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Direct Evidence that RNA Inhibits APOBEC3G ssDNA Cytidine Deaminase Activity

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Abstract

APOBEC3G (A3G) is a deoxycytidine deaminase active on ssDNA substrates. In HIV infected cells A3G interacted with reverse transcription complexes where its activity as a deoxycytidine deaminase led to mutation of the viral genome. A3G not only bound ssDNA, but it also had an intrinsic ability to bind RNA. In many cell types that can support HIV replication, A3G ssDNA deaminase activity was suppressed and the enzyme resided in high molecular mass, ribonucleoprotein complexes associated with cytoplasmic P-bodies and stress granules. Using a defined *in vitro* system, we show that RNA alone was sufficient to suppress A3G deaminase activity and did so in an RNA concentration-dependent manner. RNAs of diverse sequences and as short as 25 nucleotides were effective inhibitors. Native PAGE analyses showed that RNA formed ribonucleoprotein complexes with A3G and in so doing prevented ssDNA substrates from binding to A3G. The data provided direct evidence that A3G binding to cellular RNAs constituted a substantial impediment to the enzyme's ability to interact with ssDNA.

Keywords

APOBEC; deaminase; ribonucleoprotein; RNA; ssDNA; HIV

1. Introduction

A3G belongs to a family of cytidine deaminases that have RNA and/or DNA editing activity, that includes activation-induced deaminase (AID), APOBEC1, APOBEC2, APOBEC3A-H, and APOBEC4 [1–3]. These enzymes mediate the hydrolytic deamination of C or dC residues, thus converting C/dC to U/dU [2]. Some APOBEC3 proteins, including A3G have two zinc-dependent deaminase domains (ZDD). The N-terminal ZDD of A3G is catalytically inactive and is required for RNA binding while the C-terminal ZDD is an active deaminase domain. While each ZDD has a different function, both contributed to A3G's antiviral activity [4–6].

In 2002, subtractive hybridization experiments performed by Sheehy, *et al.* identified A3G as an anti-viral factor [7]. During viral replication, A3G extensively deaminated the viral minus strand ssDNA, converting dC to dU residues [8,9]. The fate of A3G hypermutated

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viral DNAs was either to be destroyed by DNA repair enzymes [10] or become integrated into host cell chromosomes where they potentially encoded mutant viral proteins [11–13].

Current hypotheses predicted that in order for A3G to be able to attack viral replication complexes, it must be encapsidated within viral particles during their assembly and enter cells with the virus upon infection [14,15]. This was predicted to enable A3G to gain immediate access post-entry to HIV replication complexes where it physically blocked reverse transcription [16,17] and hypermutated nascent proviral DNA through cytidine deamination [8,12]. Packaging of A3G with virions occurred rapidly after A3G synthesis [18,19]. To do so, A3G interacted with the nucleocapsid portion of the HIV Gag polyprotein as well as host cell or viral RNAs [18,20,21].

A3G expressed in the H9 T cell line, mitogen-activated CD4+ T cells, and monocytes formed high molecular mass (HMM) ribonucleoprotein complexes predominantly localized in cytoplasmic P-bodies and stress granules through the nonspecific binding to cellular mRNAs, tRNAs, and rRNAs [22–24]. In this state, A3G pre-existing in a cell that has undergone an infection by HIV did not have sufficient anti-HIV activity to inhibit the virus [23]. RNase A digestion of biochemical isolates of HMM complexes reduced A3G to low molecular mass (LMM) protein monomers and dimers [25–27] and restored A3G enzymatic activity. In fact, RNase H degradation of the HIV RNA genome was required during viral replication to activate A3G deaminase activity on single stranded proviral DNA during viral RNA reverse transcription [19]. These findings suggested that P-body and stress granule association of A3G impaired deaminase-dependent and deaminase-independent host defense activities. We have postulated that disruption of A3G binding to RNA may activate antiviral activities that are otherwise latent in an A3G expressing but HIV permissive cell [28]. We showed using a defined *in vitro* system that RNA alone was sufficient to inhibit A3G deaminase activity and that RNA complex formation with A3G inhibited the ability of the enzyme to bind to ssDNA.

2. Materials and Methods

2.1. RNAs, ssDNA and primers

Nucleic Acids used for A3G binding and deaminase assays:

ApoB 99 RNA 5'-
GGGAACAAAAGCTGGGTACCGGGCCCCCTCGAGGTCGATGC
AGACATATATGTACAATTTGATCAGTATATTAAAGATAGTTATGATTTACAA
GCT-3',
ApoB 25 RNA 5'-CAUUAUUGAUACAAUUUGAUCAGUA-3' (Sigma-Aldrich®),
ApoB 20 RNA 5'-AUGAUACAAUUUGAUCAGUA-3' (Sigma-Aldrich®),
ApoB 15 RNA 5'-ACAAUUUGAUCAGUA-3' (Sigma-Aldrich®),
ApoB 12 RNA 5'-AUUUGAUCAGUA-3' (Sigma-Aldrich®),
ApoB 10 RNA 5'-UUGAUCAGUA-3' (Sigma-Aldrich®),
7SL RNA 5'-
CCGGGCGCGGUGGCGCGGCCUGUAGUCCAGCUACUCGGGAGGC
UGAGGAGGGAGGAUCGCUUGAAACAAUAGCGAGACCCCGUCUCUG-3',
HIV-1 Gag RNA (1573-1668) 5'-GUUGGAAAUGUGGAAAGGAAGGACACCAA
AUGAAAGAUUGUACUGAGAGACAGGCUAAUUUUUAGGGAAGAUCUGGC
CUUCC UACAAGGGAAGGC-3',

41 nt ssDNA substrate 5'-

ATTATTATTATTATTATTATTATCCCAAGGATTTATTTATTTA-3' (Sigma-Aldrich®)

primer for poisoned primer extension (5'-TAAATAAATAAATCC-3') (Sigma-Aldrich®). ApoB 99 RNA, 7SL RNA, and Gag RNA all were transcribed *in vitro* using mMessage mMachine® kit (Ambion®).

2.2. 5' Radiolabeling of RNAs, ssDNA substrate and primer

RNA, ssDNA substrate or the primer used to quantify deaminase activity (250 pmol) were 5' end radiolabeled with ^{32}P - γ -ATP (6,000 Ci/mmol) using T4 polynucleotide kinase (Roche) and purified using a 15% denaturing PAGE.

2.3. EMSA with 5' radiolabeled RNA's and 41nt ssDNA substrate

The radiolabeled RNAs and ssDNA substrate were incubated at varying molar ratios of A3G to ssDNA/RNA in deaminase buffer (40 mM Tris pH 7.2, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 0.1 % Triton X-100, 2 % glycerol) at 37 °C for 20 min. The resulting complexes were resolved on either an 8% or a 5% native gel and visualized and quantified using screens and a Typhoon™ phosphorimager.

2.4. RNA competition for A3G ssDNA deaminase activity and A3G:ssDNA complex formation

The RNA used for competition studies was first incubated with 1.75 μM of purified A3G at the indicated molar ratio (0-6 μM) for 5 min at 37 °C. The ssDNA was then added for a final concentration of 0.06 μM and was incubated for 60 min in deaminase buffer at 37 °C. Deaminase activity on the ssDNA substrate was detected by a poisoned primer extension assay described previously [29], and quantified by Phosphorimager scanning densitometry. The percentage of deamination was calculated by visualizing and quantifying the primer extension products by phosphorimager densitometry and percentages were calculated by dividing the volume of the deaminated substrate (dU) by the total of deaminated and unmodified substrates (dU+dC). Alternatively, radiolabeled ssDNA substrate was used in the competition reactions and the resulting complexes were then resolved on a 5% native gel, and visualized using a Typhoon™ phosphorimager by autoradiography.

3. Results

We and others have shown that A3G binds to and deaminates short ssDNA substrates under defined *in vitro* conditions consisting only of recombinant A3G and ssDNA [27,30]. The size of the A3G:ssDNA complexes assembled on a given length of ssDNA substrate (determined by electrophoretic gel mobility shift assays, EMSA) and the size heterogeneity of complexes was largely dependent on the concentration of A3G in the reaction [31] (Figure 1A). A3G:ssDNA complex formation was not dependent on whether the ssDNA contains a 'hotspot' for dC deamination as indicated by efficient complex formation with the (dU)₃ ssDNA (Figure 1B).

A3G also has been shown to bind RNA [2,27,30,32]. EMSA analysis showed efficient assembly of ribonucleoprotein complexes formed with an AU-rich RNA previously reported to have the highest affinity binding to A3G [2] (Figure 1C). A3G [28] ribonucleoprotein complexes did not have the same EMSA banding pattern as A3G:ssDNA complexes, however the RNA and ssDNA differed in length (99 nt versus 41 nt) and had different sequences. We therefore evaluated EMSA banding pattern of HIV and 7SL RNAs (96 nt and 91 nt, respectively) that are known to bind to A3G in HIV infected cells [20,21,33]. A3G

formed ribonucleoprotein complexes efficiently with these RNAs in an A3G concentration-dependent manner (Figure 1D and E, respectively); though once again, the complexes that were formed by each RNA appeared markedly different than that observed for either ssDNA and also dissimilar from those seen with the AU-rich RNA even though they were all of the same length. The data suggested therefore that complexes formed by A3G on ssDNA are not the same as those formed on RNA.

Full length A3G was required for optimal ssDNA deaminase activity and RNA binding [32,34]. It is not known whether the catalytically active C-terminal ZDD binds to ssDNA substrates as well as RNA or whether the N-terminal non-catalytic ZDD participates in ssDNA binding or RNA binding [32,35]. If ssDNA and RNA bound to the same site on A3G, then one might anticipate that these nucleic acids would compete for A3G binding.

We conducted RNA competition analysis to evaluate the relationship of A3G ssDNA binding and ribonucleoprotein complex formation. A3G:ssDNA complexes assembled on radiolabeled ssDNA substrate as in Figure 1A were incubated with increasing concentrations of AU-rich RNA and the reactions were resolved by EMSA. RNA destabilized A3G:ssDNA complexes as evident by the RNA concentration-dependent inhibition of the formation of low mobility A3G:ssDNA complexes and the corresponding appearance of higher mobility A3G:ssDNA complexes (Figure 2A). Most of the ssDNA remained unbound to A3G at the highest concentrations of RNA tested. Consistent with the inhibition of ssDNA substrate binding, A3G deaminase activity on the ssDNA substrate was inhibited in an RNA concentration-dependent manner (Figure 2B). Both HIV Gag and 7SL RNAs inhibited ssDNA binding to A3G (Figure 2C and E, respectively) and both inhibited A3G deaminase activity (Figure 2D and F, respectively) in a RNA concentration-dependent manner. Given the diversity in sequence and markedly different predicted propensity of each of the three RNA segments tested to form stable secondary structures, the data suggested that the inhibition of ssDNA substrate binding to A3G was a general consequence of RNA binding to A3G. The data also suggested that RNA inhibition of deaminase activity resulted from the inability of A3G to bind to ssDNA when RNA was bound to A3G.

We evaluated whether RNAs of shorter lengths retained the ability to compete for ssDNA binding to A3G. AU-rich RNAs of decreasing length (25 nt, 20 nt, 15 nt, 12 nt and 10 nt) were evaluated for their ability to form ribonucleoprotein complexes with A3G. EMSA revealed efficient complex formation in an A3G concentration-dependent manner for RNAs of 25 nt to 15 nt (Figure 3A, B and C). The diversity in size of complexes formed on shorter RNAs was less than that observed with longer RNAs (Figure 1C-E) and A3G complexes formed on shorter RNAs were more sharply defined in their mobility. This was especially evident with the 15 nt RNA where A3G input increased the yield of one major EMSA complex. RNAs shorter than 15 nt were not able to form ribonucleoprotein complexes with A3G efficiently (Figure 3D and E). These data suggested that smaller RNAs had a limited capacity to bind to A3G and were less competent in the RNA-dependent oligomerization of A3G.

Given this finding, we asked whether shorter RNAs retained the ability to inhibit A3G binding to and deamination of ssDNA. RNA competition analyses were conducted as described in Figure 2 and evaluated by EMSA. The 25 nt RNA inhibited ssDNA binding to A3G in an RNA concentration-dependent manner (Figure 4A) but far less effectively than longer RNAs (Figure 2A,C,E). RNAs 20 nt and 15 nt did not inhibit ssDNA substrate binding (Figure 4B and C). As anticipated, the 25 nt RNA inhibited A3G deaminase activity in an RNA concentration-dependent manner (Figure 4D) but the 20 nt and 15 nt RNAs did not (Figure 4E and F). The data suggested that although small RNAs bound to A3G, RNAs

of 25 nt and longer were required to induce higher-order complex formation and this was associated with RNA being able to inhibit ssDNA binding to A3G and deaminase activity.

4. Discussion

A3G formed homodimers and homotetramers through protein-protein interactions in the absence of nucleic acids [26,34] but, tetramers and higher-order complexes were also formed through nucleic acid bridging of A3G subunits [21,27,31,35–37]. Tetramers and higher order complexes of A3G with ssDNA are required for deaminase activity [31]. In contrast, A3G deaminase activity was inactivated through the formation of ribonucleoproteins whether they were cytoplasmic P-bodies or stress granules or assembled on viral RNA genomes and cellular RNAs during encapsulation in the core of viral particles [19]. The mechanism whereby A3G becomes enzymatically inactivated is unknown although reactivation by RNase digestion *in vitro* [27] or by RNase H digestion of the HIV RNA genome during reverse transcription [19] suggested that RNA binding to A3G alone may have inhibited the enzyme.

Using a defined *in vitro* system consisting of native and full length human A3G, short ssDNA substrate and short RNAs we showed that RNA alone was sufficient to inhibit A3G deaminase activity on ssDNA. We demonstrated that RNA binding to A3G inhibited ssDNA binding to A3G and did so in an RNA concentration-dependent manner. RNAs of diverse sequence and secondary structure were efficient in inhibiting A3G binding to and deaminating ssDNA.

The simplest explanation for our data is that RNA competitively inhibited ssDNA binding to A3G as a consequence of both nucleic acids interacting with the same site, the C-terminal ZDD. In support of this possibility, APOBEC1 and the yeast homolog of APOBEC1, CDD1 use a single ZDD to bind to ssDNA and RNA. The RNA concentration-dependent inhibition of A3G:ssDNA complex formation shown here was consistent with a competitive mechanism of inhibition. There is however no evidence that the C-terminal ZDD alone can bind RNA. In fact the N- and C-terminal ZDD were both required for RNA binding [38] and optimal ssDNA deaminase activity [32,34]. We therefore cannot rule out the possibility that RNA and ssDNA bind to different sites on A3G and that RNA binding to A3G may have induced a conformational change in A3G that no longer was able to bind to ssDNA. The possibility that A3G forms unique conformations depending on whether it was bound to ssDNA or RNA was supported by the markedly different EMSA banding patterns for A3G complexes formed with these nucleic acids. In this case, our data also could be explained by RNA acting as an allosteric inhibitor of A3G deaminase activity. Additional kinetic experiments will be required to better understand the mechanism of RNA inhibition of ssDNA binding to A3G.

Interestingly, RNAs shorter than 25 nucleotides were not effective inhibitors and although these RNAs assembled into complexes with well-defined mobility, they were not capable of forming higher-order complexes. Therefore our data additionally suggested that homo-oligomeric ribonucleoprotein complexes of A3G were required for the RNA-dependent inhibition of A3G binding to ssDNA.

We propose that the findings presented here have important implications for the biology of A3G in host defense. Given the high abundance of cellular RNAs in the cytoplasm, it is likely that A3G will be bound to RNA and enzymatically inactive. Our *in vitro* data therefore corroborated *in vivo* data showing that A3G contained in cellular HMM complexes were enzymatically inactive and incapable of providing host defense [23,39]. There are conditions where A3G was observed to be in lower molecular mass complexes with

deaminase activity [25]. It is currently controversial whether these low molecular mass complexes of A3G can provide host defense activity sufficient to inhibit HIV infectivity [28]. Cellular mechanisms may regulate A3G interactions with cellular RNAs and these would enable the assimilation of A3G within P-bodies and stress granules to be reversible. A3G post-translational modifications such as protein phosphorylation may contribute to this mechanism [40,41]. The data presented here suggested that factors or therapeutics that can modulate A3G binding to RNA have the potential to activate A3G deaminase activity and may enable the efficacy of A3G in antiviral host defense.

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RNA inhibits A3G binding to ssDNA
RNA regulates ssDNA deaminase activity
A3G novel drug target

FIGURE 1. A3G assembles multiple complexes on ssDNA and RNA

EMSA using [^{32}P] γ -ATP 5' end-labeled 41 nt ssDNA or RNA. The concentration of ssDNA in each reaction was 0.6 μM and the concentration of A3G in each lane was 0 μM , 0.22 μM , 0.44 μM , 0.86 μM . Shifts were visualized by phosphorimager scanning densitometry by virtue of their labeled ssDNA content. The gels shown are representative of 4 independent experiments. A. EMSA using 41nt substrate ssDNA containing CCCA (hot spot motif) B. EMSA using 41nt substrate ssDNA containing UUUA. For EMSA using RNAs the concentration of RNA in each reaction was 2.0 μM whereas the concentration of A3G in each lane was 0 μM , 0.03 μM , 0.06 μM , 0.11 μM , 0.22 μM , 0.44 μM , 0.86 μM . Shifts were visualized by phosphorimager scanning densitometry by virtue of their labeled RNA content. The gels shown are representative of 3 independent experiments. C, D and E are EMSAs using 99nt ApoB RNA, 96nt HIV GAG RNA and 91nt 7SL RNA respectively.

FIGURE 2. RNA competes with ssDNA for A3G binding and deaminase activity

EMSA of RNA competition used [32 P] γ -ATP 5' end-labeled 41 nt substrate ssDNA. The concentration of ssDNA in each reaction was 0.6 μ M and the concentration of A3G was 1.75 μ M. A. The concentration of ApoB 99 RNA, HIV GAG RNA and 7SL (Alu domain) RNA (A, C and E respectively) increased from 0 μ M to 60 μ M. Each gel is representative of 3 independent experiments. The effect of apoB RNA, HIV GAG RNA and 7SL (Alu domain) RNA (B, D and F respectively) on deaminase activity was assayed in parallel reactions (set up as described above) by poisoned primer extension. The lowest arrow on the left indicates free primer, the middle arrow indicates the primer extension product for deaminated ssDNA (dU) and the upper arrow indicates the primer extension product for unmodified ssDNA (dC). The percent deaminated ssDNA (%dU/dC) was determined by phosphorimager scanning densitometry and calculated as dU divided by (dU + dC) times 100. Each gel is representative of 3 independent experiments.

FIGURE 3. Size dependence of A3G assembly on RNA

The concentration of [^{32}P] γ -ATP 5' end-labeled RNA in each reaction was 2.0 μM , whereas the concentration of A3G varied from 0 μM , 0.03 μM , 0.06 μM , 0.11 μM , 0.22 μM , 0.44 μM , 0.86 μM . Shifts were visualized by phosphorimager scanning densitometry by virtue of their labeled ssDNA content. A. 25nt ApoB RNA, B. 20nt ApoB RNA, C. 15nt ApoB RNA, D. 12nt ApoB RNA, E. 10nt ApoB RNA. The gels are representative of 4 independent experiments.

Figure 4. Size dependence of RNA competition

RNA competition for EMSA used 0.6 μM of [^{32}P] γ -ATP 5' end-labeled 41 nt substrate ssDNA and 1.75 μM of A3G. The concentration of the 25 nt, 20 nt and 15 nt apoB RNAs (A, B and C respectively) increased from 0 μM to 60 μM . RNA inhibition of deaminase activity was assayed in parallel reactions set up as described by poisoned by poisoned primer extension on 41 nt ssDNA that following incubated with A3G with or without RNA. All gels are representative of 2 independent experiments.