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Functions and Regulation of the APOBEC Family of Proteins

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Abstract

APOBEC1 is a cytidine deaminase that edits messenger RNAs and was the first enzyme in the APOBEC family to be functionally characterized. Under appropriate conditions APOBEC1 also deaminates deoxycytidine in single-stranded DNA (ssDNA). The other ten members of the APOBEC family have not been fully characterized however several have deoxycytidine deaminase activity on ssDNAs. Despite the nucleic acid substrate preferences of different APOBEC proteins, a common feature appears to be their intrinsic ability to bind to RNA as well as to ssDNA. RNA binding to APOBEC proteins together with protein-protein interactions, post-translation modifications and subcellular localization serve as biological modulators controlling the DNA mutagenic activity of these potentially genotoxic proteins.

Keywords

APOBEC; DNA deaminase; mutation; regulation; RNA editing

1. Introduction

The purpose of this review is to familiarize the reader with the proteins in the APOBEC family in order to better appreciate differences in their functional roles as well as to describe cellular and viral control mechanisms that determine APOBEC activities. The review begins with Apolipoprotein B Editing Catalytic subunit 1 (APOBEC1 or A1) because it is the founding member of the family [1]. All family members have in common a zinc-dependent

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cytidine deaminase domain (ZDD) that is identifiable through its primary amino acid motif and a conserved super-secondary structure.

1.1. Overview of the requirements for apolipoprotein B mRNA editing

Apolipoprotein B (*apoB*) mRNA (C to U editing) and the glutamate receptor mRNA (A to I editing) were the first mRNAs discovered to be edited in mammalian cells circa the late 1980s [2]. Human liver and intestine produced a long and short form of the apolipoprotein B (ApoB) protein and the discovery of *apoB* mRNA editing resulted from research to determine the molecular basis for this polymorphism. Sequencing revealed a single nucleotide difference between mRNA and the genomically encoded sequence which was attributed to post-transcriptional RNA editing [3, 4]. The cis-acting sequences required for editing site recognition flanking the cytidine to be edited, in particular the 11 nt 'mooring sequence' (Figure 1A), had already been completely defined [2] before A1 was discovered [5]. However A1 is a low-affinity RNA-binding protein [6–8] and its ability to edit mRNA could only be realized in cells or cell extracts if they contained the RNA-binding protein APOBEC1 Complementation Factor (ACF) [9–11]. A1 dimers [7, 12] and RNA-bridged dimers of ACF [13] make up the minimal composition of the 27S editosome [14, 15] (Figure 1A).

1.2. Site-specific editing

The primary editing site at nt 6666 in *apoB* mRNA is a CAA glutamine codon that is deaminated to a UAA premature stop codon. Unedited and edited mRNAs coexist at varying ratios in editing-competent cells because tissue-specific and metabolically regulated differences in editing efficiency and because edited *apoB* mRNA is stabilized through the ability of ACF to blunt nonsense codon mediated mRNA degradation [16]. The specificity of this editing event is apparent in that *apoB* mRNA contains 3,315 cytidines of which 375 are in the correct reading frame and 100 are CAA. The mooring sequence is necessary and sufficient in determining whether a 5' located cytidine is a candidate for editing [17–21]. However, editing activity itself is determined by the expression and nuclear retention of A1 and ACF [14, 22, 23] (see Sections 3.1 and 4) and has a temporal and spatial 'window of opportunity' for editosome assembly and function that occurs subsequent to pre-mRNA splicing but prior to mRNA nuclear export [20, 24].

1.2.1. RNA substrates—An open question in A1 research has been how much of the transcriptome is edited? Within apoB mRNA the cytidine at nt 6802 also is edited (converting an ACA threonine codon to an AUA isoleucine codon) [17]. Editing at C6802 is associated with editing at C6666 and therefore is unlikely to impact the biology of ApoB protein. The other example is the mRNA encoding the tumor suppressor neurofibromin which was evaluated for mooring sequences that might support editing because of the occurrence of truncated proteins in NF1 tumor tissues. *NF1* mRNA contained three mooring sequence-like motifs of which one supported mooring sequence dependent C to U editing at nt 2914 [19, 25]. Editing of cytidines at C6666 and C6802 of *apoB* mRNA and C2914 in *NF1* mRNA were mooring sequence-dependent; suggesting that the mooring sequence itself might be predictive of other mRNAs that could support C to U editing and therefore may be of utility in computational analyses of the transcriptome. Computational modeling using a

weighted matrix which considered the editing efficiencies of all natural and experimental editing sites predicted that mooring sequences existed in multiple mRNAs although no new editing events were identified [26]. Transcriptome-wide, comparative RNA sequencing revealed mooring sequence-dependent editing of cytidines to uridines within the 3' UTRs of 32 mRNAs [21]. Further deep sequencing studies and the application of advanced computational prediction of editing sites [27, 28] are likely to reveal abundant and biologically significant C to U post-transcriptional editing events as have been revealed for A to I pre-mRNA editing [29].

In the absence of A1, *apoB* mRNA is not edited and therefore A1 is the sole family member capable of recognizing and using *apoB* mRNA as a substrate [30, 31]. In tissues where A1 is expressed, deaminase activity on RNA is highly regulated through A1's: (i) interactions with ACF, (ii) shuttling between its storage form in the cytoplasm and the assembly of editosomes in the nucleus (Sections 3.1 and 4) and (iii) regulation of A1 protein expression and abundance [1]. Experimental overexpression of A1 in rat hepatoma cells resulted in promiscuous editing of cytidines in *apoB* mRNA as far as 50 nt 5' of the mooring sequence [20] and hyperediting activity on mRNAs that otherwise were not edited [32, 33]. Overexpression of A1 was associated with oncogenesis [34], which has been inferred to be due to its RNA editing capability. Recently, A1 was shown to edit ssDNA when overexpressed in an *E. coli* mutator assay [35, 36]. This raises the question of whether deregulation of A1 expression in mammalian cells could also lead to genomic DNA mutation and cancer.

1.2.2. ssDNA substrates—A1 has no known mammalian DNA substrate but it has DNA deaminase activity sufficient to induce reversion mutations when overexpressed in *E. coli* that have been placed under selection pressure [35, 36]. In addition, A1 expressed in neurons may have a protective function against herpes simplex virus that involves ssDNA deamination of the viral genome [37]. The sequence requirements for A1 deamination were lax but deamination occurred within transcribed or otherwise single-stranded regions of DNA and the dC that was deaminated had a nearest neighbor preference of a 5' T [35].

Unlike RNA editing, ssDNA editing did not require a cofactor and this appears to be true for the other APOBEC family members (Section 2). Many in the field have asked whether A1 RNA editing is a curious exception or will RNA substrates be identified for the other APOBEC family members? This question is made all the more interesting by the fact that many of the APOBEC family members bind RNA as well as ssDNA and in the RNA-bound state, were not active as ssDNA deaminases (discussed below). In this regard, A1 from various species differed in their capacity for site-specific RNA editing [38, 39]. Reptilian A1, that lacks a C-terminal dimerization and regulatory domain only deaminated ssDNA [40]. In contrast, the yeast APOBEC homolog known as CDD1 site-selectively edited *apoB* mRNA when this mRNA was expressed in yeast [41, 42]. CDD1 is a 15.5 kDa protein representing little more than the deaminase catalytic domain and yet it had deaminase activity on RNA, ssDNA and free cytosine [43]. It is therefore unclear what structural features of the APOBEC proteins determine their range of substrates.

2. The Eleven proteins in the APOBEC family

The APOBEC family of proteins are readily identifiable in amino acid similarity searches through the occurrence of the zinc-dependent cytidine or deoxycytidine deaminase domain (ZDD) (H/C)-x-E-$x_{25–30}$P-C-x-x-C (Figure 2A). Zinc is coordinated through the underlined H or C residues in the context of a super secondary structure consisting of five antiparallel beta strands forming a beta sheet that is supported through two alpha helices. Deaminase activity of this ZDD involves hydrolytic removal of the exocyclic amine from cytidine (C) or deoxycytidine (dC) to form uridine (U) or deoxyuridine (dU) [44].

2.1. Activation Induced Deaminase, AID

Upon encountering a foreign antigen, immunoglobulin genes within germinal center B cells undergo several rounds of modifications allowing antibodies to be expressed with strong antigen-binding affinity and different effector functions [45, 46]. Activation induced cytidine deaminase (AID) was discovered more than a decade ago using subtractive hybridization by comparing the transcripts from resting and activated murine B cells that underwent immunoglobulin gene diversification [47]. AID's deaminase activity [35] is responsible for dU mutations that are converted through low fidelity excision repair to a variety of point mutations or cause DNA strand breaks. This mutagenic activity leads to Somatic Hypermutation (SHM) and immunoglobulin gene recombination events known as Class Switch Recombination (CSR) and Gene Conversion (GC) [48–52].

The human AID gene maps to chromosome 12p13 and encodes a 198 amino acid protein containing a single ZDD [53] (Figure 2A). AID gene mutations have been identified in patients with a rare immunodeficiency known as Hyper IgM sydrome, HIGM (characterized by high serum levels of IgM and lack of other immunoglobulin isotypes (IgG, IgE, IgA)) [54]. This phenotype also was observed in AID knockout mice [47, 55] which demonstrated that AID was required for immunoglobulin diversification. On the other hand AID expression has been implicated in several pathologies including Non-Hodgkin B cell lymphomas and solid tumors [56]. Increased expression of alternative splice variants of AID has been reported in patients with chronic lymphocytic leukemia (CLL) [57–59]. Alternatively spliced transcripts lacking C-terminal residues or the entire exon that contains the nuclear export signal have been observed in cancer cells. Although this suggested that AID hyperactivity led to genomic instability, the role of AID deaminase activity remains to be confirmed [60, 61]. The formation of lung micro-adenomas and massive T cell lymphomas in transgenic mice ubiquitously expressing AID confirmed the hypothesis that deregulated expression of AID can lead to malignant transformation [62]. In fact, up-regulation of AID expression through the NF_KB signaling pathway in response to hepatitis C infection of hepatocytes also was associated with genomic mutations, however these mice did not develop cancers [63]. This suggested that an additional control system buffering the effects of endogenous AID expression in B cells may exist.

In support of this possibility, constitutive expression of AID in mice that was restricted to B cells was not associated with developmental defects, excessive DNA mutations or tumors even though a large amount of AID protein was evident [64]. Why AID expressed in germinal centers normally only targets variable and switch regions of immunoglobulin genes

for mutagenesis is unknown. Part of the answer might be that B cell-specific regulation inhibits the expression of AID through Ca2+/calmodulin mediated inhibition of the E2A protein that is required for AID gene transcription [65, 66]. Accumulating evidence indicates that AID selectivity for its ssDNA substrates may be regulated through AID interactions with cis-acting elements in the Ig loci or by binding to SHM- and CSR-specific protein cofactors [49, 67]. The data clearly show that AID and its ssDNA deaminase activity are essential for a responsive B cell immune system but it is also clear that AID must be highly regulated to control its oncogenic potential.

2.2. APOBEC2

APOBEC2 (A2) was discovered by a search of mouse and human EST databases for genes homologous to A1. Human A2 is located on chromosome 6 and expressed in cardiac tissue and skeletal muscle [35, 68]. Before any function was ascribed to A2, its crystal structure became the first and only full length APOBEC family member to be solved to date [69]. Although this structure has been used for homology modeling of other family members, A2 seems to be functionally quite different. While the other APOBECs are clearly capable of deaminase activity on ssDNA in the absence of cofactors, A2 was non-mutagenic in yeast or bacteria based mutator assays [35, 70]. However, a recent paper suggested that A2 DNA mutagenic activity targeted specific tumor suppressor genes and that A2 overexpression in mice resulted in liver and lung tumors [68]. The potential of A2 activity on DNA is of interest considering that A2 is essential for muscle development [71]. A2 may have a specific and unique function in muscle and heart tissue. Whether A2 acts alone or has a cofactor is not known. The data suggest that A2 may be far less prone to off target activity given its weak interaction with RNAs and lack of autonomous deaminase activity in bacterial or yeast based systems [70, 71].

2.3. APOBEC3

The main function of APOBEC3 (A3) proteins (clustered on human chromosome 22) is as sentinels in innate immunity to mobile genetic elements (i.e. endogenous retroelements and exogenous viruses) (Figure 2B). Many forms of A3 genes are found in mammals (Figure 2A): a single A3 gene in rodents, cats, pigs, and sheep, two in cows, three in dogs and horses, and seven in primates [72]. A3B, A3DE, A3F, and A3G differ from A1, AID, A3A, A3C and A3H in that they contain two deaminase domains instead of one within a single polypeptide (Figure 2A) [44, 73]. Deaminase activity on particular mobile genetic elements varies greatly between these homologs (Figure 2B). The goal of this section is evaluate these differences.

The most studied A3 family member is A3G. The function of A3G as an antiviral host factor was discovered in 2002 through cDNA transfer experiments designed to identify a host cell suppressor of the HIV-1 accessory protein known as the viral infectivity factor (Vif) [74]. Vif binds to A3G and induces its destruction via the ubiquitination and proteasome degradation pathway [75]. Viruses deficient in Vif had low infectivity if they were produced in cell lines known as ‘nonpermissive’ (express A3G) but otherwise exhibited near wild type infectivity levels when produced in ‘permissive’ cell lines [75]. Transfection of permissive cells with A3G was necessary and sufficient for conversion to the nonpermissive phenotype

for Vif-deficient HIV-1 infectivity [74]. From these findings it became clear that wild type HIV-1 expressed Vif to overcome the A3G innate immune system.

The primary antiviral mechanism of A3G required that it be encapsidated into HIV viral particles [75]. During reverse transcription A3G hypermutated minus-strand HIV DNA with mutations becoming fixed as G to A mutations upon plus-strand synthesis [76, 77]. A3G also acted on a wide variety of distantly related retroviruses that package genomic RNA. The retroviruses A3G has been reported to affect SIV [78], equine infectious anemia virus (EIAV) [79], murine leukemia virus (MLV) [80], and foamy virus (FV) [81] (Figure 2B). The interspecies transmission of SIV was likely prevented by A3G's insensitivity to SIV Vif, while MLV and EIAV virions do not have a Vif equivalent to protect the viruses against A3G.

A3F was capable of anti-HIV, SIV, EIAV and FV activity nearly equivalent to that of A3G [78, 79, 81]. However, A3F preferred a dTC sequence context for deaminase targeting opposed to the dCC preference for A3G. This enabled A3F to target DNA sequences for hypermutation other than those mutated by A3G [82]. A3DE also had antiviral effects on HIV infectivity in a Vif-sensitive manner although to a lesser degree than A3G or A3F [80]. A specific haplotype II (in Africans) of A3H had activity similar to A3G while the haplotype I, III and IV were not antiviral [83]. A recent report examined antiviral activities of all human and rhesus macaque A3s stably expressed in a Sup T1 cell line and found that A3DE, A3F, A3G, and A3H (haplotype II) from both species had similar expression patterns in T cells and activity against HIV and SIV [78]. In this study A3A, A3B and A3C were inactive against HIV (Figure 2B) [78]. In a different study, A3A had anti-HIV activity that was linked to a specific A3A protein variant. Deaminase activity in INF-α treated monocytes and monocyte derived macrophages correlated specifically to a variant that was expressed equal to wild type A3A but whose translation initiated with methionine at residue 13 instead of residue 1 [83]. It is unclear whether this variant was the key difference between the studies or if a cofactor specifically expressed in monocytes activated A3A against HIV. A3A was also a potent inhibitor of a parvovirus, AAV-2 (Figure 2B), and its activity on these viruses correlated with protein structural divergence from A3G [84].

A3G, A3F and A3B inhibited hepatitis B virus (HBV) infection (Figure 2B) [85]. HBV is considered a pararetrovirus because it reverse transcribes "pre-genomic" RNA like retroviruses, but unlike retroviruses reverse transcription occurs before viral release and therefore a DNA genome is packaged. Liver cells are the target of HBV and A3G, A3F and A3B expression in human liver was up-regulated by INF-α [85]. However, the low and variable levels of A3s in the liver may have caused low levels of mutations that imparted HBV with some selective advantages. Specifically this mutagenic activity produced a truncation mutation in the HBx gene that was linked to hepatocellular carcinoma [86].

The original A3 targets may have been endogenous retroelements [87, 88]. Endogenous retroelements' ability to copy themselves into random locations in the genome leads to genomic instability and disease [88]. There are three major classes of retroelements: (*i*) long terminal repeat (LTR) based endogenous retroviruses, and non-LTR based (*ii*) autonomous long interspersed nuclear elements (LINEs) and (*iii*) non-autonomous short interspersed

nuclear elements (SINEs). LINEs are autonomous because within their code is everything needed to reverse transcribe and re-insert their sequence into another location within the cell's genome. Conversely, SINEs are non-autonomous because they must use the machinery encoded in LINEs *in trans* for reverse transcription and genomic re-insertion [88].

Various retroelement reporter assays revealed that A3G and A3F inhibited LTR-based endogenous retroviruses (i.e. IAP, Mus-D and Ty1) [89, 90]. Hypermutations were detected in these sequences [89, 90] as well as a reduced number of reverse transcripts [89] consistent with the known A3G mechanism on exogenous retroviruses. In contrast, A3G inhibited SINE retrotransposition (i.e. *Alu* and hY) by sequestering these RNAs as ribonucleoprotein complexes (Section 4) [87, 91]. This mechanism was deaminase-independent consistent with A3G being enzymatically inactivated by cellular RNAs in HMM (Section 3.3 and 4) [75, 87, 91].

The expansion of A3s in primates correlates well with the decreased presence of active retroelements in humans [88]. There are seven A3s in humans and the only currently active retroelements in humans are non-LTR based retroelements (i.e. LINEs and SINEs). Conversely, mice have only one APOBEC3 protein and their genomes contain active LTR-based and non-LTR based retroelements [88, 89]. Moreover, mice carry 50–60 times more active LINE-1 retroelements in their genomes than humans and the proportion of LINE-1 causing disease is 35% greater in mice compared to humans [88]. In this regard all human A3s inhibit LINE-1 and/or *Alu* retrotransposition, with the one exception that A3G does not inhibit LINE-1 (Figure 2B) [92–97]. A3DE, A3F and A3H were localized to the cytoplasm and were able to block either LTR-based or non-LTR based retroelements in manners similar to A3G [89, 94, 95, 97, 98]. In contrast, A3A, A3B and A3C were capable of nuclear localization and may be able to directly inhibit nuclear reverse transcription of retroelements [92–96]. Overall these multiple fronts of defense highlight the diversity within human A3 genes as a key determinant in combating the genotoxic threat posed by endogenous retroelements. However, as with A1, A2 and AID, up-regulation of nuclear A3A and A3B deaminases may become genotoxic and pose a risk of inducing cancer [85, 99]. This threat along with the variable activities and expression levels on different mobile genetic elements underscores the need to further understand cell type-specific regulation of A3 proteins.

2.4. APOBEC4

APOBEC4 (A4) has no ascribed function and was discovered by a computational homology search that revealed its location on human chromosome 1 and inspection of the ESTs suggested that it is expressed in testes [100]. The A4 sequence is distinctly divergent from other APOBEC genes and contains a significant alteration in the presumptive deaminase domain compared to the consensus ZDD motif (PCx_6C instead of PCxxC) [100] (Figure 2A). A recent report showed that A4 was non mutagenic when expressed in yeast and bacteria [70]. As suggested for A2, A4 might require a specific cofactor for mutagenic activity or it may have a deaminase-independent function.

3. Subcellular compartmentalization of APOBEC proteins regulates their activity

3.1. A1 in 27S editosome and 60S pre-editosomal complexes

A1 distribution in both the nucleus and cytoplasm is determined in part by an N-terminal nuclear localization signal and a C-terminal cytoplasmic retention signal (Figure 3A) [101, 102]. Although RNA editing can occur in the cytoplasm when A1 is overexpressed [103], cytoplasmic A1 editing activity is normally suppressed and RNA editing is restricted to the cell nucleus within a temporal and spatial window that occurs subsequent to pre-mRNA splicing and prior to mRNA nuclear export [24, 104].

Glycerol gradient velocity sedimentation of nuclear extracts revealed A1 and ACF co-sedimenting with editing activity as 27S complexes [14, 105]. A1 and ACF in cytoplasmic extracts co-sedimented as 60S complexes whose RNA editing activity could be activated *in vitro* and converted to 27S complexes by incubating 60S complexes at 30 °C [105] or by treating them with elevated monovalent salt concentrations [15, 106]. Other than A1, ACF and RNA, the molecular composition of 27S and 60S complexes is unknown. It is also not known whether the interaction of A1 with ACF in inactive 60S complexes is different than that in 27S complexes which are editing competent.

The proportion of *apoB* mRNA edited in tissues is determined by shuttling of A1 and ACF from cytoplasmic 60S complexes into the nucleus where they are recovered as 27S complexes [14, 16, 101, 107]. This process is regulated tissuespecifically, during development and in response to metabolic regulation (reviewed in [108]). Though different from A1, ACF contains nuclear localization and nuclear export signals [16, 107] and therefore it is possible that A1 and ACF may shuttle independently. ACF shuttling occurred in the absence of A1 but the converse situation is not known. Nuclear retention of A1 and ACF was strongly influenced by ACF phosphorylation that in turn was regulated by insulin and ethanol through protein kinase C [14, 22, 109, 110]. The distribution of A1 and ACF in cytoplasmic 60S or nuclear 27S complexes was regulated through the abundance of ACF and determined in part by leptin inhibition of ACF gene transcription [109] and through the expression of ACF variants (due to alternative pre-mRNA splicing) that have different affinities for A1 and different levels of nuclear retention [43, 111, 112]. These findings underscore the importance of subcellular localization of A1 and its organization as low or high molecular mass complexes as key regulatory determinants for A1 mRNA editing activity.

3.2. AID Cytoplasmic Retention

Controlling the abundance of AID in different cellular compartments is one of the major regulatory mechanisms restricting its contact with the genomic material [113–115]. Despite its function in the nucleus, AID is predominantly localized in the cytoplasm, even when it is overexpressed (Figure 3B) [116]. This pattern is produced by the action of an active transport system [117]. When the export is blocked with a CRM-1 specific inhibitor, Leptomycin B (LMB), the nucleo-cytoplasmic shuttling feature of AID becomes more obvious with the majority of AID sequestered in the nucleus [118, 119] (Figure 3B).

While findings from different groups confirmed the existence of a CRM-1 dependent nuclear export signal (NES) at the C-terminus of AID, there is no consensus about the exact location and nature (classical bipartite or conformational NLS) of the N-terminal nuclear localization signal (NLS) [117, 119]. AID has been shown to interact with several importin alpha isoforms (impα1, impα3, and impα5) [117], suggesting that the generic impα/β import pathway may be mediating AID nuclear entry. CTNNBL1 (catenin, beta-like 1), an NLS-binding protein containing architectural homology and binding affinity to impα5 but not to imp β, was also associated with AID when AID was overexpressed in HEK293T cells [120]. For this reason a role for CTNNBL1 in nuclear localization of AID as either an adapter protein mediating AID-impα5 interaction or AID association with spliceosome components has been proposed [120].

A cytoplasmic retention signal may contribute to the steady state cytoplasmic localization of AID [117]. Under conditions rendering both NLS and NES inactive (combination of oxidative stress, N-terminal tagging of AID, mutagenesis or LMB treatment), AID remained in the cytoplasm, rather than as predicted from AID's size, passively diffusing into the nucleus and displaying a homogenous cellular distribution [117]. Mutational analysis revealed a retention signal overlapping with the NES but distinguishable from it with the specific amino acid substitutions (D188A, L198S) [117].

Protein factors have been shown to stabilize or destabilize AID in various cellular fractions [121, 122]. Heat-shock protein 90 bound to the N-terminal region of AID and stabilized cytoplasmic AID by shielding it against E3 ubiquitin ligase mediated proteosomal degradation [121]. Moreover, analysis of the half-life of different AID mutants demonstrated that, compared to wild type AID, nuclear export deficient AID was 3-fold less stable whereas nuclear import impaired AID had a 3-fold longer half-life suggesting that cytoplasmic AID is more stable [122].

3.3. APOBEC3 cytoplasmic ribonucleoprotein complexes

A3G is strongly retained in the cytoplasm of interphase cells and remains excluded from chromosomes during mitosis (Figure 3C and D). This localization is due to a cytoplasmic retention signal between amino acids 113–128 [123]. Interestingly this region is also crucial for Vif, Gag and RNA interactions, but A3G binding to RNA was not required for cytoplasmic retention [123–125]. The N-terminal half of A3G expressed in a quail cell line was not restricted to the cytoplasm (in contrast to what was observed in mammalian cells) suggesting that there may be a mammalian specific factor involved in the cytoplasmic retention of A3G [126].

A3G is present in two distinct cytoplasmic forms: low molecular mass (LMM) and RNA-bound, high molecular mass (HMM) complexes. Cytokines IL-2, IL-15, and IL-7 stimulated the formation of HMM [127] but poly(I:C) and TNF-α increased A3G expression as LMM [128]; suggesting that these various forms of A3G may have functional significance. Vif also has been suggested to facilitate HMM formation [129]. On the other hand, HMM formation was not a common feature among all A3s. A3DE, A3F and A3H all have similar antiviral profiles to A3G and share in the ability to form HMM complexes and/or associate with *Alu* RNA [97]. On the other hand, A3A in INF-α activated PBMCs did not have to be

treated with RNase to activate its deaminase activity, suggesting that it is not inhibited by RNA in HMM complexes [83].

Immunocytochemistry revealed a homogeneous cytoplasmic distribution of A3G along with concentrated foci determined to be P-bodies and stress granules (Figure 3) [87, 98, 130]. These cytoplasmic aggregates are composed of various cellular RNAs and their associated proteins and function as RNA and ribonucleoprotein degradation or recycling centers. A3G promoted dissociation of miRNA-targeted mRNA from P bodies, thus allowing for translation of these mRNAs [131] and the interaction of A3G with Ago1 and Ago2 (proteins associated with the RNA interference pathway) [132] adds credence to the idea that A3G may function as an RNAi regulator in P bodies. Collectively, these data support a model whereby the oligomeric state and/or localization of A3 proteins is influenced by both cellular and viral factors which, in turn, may be a manifestation of how cells regulate the functionality of these proteins.

4. RNA binding to APOBEC as a cellular regulator of ssDNA deaminase activity

Although there may be several different HMM complexes with which A3G associates, they all share a common characteristic that RNA binding to A3G inhibits ssDNA deaminase activity [75, 133]. Both A1 and A3G were retained as large cytoplasmic complexes that were catalytically inactive. The catalytic activity of A3G that was sequestered in ribonucleoprotein complexes could be restored *in vitro* by RNase digestion of HMM [134]. Most of the interactions of A3G with other proteins within these complexes were through RNA bridging as RNase digestion reduced megaDalton size A3G ribonucleoprotein complexes to dimers and mononers. Interestingly, A3G-RNA complexes in viral particles were inactive until RNaseH activity of reverse transcriptase degraded the RNA of the DNA-RNA hybrid [135]. Although A3G has the ability to bind RNAs nonselectively, several studies have shown that A3G (and A3F) can select for cellular and viral RNAs that become incorporated with it into viral particles [136–140]. A3G:7SL and A3G:viral RNA interactions may facilitate the association of A3G and the nucleocapsid (NC) region of HIV-1 Gag; a requirement for A3G viral packaging [141–143]. RNAs of diverse sequence and propensity for secondary structure, and as short as 25 nt bound A3G *in vitro* and prevented the formation of A3G:ssDNA complexes necessary for catalytic activity [144]. A3C also was incorporated into HIV particles and although it did not associate with 7SL RNA or HIV NC, it did interact with the matrix (MA) region of HIV Gag in what may be a 5.8S ribosomal RNA-dependent manner [145, 146]. Furthermore, AID is bound to mRNA in the cytoplasm of cells [147] and *in vitro* studies have shown AID to have very little catalytic activity unless pre-treated with RNase [148].

RNA binding is emerging as a general means of inactivating ssDNA deaminase activity in the APOBEC family; however the mechanism whereby RNA inhibits deaminase activity is unknown. For A3G, deaminase activity resides in the C-terminal ZDD while RNA binding has been suggested to occur through an interaction with the N-terminal ZDD [149, 150]. RNA binding may regulate deaminase activity allosterically by binding to the N-terminus of A3G and inducing a conformational change in A3G so it can no longer bind ssDNA, or

competitively by directly displacing ssDNA from the C-terminal catalytic domain (Figure 1B) [144]. This model is not directly applicable to enzymes with a single ZDD although RNA inhibition of the deaminase activity of these APOBEC proteins also may be because RNA and ssDNA interactions with the enzyme are mutually exclusive.

5. Concluding Remarks

Other than A2 and A4, binding to an appropriate nucleic acid by APOBEC proteins results in deamination. When limited in frequency and targeted to specific genes, this mutagenic activity can be beneficial to organisms as well as to viral pathogens. There are however clear indications that APOBEC mutagenic activities and nucleic acid binding capabilities can be genotoxic for retroviruses and are used as such in host cell defense. Excessive APOBEC activity and/or off-target mutations within the cellular genome can be genotoxic and oncogenic. Thus cells have multiple mechanisms that regulate the expression of APOBEC proteins, control their enzymatic activity and restrict their access to DNA or RNA substrates.

Use of RNA as a substrate is considered unique to A1. Even though RNA is not commonly used as a substrate, the majority of APOBEC family members bind to both RNA and ssDNA. An emerging theme is that RNA binding to APOBEC enzymes and the resultant homo-multimerization of these enzymes inhibits their ssDNA binding and deaminase activities. Typically RNA binding sequesters APOBEC as large ribonucleoprotein aggregates that are compartmentalized within the cell cytoplasm or within viral particles. Ribonucleoprotein complexes form rapidly after APOBEC translation and are typically not RNA sequence-specific. Thus the availability of free and active APOBEC deaminases is anticipated to be limited under most circumstances. While this may diminish deaminase-dependent antiviral activity, APOBEC binding to retroviral RNAs may impair their ability to reverse transcribe or translate and thereby provide deaminase-independent antiviral activity for the host cell. Inhibition of deaminase activity is reversible and involves release of APOBEC from its interaction with RNA. The mechanism whereby some cell types maintain APOBEC in an RNA-depleted and active state is not known.

The identification of RNA editing substrates has largely depended on prior knowledge of protein isoforms. Proof that an APOBEC family member can edit these RNAs has relied on the identification of a cell type or cell extract that expressed an appropriate complementation factor. Although RNA editing by AID has been suggested since the discovery of the enzyme’s role in SHM and CSR, no edited transcript has been found and the AID RNA editing hypothesis has fallen out of favor. Future discoveries may reveal a broader RNA editing phenotype for this family but current research is focused on APOBEC ssDNA deaminase activities where RNA plays a role as a regulatory cofactor.

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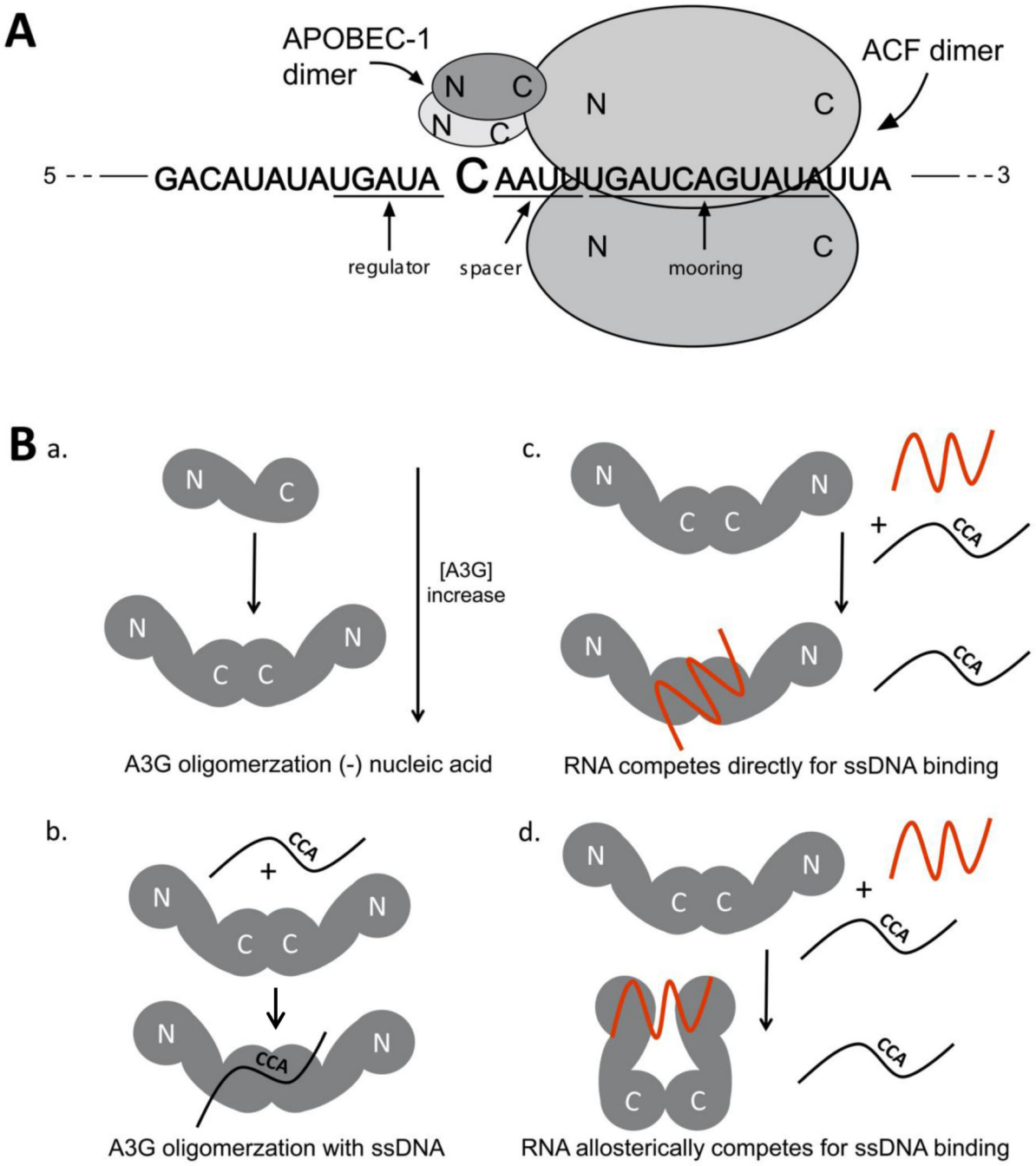


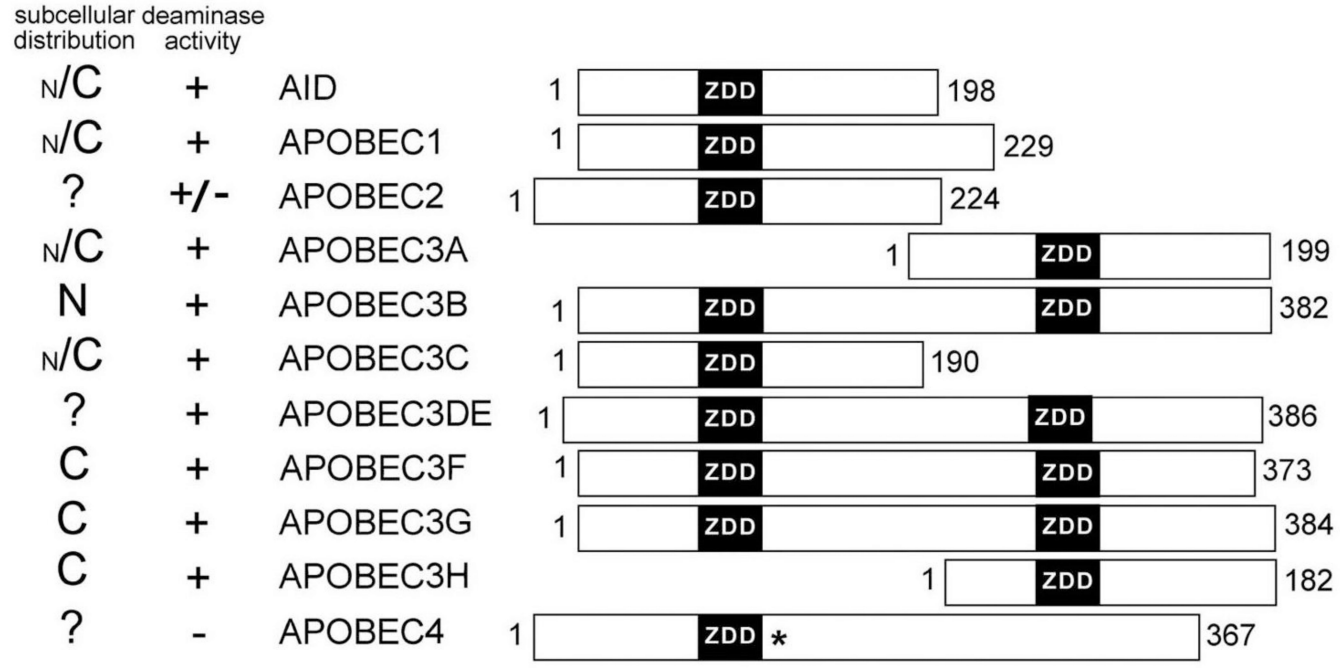
Figure 1. Models for A1 and A3G complexes with nucleic acids

A. The tripartite apoB mRNA editing motif consisting of the mooring sequence, and spacer 3' of the C to be edited and the enhancer element 5' of the edited C is shown with a cartoon of an editosome assembled upon it. An A1 C-terminal dimer is positioned for site-selective C to U editing by virtue of its association with ACF dimers that are shown bound to the mooring sequence. For the purposes of presentation only one A1 dimer is shown bound to one ACF through C-terminal to N-terminal (respectively) interactions (although the precise stoichiometry is unknown). Most of the three RNA recognition motifs comprising greater

than the N-terminal half of ACF are required for optimal A1 binding. ACF dimerization requires only the N-terminal half of ACF.

B. a. An A3G monomer is shown containing an N- and C-terminal ZDD (label as ‘N’ and ‘C’). Nucleic acid deficient A3G forms a heterogeneous mixture of oligomers consisting mostly of dimers in solution and an A3G concentration-dependent small population of monomers and tetramers. **b.** A3G dimers to bind ssDNA substrates (black line with the CCA editing site) and must form at minimum a tetramer for enzymatic activity. **c.** RNA (red line) competes for ssDNA binding by displacing ssDNA and binding at the same site or **d.** RNA competes for ssDNA binding by binding at a distal site and causing an allosteric change in A3G conformation, preventing ssDNA binding.

A



B

	Exogenous Retroviruses					DNA Viruses		Endogenous Retroelements		
	HIV	SIV	EIAV	MLV	FV	HBV	AAV	LTR	LINES	SINES (*Alu*)
A3A	+ (monocytes)	-	-	+	ND	-	+	+	+	+
A3B	-	-	-	-	-	+	-	+	+	+
A3C	-	-	-	-	-	-	-	+	+	+
A3DE	+	+	-	-	ND	ND	ND	ND	+	+
A3F	+	+	+	-	+	+	-	+	+	+
A3G	+	+	+	+	+	+	-	+	-	+
A3H	+	+	-	-	ND	-	ND	ND	+	+

Figure 2. APOBEC family localization and activity

A. Bar diagrams of human APOBEC proteins and their relative alignments according to exon junctions. The ZDD motifs (black) and number of amino acids for each protein are indicated. The (*) next to the ZDD for APOBEC4 indicates that it is divergent from the consensus ZDD. The relative subcellular distribution is on the left with N for nuclear and C for cytoplasmic, and '?' if localization is unknown. The size of N or C indicates relative distribution. The deaminase activity is indicated by a + or − and the +/− for APOBEC2 indicates mixed results depending on the system (see text). **B.** A chart of A3 proteins' activity on different mobile genetic elements, with + for active and − for inactive and ND

when activity has been not determined. The (monocytes) under the A3A HIV activity indicates that it has only been shown to be active in monocyte derived cells. The information in **A** and **B** was compiled from references within the text.

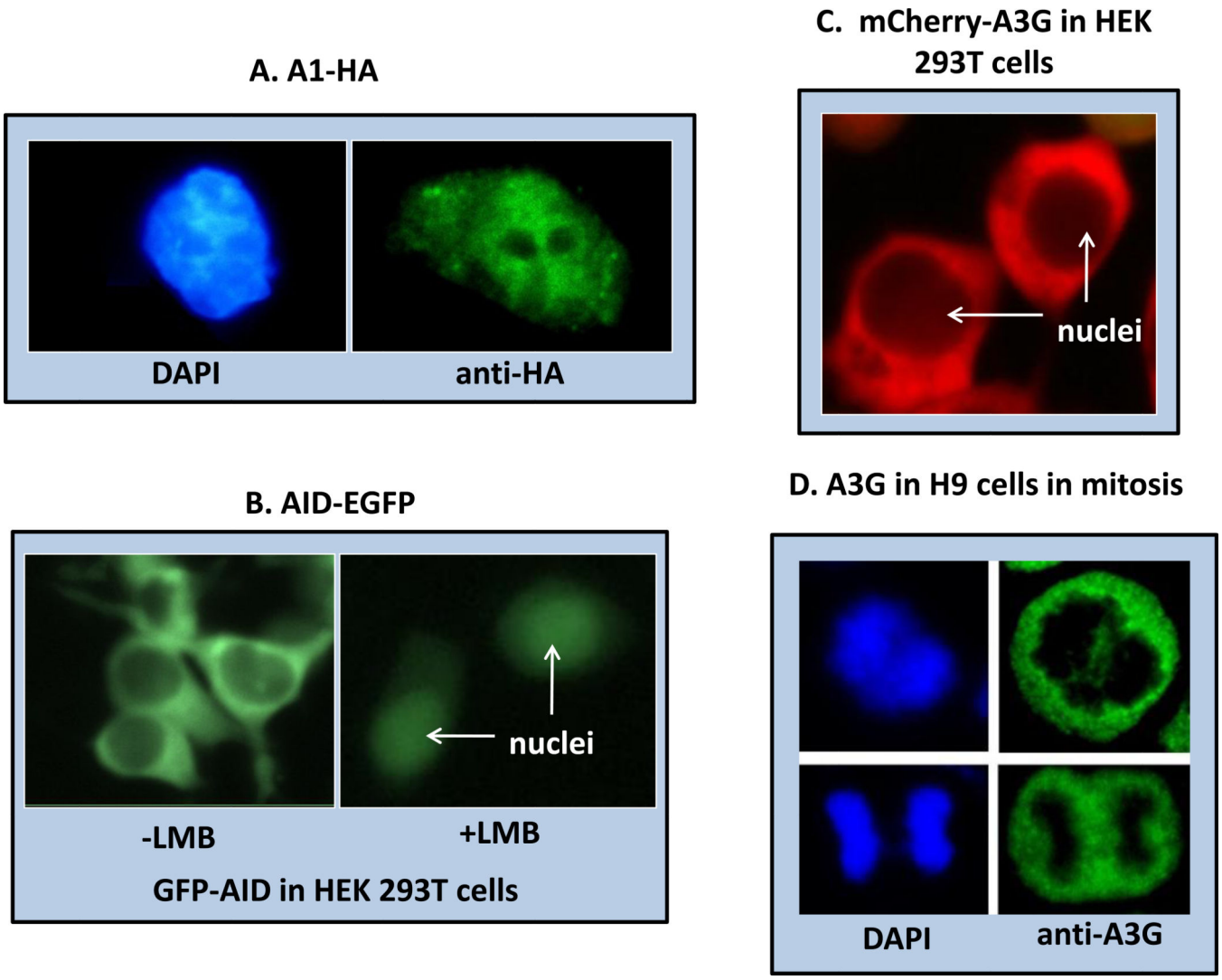


Figure 3. Localization of the APOBEC proteins by *in situ* fluorescence

A. APOBEC-1 (A1) with a C-terminal HA tag was expressed by transfection in McArdle rat hepatoma cells. Immunocytochemical localization of the HA tag in fixed cells shows that A1 is distributed throughout the cytoplasm and nucleus (DAPI) but is not localized in the nucleolus. A1 shuttles between the cytoplasm and nucleus. **B.** Activation Induced Deaminase (AID) as a C-terminal GFP chimeric protein was expressed in transfected human embryonic kidney cells 293T and visualized in live cells. AID appears predominantly in the cytoplasm (left panel, four transfected cells shown) but its rapid shuttling activity can be demonstrated by inhibiting its CRM1-dependent nuclear export with Leptomycin B (LMB) (right panel, two transfected cells shown). **C.** A3G as an N-terminal mCherry chimeric protein was expressed in transfected HEK293T cells and visualized in live cells. A3G is restricted to the cytoplasm because it has no nuclear localization signal but does have a cytoplasmic retention signal. **D.** Natively expressed A3G in synchronized H9 cells after fixing and staining with anti-A3G polyclonal antibody. DAPI stained mitotic chromosomes

(left top, anaphase; left bottom, telophase) are segregated in the mitotic cells from cytoplasmic A3G. Original magnification was 40X.