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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 nt 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.

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 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.

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2. Materials and methods

2.1. RNAs, ssDNA and primers

Nucleic acids used for A3G binding and deaminase assays:

ApoB 99 RNA 5′-GGGAACAAAAGCTGGGTACCGGGCCCCCCCTC-GAGGTCGATGC AGACATATATGTACAATTTGATCAGTATATTAAAGAT AGTTATGATTTACAAGCT-3′, ApoB 25 RNA 5-′CAUAUAUGAUACAAU UUGAUCAGUA-3′ (Sigma–Aldrich®), ApoB 20 RNA 5′-AUGAUA CAAUUUGAUCAGUA-3′ (Sigma–Aldrich®), ApoB 15 RNA 5′-ACAA UUUGAUCAGUA-3′ (Sigma–Aldrich®), ApoB 12 RNA 5′-AUUUGAUC AGUA-3′ (Sigma–Aldrich®), ApoB 10 RNA 5′-UUGAUCAGUA-3′ (Sigma–Aldrich®), 7SL RNA 5′-CCGGGCGCGGUGGCGCGCGCCUGUA GUCCCAGCUACUCGGGAGGCUGAGGAGGGAGGAUCGCUUGAAACA AUAGCGAGACCCCGUCUCUG-3′, HIV-1 Gag RNA (1573-1668) 5′-GUUGGAAAUGUGGAAAGGAAGGACACCAAAUGAAAGAUUGUACUG AGAGACAGGCUAAUUUUUUAGGGAAGAUCUGGCCUUCCUACAAGG GAAGGC-3′, 41 nt ssDNA substrate 5′-ATTATTATTATTATTATTATTC CCAAGGATTTATTTATTTA-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 (6000 Ci/mmol) using T4 polynucleotide kinase (Roche) and purified using a 15% denaturing PAGE.

2.3. EMSA with 5′ radiolabeled RNA's and 41 nt 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™ phosphorim ager.

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 purifiedA3G 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] (Fig. 1A).

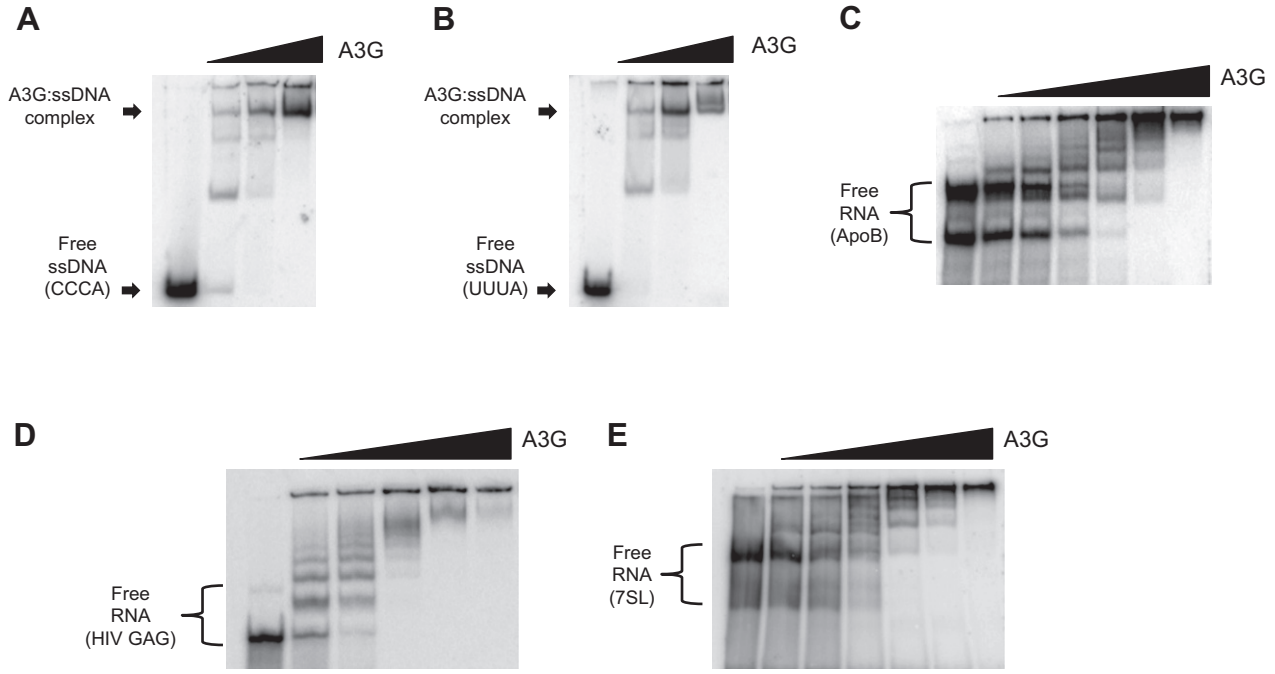

Fig. 1. A3G assembles multiple complexes on ssDNA and RNA.EMSA using [^{32}P] γ-ATP 5′ end-labeled 41 nt ssDNA or RNA. The concentration of ssDNA in each reaction was 0.6 μM and the concentration of A3G in each lane was 0, 0.22, 0.44, 0.86 μM. Shifts were visualized by phosphorimager scanning densitometry by virtue of their labeled ssDNA content. The gels shown are representative of four independent experiments. (A) EMSA using 41 nt substrate ssDNA containing CCCA (hot spot motif). (B) EMSA using 41 nt 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, 0.03, 0.06, 0.11, 0.22, 0.44, and 0.86 μM. Shifts were visualized by phosphorimager scanning densitometry by virtue of their labeled RNA content. The gels shown are representative of three independent experiments. (C, D and E) are EMSAs using 99 ntApoB RNA, 96 nt HIV GAG RNA and 91 nt 7SL RNA, respectively.