Cytidine Deaminases as a Weapon Against Retroviruses and a New Target for Antiviral Therapy

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Abstract: APOBEC3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G) was identified as an anti-HIV-1 (human immunodeficiency virus type 1) cellular factor in target CD4 T cells. It is a member of the APOBEC family of cytidine deaminases consisting of APOBEC1, APOBEC2, APOBEC3 (A to H), and AID (activation induced deaminase). During reverse transcription, it deaminates dC to dU in nascent minus-strand viral DNA, resulting in G-to-A hypermutation in the plus strand DNA to inhibit the replication of HIV-1. On the contrary, HIV-1 Vif protein counteracts this enzyme by the ubiquitin-proteasome pathway to enable HIV-1 replicate in target cells. Vif forms an E3 ligase complex with cellular proteins including Cullin5, ElonginB, and ElonginC (Vif-BC-Cul5) and functions as a substrate recognition subunit of the complex to target APOBEC3G for ubiquitin-proteasome dependent degradation in virus-producing cells. APOBEC3G has also been shown to have a broad antiviral activity on a wide variety of viruses which include not only retroviruses such as other lentiviruses, murine leukemia virus (MLV), and human T-cell leukemia virus type 1 (HTLV-1) but also other viruses such as hepatitis B virus (HBV) and adeno-associated virus. Furthermore, other members of the APOBEC family also show a broad antiviral activity, but target virus specificities vary among APOBEC members. On the other hand, viruses have their own mechanisms to escape from APOBEC. These expanding evidences suggest that the APOBEC family of cytidine deaminases plays an important role in antiviral innate immunity and might be a novel target for an antiviral therapy. Here we review the present understanding of APOBEC3 proteins as an antiviral innate immunity and battles between APOBEC3 and viruses.

Key Words: APOBEC3G, HIV-1 Vif, retrotransposon, murine leukemia virus, innate immunity.

1. INTRODUCTION

Human immuneodeficiency virus type 1 (HIV-1) Vif protein plays an essential role in the viral life cycle [1]. A curious feature of Vif is that it must be expressed in cells producing HIV-1, but its pro-infectivity effects are not manifested until the next target cell is infected by the virus. This mystery was resolved by the identification of APOBEC3G as an anti-HIV-1 host factor [2]. APOBEC3G is incorporated into budding virions lacking Vif [3]. After infection of target cells, during reverse transcription, it deaminates dC to dU in nascent minus-strand viral DNA, resulting in G-to-A hypermutation in the plus strand DNA to inhibit viral replication (Fig. **1a**) [4-7]. Vif antagonizes APOBEC3G *via* the ubiquitin-proteasome pathway in virus-producing cells (Fig. **1b**) [8-10]. Other members of the APOBEC family also have antiviral activity against HIV-1, although sensitivity to Vif varies among the molecules (Table **1**) [3]. Antiviral function of the APOBEC family extended not only to retroviruses, including human T-cell leukemia virus type 1 (HTLV-1) [11, 12], murine leukemia virus (MLV) [5, 13], and foamy viruses [14, 15], but also to hepatitiss B virus (HBV) [16, 17], adeno-associated virus [18], and retrotransposons [19-21]. On the other hand, viruses have their own strategy to escape from APOBEC proteins [14, 15, 22, 23]. In this review, the recent progress in research on the APOBEC family proteins is discussed.

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2. APOBEC FAMILY PROTEINS

The APOBEC superfamily consists of APOBEC1 [24], APOBEC2 [25], APOBEC3 (A to H) [26], and AID [27]. APOBEC proteins contain either one or two cytidine deaminase motifs with the consensus sequence His-X-Glu-X_{23-28}-Pro-Cys-X_{2-4}-Cys (where X stands for any amino acid) (Fig. **2a**). APOBEC proteins are distributed only within the vertebrate lineage. The most ancient family members are AID and APOBEC2, of which the former is slightly more conserved than the latter between the fish and the human proteins (70-74% versus 68-70% similarity) [28]. APOBEC1, a prototypic enzyme of the APOBEC superfamily, edits the messenger RNA of apolipoprotein B as an RNA-editing enzyme [24, 29, 30]. AID, the second member functionally identified, plays an essential role in class-switch recombination and somatic hypermutation for antibody diversification in B cell development [31]. APOBEC2 is expressed in cardiac and smooth muscles, but its precise function remains unclear (Table **1**) [25, 32]. Recent studies on mouse APOBEC2 have not provided any evidence of a deaminating activity on cytidine, whether as a free nucleotide or in DNA [33], and gene targeting experiments have revealed that APOBEC2 (as well as APOBEC3) is not essential for normal mouse development, survival, and fertility [33]. The genes encoding APOBEC3 are clustered on human chromosome 22 [26]. The physiological roles of APOBEC3 proteins are still unknown, although it is clearly shown that they function as an innate immunity defense against retroviral infections. Down the vertebrate evolutionary tree a dramatic reduction in the number of APOBEC3 family members can be seen. Rodents such as

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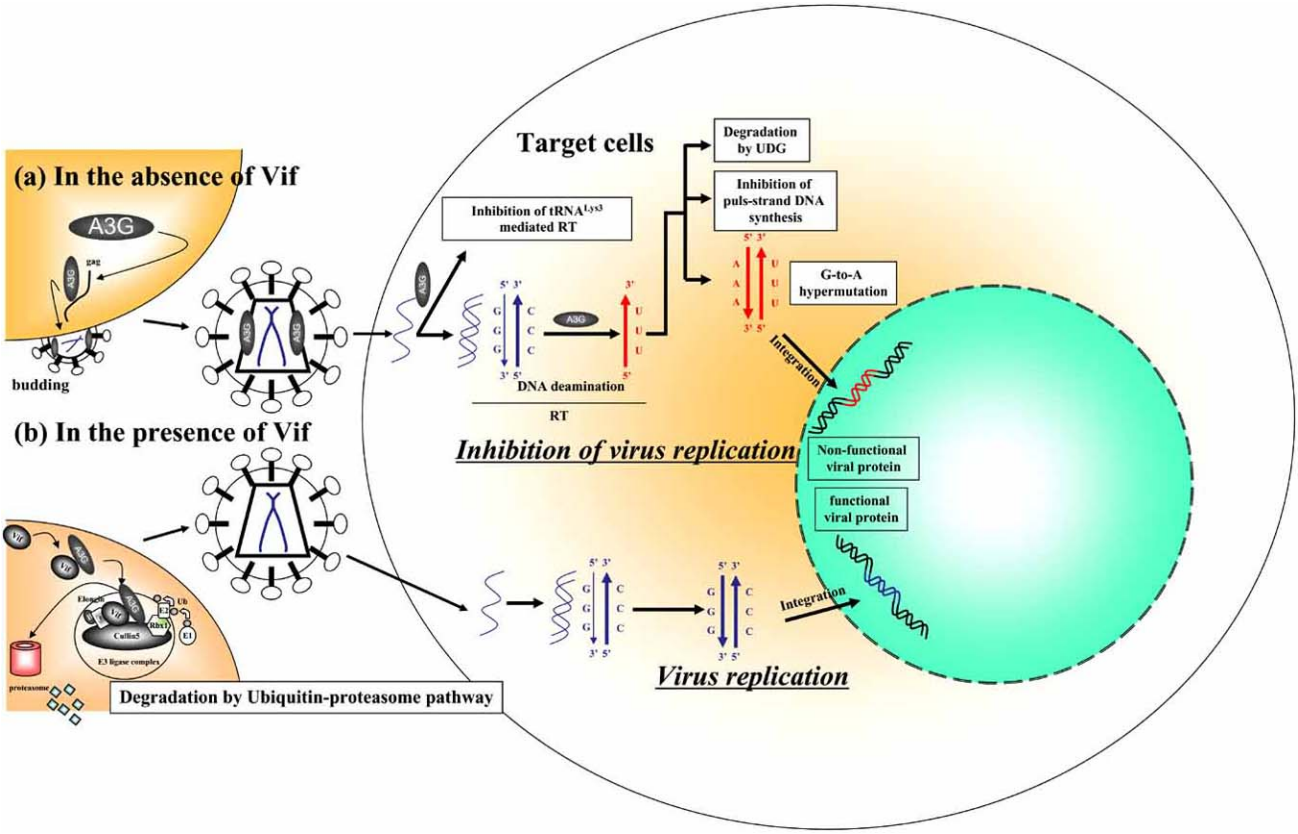

Fig. (1). APOBEC3G and Vif are key proteins in retroviral infection.

This figure presents a model how the APOBEC and Vif can influence the viral infectivity. (a) In the absence of Vif, APOBEC3G protein can bind NC region of Gag and be incorporated into budding virions. In target cells, APOBEC3G inhibits the synthesis of viral DNA mediated by $tRNA^{Lys3}$ as a primer and mediates the extensive deamination of cytidine residues in the minus-single-stranded viral DNA during reverse transcription, accordingly inhibiting viral replication. (b) In the presence of Vif, APOBEC3G is neutralized by Vif. Vif defeats the antiviral activity of APOBEC3G principally by recruiting the E3 ligase complex (Cullin5, Elongin B, Elongin C, and Rbx1) and functioning as a substrate recognition subunit to induce polyubiquitylation of this protein, leading to their accelerated degradation by the 26S proteasome.

bers can be seen. Rodents such as mice and rats, besides expressing AID, APOBEC1, and APOBEC2, encode only one APOBEC3, in contrast to humans and chimpanzees, who have at least eight APOBEC3 genes (A to H). The selective pressures that drove APOBEC3 gene expansion suggest that the APOBEC3 family may have developed to prevent genetic instability and then switched their activity against exogenous invading genetic elements [34]. Indeed, the expansion of APOBEC3 genes in primates corresponds to the drop in retrotransposons activity [35]. APOBEC3C, 3F and 3G are expressed in numerous tissues, including human spleen, peripheral blood lymphocytes, ovary, and testis (Table **1**) [26]. Only reduced levels of APOBEC3B are present in the principal cellular targets of HIV, although APOBEC3B is prevalent in various cancer cell lines [26, 36]. Recently, APOBEC4, a novel member of the APOBEC family, has been identified by computational analysis and presents orthologues in mammals, chickens, and frogs, but not in fishes [37].

3. STRUCTURE OF APOBEC3G

APOBEC3G has two zinc finger domains, which are important for the biochemical and biological activities of the protein (Fig. **2a**). A short α-helical domain is followed by a catalytic domain, a short linker peptide, and a pseudocatalytic domain. The entire unit is duplicated to form the domain structure helix1-catalytic domain1-linker1-pseudocatalytic domain1-helix2-catalytic domain2-linker2-psudocatalytic domain2. Each catalytic domain contains the conserved motif His-X-Glu-X_{23-28}-Pro-Cys-X_{2-4}-Cys in which His and Cys residues coordinate Zn^{2+} and Glu serves as a proton shuttle in the deamination reaction [38, 39]. Catalytic domains 1 and 2 contain conserved aromatic residues located between the His-X-Glu and Pro-Cys-X_{2-4}-Cys residues (Phe-70 and Tyr-91 in catalytic domain1 and Phe-262 and Phe-282 in catalytic domain2). There is general agreement that encapsidation of APOBEC3G is dependent on the nucleocapsid domain (NC) of Gag [40-44]. Several studies have shown that APOBEC3G interacts with NC and that catalytic domain 1 is essential for its encapsidation. NC serves to capture the viral genomic RNA. From the experiments using APOBEC3G deletion and point mutants, Zn^{2+} coordination residues of catalytic domain 1 are required for RNA binding and thus it is considered that the RNA can contribute to APOBEC3G encapsidation. The catalytic domain 2 of APOBEC3G determines antiviral as well as deaminase activ-

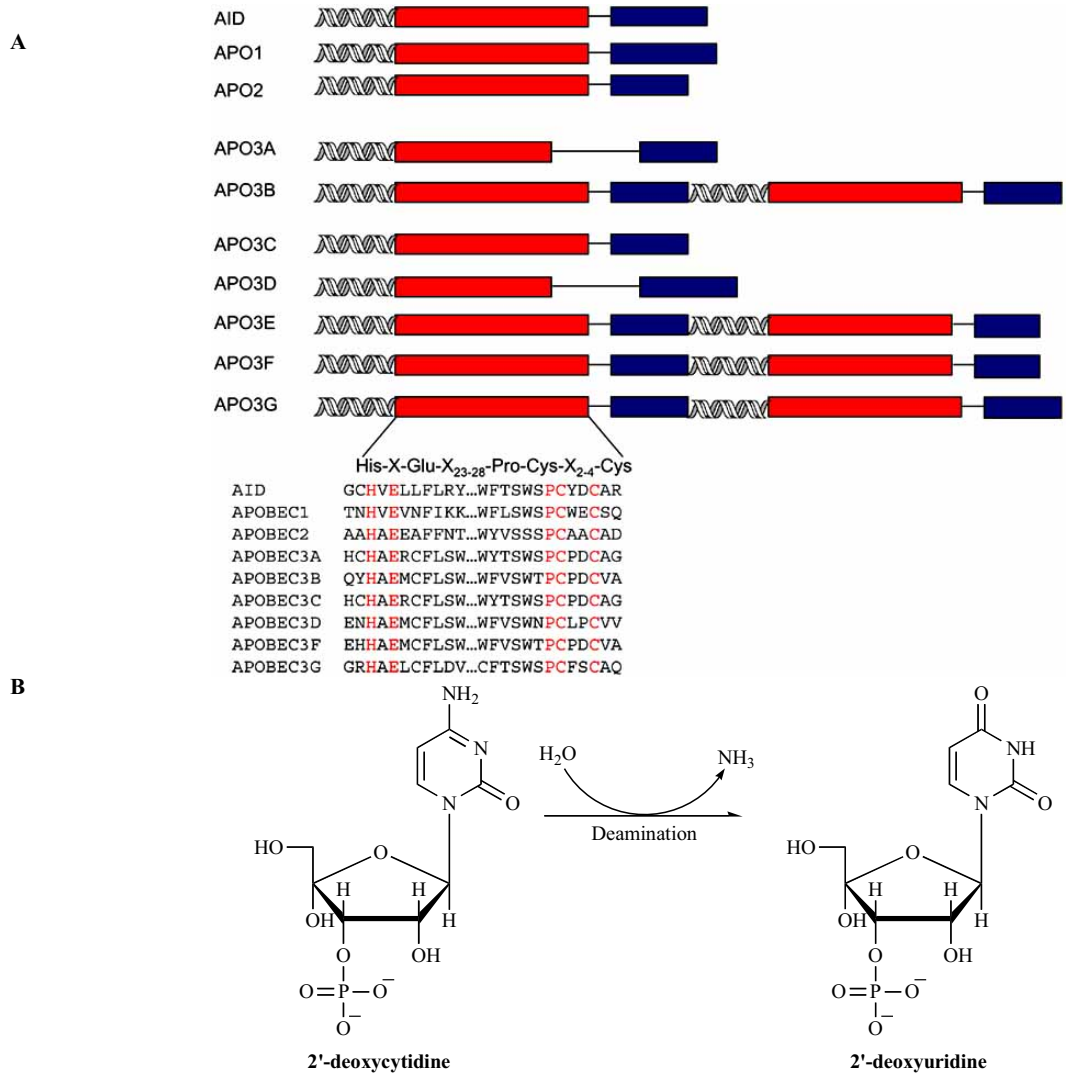

Fig. (2). The human APOBEC family of cytidine deaminases.

(a) Schematic domain organization is shown, with the catalytic domain in red and the pseudocatalytic domain in blue in each protein. The consensus amino acid sequence is shown below. The primary amino-acid sequence of the catalytic domain of APOBEC3G is shown along with the corresponding domains of other representative family members. (b) The cytidine deamination reaction catalyzed by APOBEC. The 2'-deoxycytidine is changed to 2'-deoxyuridine by adding the water and removing an amine group.

ity [6, 45-47]. The mechanism of cytidine deamination by APOBEC3G is similar to that of APOBEC1 and AID. Cytosine and cytidine deaminases are characterized by a zinc-binding deaminase motif with the consensus amino-acid sequence described above His-X-Glu-X_{23-28}-Pro-Cys-X_{2-4}-Cys. Based on the crystal structures of bacterial and yeast cytidine deaminases, the His and Cys residues probably coordinate the zinc ion that is necessary for catalytic activity and the Glu residue helps to produce the hydroxide ion that is required for amine-group removal (Fig. **2b**).

4. ANTIVIRAL ACTIVITY OF APOBEC3G

APOBEC3G is initially identified as an anti-HIV-1 host factor and its antiviral function is antagonized by HIV-1 Vif. As a cytidine deaminase protein, APOBEC3G was investigated for its mutagenic effects on viral RNA and/or DNA and it was found that APOBEC3G induced consistent mutations in a positive strand of the retroviral DNA [4-7, 48]. High levels of dG to dA hypermutation occurred preferentially as a GG to AG substitution. The increased G to A mutations observed were ascribable to the C to U transitions introduced on the complementary negative-strand DNA by the cytidine deaminase activity of APOBEC3G (Fig. **2b**). Introduction of deoxyuracils in the proviral DNA can trigger a basic site generation by the cellular uracil-DNA glycosidase (UDG) and their subsequent cleavage by specific AP-endonucleases, leading to viral DNA degradation. Moreover, the presence of deoxyuracils in the viral negative strand can determine aberrant initiation of second-strand DNA synthesis [49]. Abnormally high percentages of G to A transitions in the retroviral genome lead to non-functional viral proteins [50] (Fig. **1**). Several studies suggested that APOBEC3G

Table 1. Antiviral Activities of APOBEC Family Members

	Expression	Antiviral Activity						HIV-1 Vif Sensitivity
		HIV-1	SIV	MLV	HBV	Retrotransposon		
						LTR	non-LTR	
Human								
APOBEC1	Gastrointestinal tissues	-	ND	-	ND	ND	ND	ND
APOBEC2	Heart, skeletal muscles	-	-	ND	ND	ND	ND	ND
AID	Activated B cells	-	-	-	ND	ND	ND	ND
APOBEC3A	Keratinocytes	-	ND	-	ND	+	+	ND
APOBEC3B	T cells, keratinoocytes	+	+	+	+	+	+	-
APOBEC3C	Many tissues, various kinds of cancer cell lines	-	+	-	ND	-	+/-	-
APOBEC3DE	ND	+	+	-	ND	+	-	+
APOBEC3F	Many tissues, co-expressed with APOBEC3G	+	+	-	+	+	+	+
APOBEC3G	Many tissues, co-expressed with APOBEC3F	+	+	+	+	+	-	+
APOBEC3H	ND	-	-	-	-	ND	-	ND
Murine								
APOBEC3		+	+	-	ND	+	-	-
Rat								
APOBEC1		+	ND	ND	ND	ND	ND	-

HIV-1 indicates human immunodeficiency virus type 1; SIV, simian immunodeficiency virus; MLV, murine leukemia virus; HBV, hepatitis B virus; ND, not done: AID, activation-induced cytidine deaminase.

also has a deaminase-independent antiviral activity [47, 51]. APOBEC3G reduces the synthesis of the DNA by reverse transcriptase, >50% inhibition of early and >95% inhibition of late viral DNA production, and >95% reduction of viral infectivity, independent of DNA deamination. The inhibition of the production of early minus sense strong stop DNA is also correlated with a similar inability of $tRNA^{Lys3}$ to prime reverse transcription.

Recent studies by the Greene laboratory also revealed another model of antiviral activity of APOBEC3G [52]. APOBEC3G exists in two forms in target cells. One is a low-molecular-mass (LMM) form that is enzymatically active and another is a high-molecular-mass (HMM) ribonucleoprotein complex, including staufen-containing RNA-transporting granules, Ro ribonucleoprotein, and so on, that is inactive [53]. LMM exists predominantly in peripheral-blood-derived resting CD4 T cells and monocytes, which are refractory to HIV-1 infection as a result of an early post-entry block (Fig. **3a**). If CD4 T cells are activated or if monocytes are induced to differentiate into macrophages, APOBEC3G is transformed from the LMM into the HMM complex and enables HIV-1 infect target cells without any trouble. The HMM complex form of APOBEC3G cannot inhibit the reverse transcription of incoming virions and therefore viral DNA synthesis can progress (Fig. **3b**) [52].

5. ANTIVIRAL ACTIVITY AGAINST RETROTRANSPOSONS.

Retrotransposons are transposable elements that account for nearly one third of the human genome. The major classes of endogenous retroelements in mammals include autonomous long interspersed nucleotide elements (LINEs), nonautonomous short interspersed nucleotide elements, and elements with long terminal repeats (LTRs). These retroelements are mobile through retrotransposition, an intracellular process involving reverse transcription. They occurred in a high copy number in ancestral genomes and likely played an important role in genome evolution [54].

LTR retrotransposons include murine intracisternal A-particle (IAP), murine MusD, and yeast Ty1 are structurally similar to retroviruses. They form virus-like particles (VLPs) that are assembled and bud at the endoplasmic reticulum membrane. In contrast, non-LTR retrotransposons occupy nearly 20% of the human genome and their replication cycle is different from that of LTR retrotransposons. Reverse transcription occurs within the nucleus without the formation of VLPs. Several groups have demonstrated that human

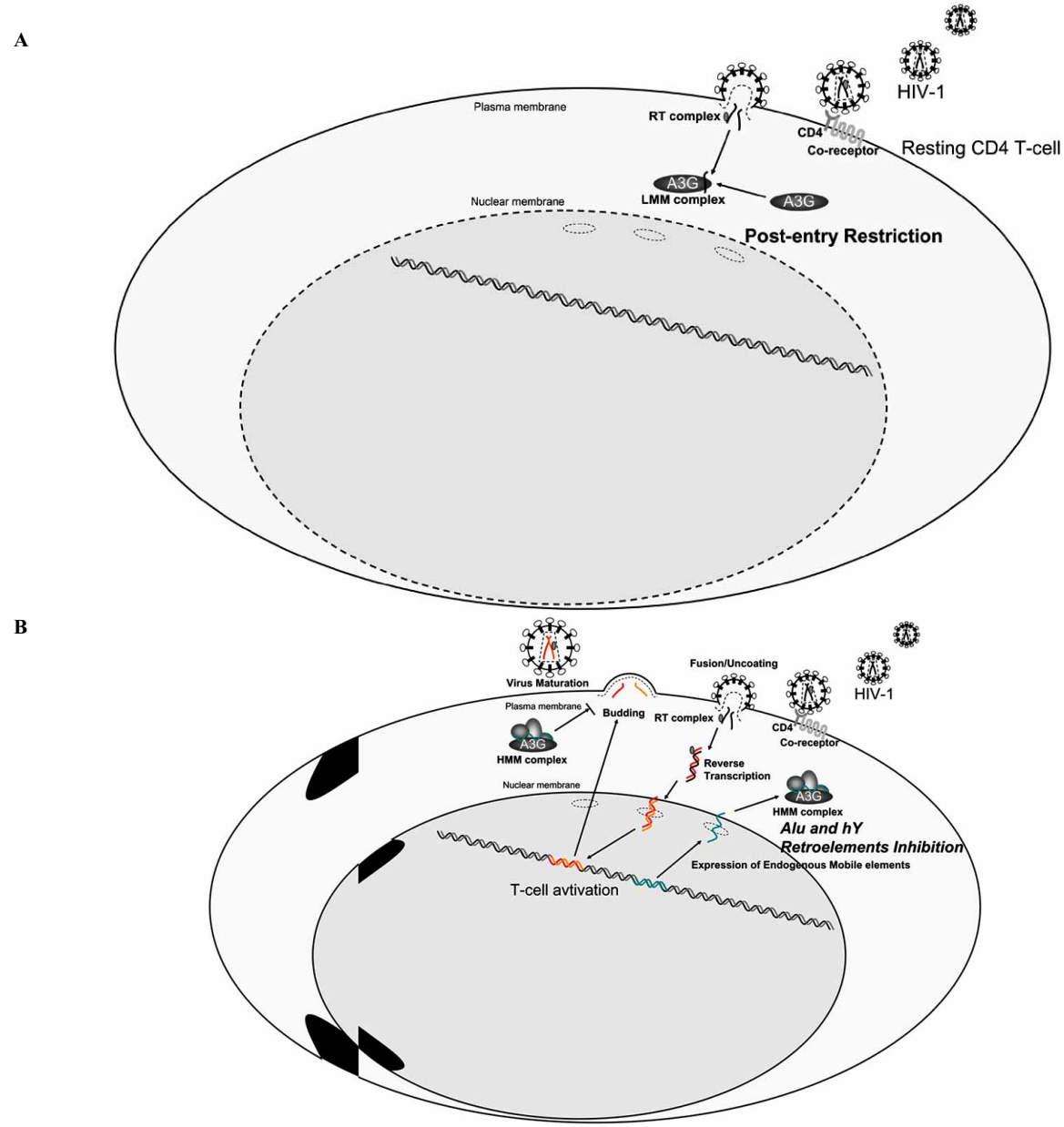

Fig. (3). APOBEC3G exists in two forms.

(a) In resting CD4+ T cells and monocytes, APOBEC3G exists exclusively in an enzymatically-active LMM form. This form of APOBEC3G results in a potent post-entry block to viral infection. (b) In activated CD4+ T cells, APOBEC3G is predominantly in an enzymatically-inactive HMM complex. This form of APOBEC3G fails to attack the incoming virion. These differences would be influenced by the lymphoid microenviroments containing cell-cell contact (especially TCR signaling) and some cytokines (IL-2, 15 etc) that promote the assembly to HMM complexes.

APOBEC3 proteins can inhibit the activity of mouse endogenous retroviruses [18, 21, 55, 56] and yeast Ty1 retrotransposons [19, 20]. APOBEC3A, B, C, and F can inhibit human long interspersed nucleotide elements 1 (L1) (Table **1**) [18]. The retrotransposition of Alu, the most prominent nonautonomous retroelements, and hY RNAs requires the L1 machinery. APOBEC3A and B can also inhibit L1-mediated Alu retrotransposition by entering the nucleus and directly interfering with L1 activity [18]. In the case of APOBEC3G, it inhibits retrotransposition of LTR retrotranspsons such as IAP, MusD [21], and Ty1 [19, 20] by inducing G to A hypermutation, but it does not affect retrotransposition of L1. On another front, APOBEC3G sequesters Alu and hY in cytoplasmic HMM complexes away from the nuclear L1 machinery, thereby interdicting the retrotransposition cycle [53]. These findings suggest that APOBEC family members function as an innate cellular defense against not only exogenous but also endogenous retroelements.

6. ANTI-HIV-1 ACTIVITY OF APOBEC3 FAMILY PROTEINS

The initial report indicated that other members of the APOBEC family, including APOBEC3B, 3C, and 3F, do not have an anti-HIV-1 activity [3]. However, subsequent studies have demonstrated that some of these proteins have a potent anti-HIV-1 activity. Similarly to APOBEC3G, APOBEC3B and 3F are found to inhibit the replication of HIV-1 [57-60] and many other viruses, such as simian immunodeficiency virus (SIV) and HBV [58]. APOBEC3DE has an antiviral effect against HIV-1 and SIV, but not MLV. Its effect is weaker than that of APOBEC3G and 3F [61]. APOBEC3C blocks the replication of SIV but not HIV-1 [62] and APOBEC3A blocks the replication of adeno-associated virus and retrotransposons as described above [18]. Only APOBEC3H is not proven to have any antiretroviral activity.

7. ANTI-APOBEC ACTIVITY OF VIF

Expression of HIV-1 Vif is later in the viral life cycle and antagonizes APOBEC3G by targeting it for ubiquitin-dependent degradation. Vif forms an E3 ubiquitin ligase complex with cellular proteins including Elongin B, Elongin C, Cullin-5, and Ring-box-1 (Vif-BC-Cul5) [63-65]. The C-terminal domain of Vif is characterized by a SOCS box motif, represented by the conserved BC box motif SLQ(Y/F)LA ΦΦΦΦ(Φ being any hydrophobic amino acid) and a down stream proline-rich region, typical of members of the active E3 ligase complexes [66]. Interaction of Vif with the cellular Cullin5-based E3 ubiquitin ligase requires a conserved BC box and upstream residues that are parts of the conserved H-X_5-C-X_{17-18}-C-X_{3-5}-H (HCCH) motif. The BC box motif is important for binding of Vif to Elongin C, whereas the HCCH motif plays a vital role in the Vif-Cullin-5 interaction [67]. The HCCH motif bears many similarities to the zinc-finger domain, that is, apo-peptide itself is α-helical but zinc binding induces a conformational change to β sheet conformation. Therefore, it is important to bind Cullin5 for HCCH motif of Vif to capture Zn^{2+}. On the other hand, Vif binds APOBEC3G through an N-terminal domain. Vif counteracts human APOBEC3G (hA3G) but not rhesus macaque APOBEC3G (rhA3G) or African green monkey (AGM) APOBEC3G (agmA3G) because of a failure to bind the nonhuman primate proteins. The species specificity of the interaction is controlled by amino acid 128 of APOBEC3G, which is Asp in hA3G and Lys in agmA3G [68-71]. Substitution of Asp in hA3G for Lys at positon 128 (D128K), found in agmA3G, results in Vif-resistant hA3G. This has been suggested to be either because D128K-APOBEC3G is no longer able to interact with HIV-1 Vif or a subsequent downstream step is inhibited. The Landau lab suggested that amino acids DRMR at position 14 to 17 in HIV-1 Vif interact with the positively charged residue at position 128 of hA3G because of their negative charges [72]. However, the Pathak lab demonstrated that two distinct Vif determinants, amino acid sequence ^{14}DRMR^{17} and ^{40}YRHHY^{44}, are essential for binding to APOBEC3F and APOBEC3G, respectively [73].

8. ESCAPE OF OTHER RETROVIRUSES FROM APOBEC PROTEINS

The big question since the identification of APOBEC3G has been why MLV is able to replicate in murine cells expressing APOBEC3 even though the virus does not have a Vif protein. A key step for APOBEC3 proteins in blocking viral replication is their encapsidation. Murine APOBEC3 cannot inhibit MLV, because it can not be incorporated into MLV virions and specific exclusion of murine APOBEC3 from MLV virions is attributed to the resistance of MLV to the murine enzyme [13, 57]. The Cullen lab shows that the inability of muirne APOBEC3 to bind MLV Gag is attributed to this exclusion [74]. However, we demonstrates that two novel mechanisms are involved in the resistance of MLV to murine APOBEC3; 1) viral RNA excludes murine APOBEC3 from MLV virions and 2) viral protease cleaves murine APOBEC3 in virions [22]. Recently, Mason-Pfizer Monkey Virus has been shown to selectively exclude simian APOBEC3G [23] and these mechanisms might also be involved in this resistance. In contrast, foamy virus has a unique protein, named Bet, of which function has been unclear until recently. Several groups have reported that Bet has no similarity in its sequence with HIV-1 Vif but can antagonize APOBEC3 by the non-proteasomal degradation pathway. These evidences suggest that virus has its own system to escape from the antiviral function of APOBEC3.

9. APOBEC3G AS A POTENTIAL ANTI-VIRAL THERAPEUTIC TARGET

Until now, the most successful therapeutic strategy against HIV-1 is the highly active antiretroviral therapy (HARRT), which combines different reverse transcriptase inhibitors and protease inhibitors. However, HAART is not sufficient for a complete eradication of HIV-1 in infected patients and in recent years the problem of multi-drug-resistant viral strains has dramatically increased. APOBEC3G and 3F represent a natural protection against HIV-1 infection, but these host defenses have been overcome by the HIV-1 Vif protein, which indicates that advances in APOBEC3 biology will reveal promising new targets for anti-HIV-1 drugs. We assume three potential challenges to develop new anti-HIV-1 drugs for APOBEC3G. 1) Inhibition of Vif interaction with APOBEC3G/3F. Recently, some groups including us pay attention on identifying small-molecule inhibitors that selectively disrupt the association of Vif with APOBEC3G/3F or block the recruitment of an E3 ligase by Vif. These drugs would conserve the intracellular levels of APOBEC3G/3F, enabling them to exert their potent antiviral activity. 2) Induction of APOBEC3G/3F expression enough for Vif not to inhibit. The induction of APOBEC3G expression in T cells by interleukin-2 (IL-2) and -15, and to a lesser extent -7 [75], and in macrophages by interferon (IFN) alpha [76] gives suggestions that APOBEC3G expression might be safely boosted beyond Vif counteracting it. Significant upregulation of APOBEC3G expression induced by IFN stimulation in human hepatocytes could be involved in host defense mechanisms directed against hepatitis B viruses (HBV). This understanding of the potential participation of APOBEC3 proteins against HBV would be applied to anti HIV-1 therapy. 3) The discovery that LMM APOBEC3G is a post-entry restriction factor indicates a new potential therapeutic strategy. Described above, APOBEC3G exists in two forms which convert from LMM to HMM following T-cell activation. Induction of LMM APOBEC3G might protect cells against viral infection.

CONCLUSIONS

Since the identification of APOBEC3G as an anti-HIV-1 host restriction factor, we obtain many scientific evidences that the APOBEC family proteins plays an important roles in the innate immunity against a wide variety of viruses and that each virus has its own escape mechanism from these enzymes. However, details of the antiviral activity of each protein and mechanisms of each virus escaping from these enzymes remain unclear. More scientific information obtained by further investigation on these will provide us with a clue leading to the development of new antiviral drugs.

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