occur during an infection with wild-type virus expressing functional Vif. Although this theoretically could serve a host defense role, it is uncertain whether the deaminase-dependent mutagenic activity of A3G is sufficient (e.g. in activated or resting T cells) to exceed a mutagenic threshold for inactivating the HIV genome if the deaminase-independent activity is not sufficient to block viral replication. It should be of concern that the discovery of the antiviral mechanisms has relied on overexpressing A3G or A3G mutants, or on experimentally ablating A3G or Vif. Logically, there is a difference between these engineered systems expressing mRNA and protein from cDNAs, and a native setting in which A3G and Vif genes are both expressed and regulated during an inflammatory response. An alternative might be the development of target-specific molecular probes that enable evaluation of the function of A3G and Vif in native cells following infection with wild-type virus. Although off-target and toxic artifacts are possible within a drug-treatment experimental design, these research reagents arguably hold greater potential than our current approaches to reveal physiological mechanisms that are relevant in patients with HIV/AIDS.

The interaction of A3G with RNA might confound the antiviral mission

A3 proteins are not essential for cell survival [22], but they could have important functions related to their ability to bind RNA, including the regulation of microRNA functions [23] and suppression of endogenous retroviral elements [24–27]. In fact, A3G/A3F-mediated mutation of viral genomes might have contributed to the rapid evolution of primate retroviral and endogenous retroviral-like elements [28]. Species-specific and APOBEC3 homolog-specific [29] sequence preferences might have contributed to the present day primate species tropism of HIV/simian immunodeficiency virus [30–33].

A question that has become increasingly perplexing is why does pre-existing cellular A3G fail to deliver a preemptive antiviral strike on incoming viruses? Why is it necessary to slip a few A3G subunits into the viral particle like the proverbial Greeks in the Trojan Horse? The answer to the question might be that cellular A3G cannot gain access the nucleoprotein core of the virus where reverse transcription takes place unless it is encapsidated or the nascent ssDNA is itself shielded from cellular A3G [34]. In addition, cells might regulate A3G for its interaction with cellular RNAs, and this might limit the availability of A3G and/or its access to nascent single-stranded proviral DNA.

Box 2. Ribonucleoprotein complexes containing A3G

Purified A3G forms homomultimers in a concentration-dependent manner through protein–protein interactions [73,74]. Binding of either RNA or ssDNA to A3G will promote higher order oligomerization of A3G subunits, as evidenced under defined *in vitro* conditions by native gel shift analyses [43] and atomic force microscopy [45]. RNP complexes containing A3G referred to as HMM complexes colocalize with P-bodies and stress granules [44,45,75–80], where cells degrade or recycle RNAs and RNP proteins. Glycerol gradient sedimentation or size exclusion chromatography of cell extracts have demonstrated that A3G associates with these MDa-sized RNPs within minutes of its translation [17,18].

Whereas HMM A3G predominates in cells that are permissive to HIV infection, cell types considered to be refractory to HIV infection (e.g. resting CD4+ T cells, monocytes and mature dendritic cells [75,77,80]) maintain A3G as a component of LMM complexes. Through mechanisms that are not understood, cytokines, dsRNAs and other growth factors regulate the expression of A3G and interconversion of LMM and HMM complexes. The significance of the interconversion of the aggregation states of A3G to its antiviral activity is currently an underdeveloped issue that needs further investigation [37,38].

All or most of the conversion of LMM to HMM complexes is attributable to RNA-bridged A3G oligomerization, and to date no protein components of P-bodies or stress granules have been shown to bind directly to A3G. As part of HMM complexes, A3G has low or no deaminase activity, but RNase digestion of isolated HMM complexes restores deaminase activity [74,75].

At the heart of this conundrum is the seemingly contradictory concept that the intrinsic ability of A3G to bind RNA is essential for the deaminase-independent antiviral mechanism by which A3G enters the viral particle (Box 1), and yet binding to viral RNA in the capsid or binding to cellular RNAs inhibits A3G deaminase-dependent mutagenesis of ssDNA during reverse transcription (Box 2). Different cells can regulate the interaction of A3G with cellular RNAs in contrasting ways to generate low molecular mass (LMM) forms of A3G (relatively free of RNA) and high molecular mass (HMM) aggregates of A3G bound to RNA in ribonucleoprotein (RNP) complexes (Box 2, Figure 1). The larger aggregates colocalize with cytoplasmic P-bodies and stress granules that function in RNA and RNP protein degradation, and/or serve as temporary storage depots. In HMM aggregates, A3G is believed to have little or no antiviral activity, yet Vif mediates A3G degradation in both the LMM and HMM forms [18].

The higher level of deaminase activity in LMM A3G and its prevalence in nonpermissive cells led to the hypothesis that LMM A3G is necessary and sufficient for cells to be refractory to HIV infection; however, this has since been retracted [35,36]. Recent experiments testing this hypothesis showed that an aggressive knockdown of LMM A3G in resting CD4+ T cells by RNA interference or Vif-mediated degradation of A3G did not render these cells permissive to HIV infection [37,38]. The implication of the new data is that LMM A3G pre-existing in cells is not sufficiently available or active to inhibit HIV replication.

The new data raise an important question: if A3G in nonpermissive cells is not sufficient to inhibit viral replication, does it have no activity at all, or does it have a low level of mutagenic activity? This is an important question because earlier studies (reviewed in [39]) warned that, rather than providing antiviral host defense, A3G/A3F activities might diversify the viral genome, a tenet predicted by evolutionary biologists studying retroviruses and retroviral elements [28]. Recent papers have confirmed this possibility under experimental conditions, where A3G/A3F promoted mutations that benefited HIV [40,41] and induced a drug-resistant phenotype [42]. The available data support the possibility that the activity of LMM A3G in infected nonpermissive cells is sufficient to promote mutations in the HIV genome. Moreover, the new data suggest that the mutational activity might not be sufficient to inhibit viral replication either during the early or late stages of the viral life cycle (Figure 1).

Embracing the uncertainty with a view toward proactive intervention

The important question before us is: what level of A3G mutagenic activity might benefit the virus compared with what level is necessary to inhibit viral replication? The simplest answer might be that a little DNA deaminase activity benefits the virus and a high level of activity destroys the virus. Unfortunately, we do not know what these levels are, and the location of mutations within the viral genome can be more important than the number of mutations. To explore this possibility, future research should determine the naturally occurring mechanisms regulating the interconversion of HMM and LMM, and how this might determine A3G mutagenic activity. Based on the results, we should gain an understanding of the paradoxical situation by which A3G host defense mechanisms are sequestered in RNPs and inactivated in permissive cells during HIV infection. The results will have a significant effect on translational research and therapeutic development using A3G/Vif as a target.

From one point of view, the available data suggest that if naturally expressed A3G activity is too low to provide host defense, inhibiting Vif alone might have limited immediate therapeutic value, and in fact could have the unintended long-term consequence of aiding the virus. Conceptually, one therapeutic strategy might be to inhibit A3G deaminase activity and allow Vif to destroy A3G. This might potentially reduce the emergence of viral resistance in patients who are also receiving other antiretroviral therapies. The significant challenge here is that deaminase inhibitors that are selective for A3G/A3F would have to be developed, so that they will not be cytotoxic to intermediary metabolism. Skeptics will suggest that inhibiting a host defense factor because it might induce beneficial mutations in the virus ignores the greater possibility that A3G mutations might overall be deleterious to the virus.

An alternative approach is to activate the mutagenic efficiency of HMM A3G, and enhance its access to viral replication complexes. Could such activators enable cells to 'fight back' even though Vif is destroying A3G? In this case, combining A3G activators with Vif inhibitors might address the need both to reduce viral infection and to lessen the frequency with which drug resistant strains emerge. Similar to deaminase inhibitors, deaminase activators will need to be A3G/A3F-specific.

It is vital that efforts continue to be made to explore the cellular and viral factors that regulate the double agent

A3G. Undoubtedly, the development of novel probes and research reagents will be crucial to our understanding of mechanisms that determine what role A3G plays in infected cells. Despite progress in these areas, the therapeutic value of A3G inhibitors or activators, with or without Vif antagonists, will not be fully appreciated until such compounds are tested in clinical trials.

Conflict of interest statement

H.C. Smith is a full time faculty member in the Department of Biochemistry and Biophysics and the Center for RNA Biology at the University of Rochester, School of Medicine and Dentistry, Rochester, NY. He is also founder of OyaGen Inc. and a consultant for the company as its chief scientific officer. OyaGen Inc., is a therapeutic development company seeking novel therapeutics using APOBEC editing mechanisms as targets (www.oyageninc.com)

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