Deaminase-independent inhibition of HIV-1 reverse transcription by APOBEC3G

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ABSTRACT

APOBEC3G (A3G), a host protein that inhibits HIV-1 reverse transcription and replication in the absence of Vif, displays cytidine deaminase and single-stranded (ss) nucleic acid binding activities. HIV-1 nucleocapsid protein (NC) also binds nucleic acids and has a unique property, nucleic acid chaperone activity, which is crucial for efficient reverse transcription. Here we report the interplay between A3G, NC and reverse transcriptase (RT) and the effect of highly purified A3G on individual reactions that occur during reverse transcription. We find that A3G did not affect the kinetics of NC-mediated annealing reactions, nor did it inhibit RNase H cleavage. In sharp contrast, A3G significantly inhibited all RT-catalyzed DNA elongation reactions with or without NC. In the case of (−) strong-stop DNA synthesis, the inhibition was independent of A3G’s catalytic activity. Fluorescence anisotropy and single molecule DNA stretching analyses indicated that NC has a higher nucleic acid binding affinity than A3G, but more importantly, displays faster association/disassociation kinetics. RT binds to ssDNA with a much lower affinity than either NC or A3G. These data support a novel mechanism for deaminase-independent inhibition of reverse transcription that is determined by critical differences in the nucleic acid binding properties of A3G, NC and RT.

INTRODUCTION

Human APOBEC3G (A3G) is a host cytidine deaminase, which was first identified by Sheehy et al. (1) as the cellular factor that blocks HIV-1 replication in the absence of the viral Vif protein. Cellular expression of A3G results in its incorporation into vif-deficient HIV-1 particles, whereas its presence in WT virions is dramatically reduced by Vif-induced degradation via the ubiquitination-proteosome pathway (2).

Initial studies suggested that the deamination activity of A3G contributes to its antiviral activity and is associated with G to A hypermutation (3–6). However, more recent results indicate that a deaminase-independent mechanism might also be involved in A3G’s antiviral activity: (i) Deamination activity is not absolutely correlated with antiviral activity against HIV-1 (7–15); (ii) Several reports (though not all) indicate that hepatitis B virus may be inhibited by A3G without significant detection of G to A hypermutation (16–18) and (iii) Other APOBEC proteins block replication of mouse mammary tumor virus (19) and several retrotransposons (14,20–24) in the absence of editing activity.

Prior to the discovery of A3G, it was already known that Δvif viruses produced in ‘nonpermissive’ cells (PBMCs or certain T-cell lines, e.g. H9) were 100- to
1000-fold less infectious than WT (25–27) and were deficient in their ability to complete reverse transcription (28–31). Moreover, similar results were obtained in endogenous reverse transcription assays (30,32,33). More recent analysis of HIV-1-infected cells expressing A3G or A3F has confirmed the initial observations (5,10,13,15,34–38).

Efficient and specific reverse transcription depends on the viral nucleocapsid protein (NC), which functions as a nucleic acid chaperone (39–43). This means that NC can catalyze nucleic acid conformational rearrangements that lead to the most thermodynamically stable structures (44). Like NC, A3G has two zinc finger domains and binds nucleic acids (45). However, whereas A3G has a strong preference for binding single-stranded (ss) nucleic acids (6,12), NC binds ss or double-stranded (ds) RNA and DNA (46) [for further information on NC’s nucleic acid binding properties, see Ref. (47)]. The two proteins also differ in their effects on virus replication: NC acts as a positive factor, whereas A3G is an inhibitor in the absence of Vif.

We previously reported the successful preparation of highly purified, catalytically active A3G expressed in a baculovirus system and demonstrated that the availability of a pure protein (without contamination by other proteins, either host or viral) was invaluable for rigorous analysis of the biochemical properties of A3G (12). In the course of this study, we unexpectedly found that A3G does not interfere with NC binding to ssRNA (and vice versa) (12). This suggested that inhibition of reverse transcription by A3G is likely to be unrelated to an effect on NC chaperone function.

To test this hypothesis and to probe the mechanism that might be involved, we took advantage of defined biochemical assay systems that we have developed over the years for studies on viral DNA synthesis (48–51). Thus, using our highly purified A3G as well as purified NC and RT, we investigated the effect of A3G on a series of reconstituted reactions that occur during reverse transcription. This allowed us to perform an independent analysis of individual steps in the pathway, which is not possible in cell-based systems. We found that A3G inhibited all reverse transcriptase (RT)-catalyzed DNA elongation reactions, but not RNase H activity or NC’s ability to promote annealing. These observations could be explained by critical differences in the binding properties of NC, A3G and RT, as measured by single-molecule DNA stretching and fluorescence anisotropy (FA). Our findings are unique and provide strong support for a novel mechanism that could account for the observed deaminase-independent A3G-mediated antiviral activity.

**MATERIALS AND METHODS**

**Materials**

Purified tRNA Lys from human placenta was obtained from Bio S&T (Lachine, Quebec, Canada). DNA and RNA oligonucleotides were purchased from Lofstrand (Gaithersburg, MD), Integrated DNA Technologies (Coralville, IA), Oligos Etc., Inc. (Wilsonville, OR). [γ-32P]ATP (3000 Ci/mmole) and [γ-32P]dCTP (6000 Ci/mmole) were purchased from GE Healthcare (Piscataway, NJ). HIV-1 RT was obtained from Worthington Biochemical Corp. (Lakewood, NJ). Calf intestinal phosphatase, T4 polynucleotide kinase, and Vent DNA polymerase were obtained from New England Biolabs (Beverly, MA). SUPERaseIn, an RNase inhibitor, was purchased from Ambion, Inc. (Austin, TX). Recombinant wild-type HIV-1 NC (55-amino-acid form) was a generous gift from Dr Robert Gorelick and was prepared as described previously (52,53). Recombinant enzymatically active A3G and the deaminase-deficient A3G mutant (C291S) were expressed in a baculovirus expression system and purified as previously described (12). A3G preparations were confirmed to be free from contamination with RNases (data not shown) and no RNA degradation was apparent in any of the experiments (e.g. see Figure 2A).

**Methods**

**Plasmid construction.** All plasmid sequences were derived from the HIV-1 pNL4-3 clone (54). Plasmid pUL (viral insert from nt 566 to 1419) was constructed from the previously described pRUG plasmid (51).

**Preparation of RNA.** The DNA templates for in vitro RNA transcription were derived from plasmid pRUG [for transactivation response element (TAR) RNA, nucleotide(s) (nt) 1–59] or pUL (for RNA UL244, nt 113–244). DNA fragments containing both the T7 promoter and the DNA sequence equivalent to the desired vRNA were amplified by PCR using Vent DNA polymerase and the following primers: forward primer (5′-caatgctaattgtgggataataactgt, located at the start of the ampr gene in pUL; reverse primers, 5′-gtgctgctgtaggag (RNA UL244) and 5′-ggctccagactgacag (TAR RNA). The DNA fragments were gel-purified and transcribed using an Ambion MEGAscript kit (Ambion Inc., Austin, TX). Gel-purified RNAs were dephosphorylated by calf intestinal phosphatase and were then labeled at their 5′ ends with [γ-32P]ATP, using T4 polynucleotide kinase (55). Unincorporated nucleotides were removed by passing the reaction mixture through NucAway Spin Columns (Ambion). RNA 244 (51) and acceptor RNA 148 (49) were prepared as described.

**Reverse transcription assays.** Reaction components were given for 20 μl unit reactions, which were scaled up as needed. SUPERaseIn at a final concentration of 0.5 U/μl was added to all RNA-containing reaction mixtures. Incubation/preincubation was always at 37°C. Six percent native or denaturing polyacrylamide gels were used for annealing or extension assays, respectively, except as noted. Radioactivity was quantified by using a Typhoon PhosphorImager and ImageQuant software.

**tRNA Lys annealing to viral RNA (vRNA).** Reaction mixtures contained buffer (50 mM Tris·HCl (pH 8.0), 75 mM KCl, 0.1 mM MgCl₂, 1 mM DTT), 0.2 pmol of purified human placental tRNA Lys and 0.1 pmol of 5′-32P-labeled RNA UL244 and were incubated in the
absence or presence of A3G (80 nM) with or without HIV-1 NC (7 nM/NC, 0.2 μM). Prior to loading on the gel, the samples were treated with Proteinase K (0.5 mg/ml).

(−) Strong-Stop DNA [−(−) SSDNA] synthesis. Template RNA 244 (0.1 pmol) and 0.1 pmol each of human tRNA\textsubscript{Lys} or \textsuperscript{32}P-labeled D18 primer, [complementary to the 18-nt primer-binding site (PBS)] were heat-annealed as described (51). A3G or heat-denatured (hd)A3G was then added to buffer designated as 'reaction buffer' (50 mM Tris–HCl (pH 8.0), 75 mM KCl, 7 mM MgCl\textsubscript{2} and 1 mM DTT) and the mixture was preincubated for 5 min. Primer extension was initiated by adding HIV-1 RT (0.1 pmol) and 50 μM each of dATP, dTTP and dGTP plus 10 μCi of [\textalpha\textsuperscript{32}P]dCTP (tRNA\textsubscript{Lys}) or 50 μM each of all four dNTPs (D18).

Annealing and minus-strand transfer. Minus-strand annealing was performed as described previously (56) in the presence or absence of A3G (80 nM) with or without HIV-1 NC (3.5 nt/NC, 0.4 μM). For minus-strand transfer assays, reaction mixtures contained the components present in annealing reactions as well as HIV-1 RT (0.4 pmol), all four dNTPs (each at 50 μM), and 1 mM MgCl\textsubscript{2}.

Initiation of plus-strand DNA synthesis. The assay was performed using a modified version of a previous protocol (57). Briefly, the 15-nt polypurine tract (PPT) primer was heat-annealed to a 35-nt minus-strand primer-binding site (PBS) and \( A \times \text{dCTP} \times \text{tRNALys} \) or \( A \times \text{dATP} \times \text{dTTP} \times \text{dGTP} \times \text{tRNA} \) mixture was preincubated for 5 min. Primer extension was assayed using a modified version of a previous protocol (expression).

RESULTS
Effect of A3G on primer placement and (−) SSDNA synthesis

To determine the mechanism by which A3G inhibits HIV-1 DNA synthesis, we analyzed the effect of A3G on single steps in the reverse transcription pathway (Figure 1) (43). Annealing and DNA synthesis were assayed in a series of reconstituted model systems. Our goal was to determine whether A3G interfered with the nucleic acid chaperone activity of NC, the catalytic activity of RT, or both during these reactions. Note that in most of the assays, the concentration of A3G did not exceed 80 nM, since A3G precipitates at high concentrations (12).

To evaluate the effect of A3G on the first step in reverse transcription (primer placement), we investigated...
the time course of NC-dependent annealing of human tRNA\text{\textsubscript{Lys}}\text{\textsubscript{3}} to the PBS in a short vRNA template (RNA UL244) (Figure 2A and B). In the absence of NC and A3G, no annealing was detected. As shown in Figure 2B, the rates of annealing in the presence of NC were similar for reactions with and without A3G and the end point values at 64 min were 70% and 66%, respectively. Addition of NC and hdA3G resulted in a slightly enhanced rate of annealing, but the end point value was very close to the values with and without native A3G. These results demonstrate that A3G did not interfere with NC-mediated formation of the vRNA–tRNA complex.

Figure 1. Schematic diagram of the events in reverse transcription. Step 1. Reverse transcription is initiated by a cellular tRNA primer (tRNA\text{\textsubscript{Lys}}\text{\textsubscript{3}}), in the case of HIV-1, following annealing of the 3’ 18 nt of the tRNA to the 18-nt PBS near the 5’ end of the genome. RT catalyzes synthesis of (+) SSDNA, which contains copies of the R sequence and the unique 5’ genomic sequence (U5). Step 2. As the primer is extended, the RNase H activity of RT degrades the genomic RNA sequences that have been reverse transcribed. Step 3. (+) SSDNA is transferred to the 3’ end of vRNA (minus-strand transfer). Step 4. Elongation of minus-strand DNA and RNase H degradation continue. Plus-strand synthesis is initiated by the 15-nt PPT immediately upstream of the unique 3’ genomic sequence (U3). Step 5. RT copies the u3, u5 and r regions in minus-strand DNA, as well as the 3’ 18 nt of the tRNA primer, thereby reconstituting the PBS. The product formed is termed (+) SSDNA. Step 6. RNase H removal of the tRNA and PPT primers from minus- and plus-strand DNAs, respectively. Step 7. Plus-strand transfer, facilitated by annealing of the complementary PBS sequences at the 3’ ends of (+) SSDNA and minus-strand DNA, is followed by circularization of the two DNA strands and displacement synthesis. Step 8. Minus- and plus-strand DNAs are elongated, resulting in a linear dsDNA with a long terminal repeat (LTR) at each end. vRNA is shown by an open rectangle and minus-and plus-strand DNAs are shown by black and gray rectangles, respectively. The tRNA primer is represented by a short open rectangle (3’ 18 nt of the tRNA) attached to a “clover-leaf” (remaining tRNA bases). Minus- and plus-strand sequences are depicted in lower and upper case, respectively. The very short white rectangles represent fragments produced by RNase H cleavage of genomic RNA. Adapted from reference (43) with permission from Elsevier.

Figure 2B, the rates of annealing in the presence of NC
SSDNA synthesis. To address this question, we used purified deaminase-deficient A3G C291S protein (12) and then compared the effect of WT A3G or C291S on D18-primed (-) SSDNA synthesis. As shown in Figure 4, the rates and extents of (-) SSDNA synthesis in the presence of WT or mutant A3G were virtually the same, in accord with the observation that WT and C291S proteins have similar $K_d$ values for binding to ss nucleic acids (12). The data of Figure 4 are significant and demonstrate that the inhibitory effect of A3G on (-) SSDNA synthesis is independent of A3G deaminase activity in our assay system.

Effect of A3G on minus-strand transfer reactions

Synthesis of a full-length minus-strand copy of the vRNA genome is achieved by transfer of (-) SSDNA to the 3′ end of vRNA ('acceptor RNA') followed by RT-catalyzed extension of the annealed DNA (Figure 1, step 3). This process (minus-strand transfer) is facilitated by base pairing of the complementary repeat regions at the 3′ ends of the nucleic acid substrates and is dependent on RNase H cleavage of vRNA sequences annealed to (-) SSDNA (43).

In Figure 5A, we show the time course of RNase H cleavage with the heat-annealed 59-bp TAR RNA/DNA hybrid, which was incubated with and without NC, in the absence or presence of A3G. Reactions containing A3G appeared to have slightly faster cleavage rates. However, the overall cleavage pattern was the same under all four conditions. These results indicated that A3G does not interfere with RNase H cleavage and is consistent with A3G’s limited ability to bind to an RNA–DNA hybrid (6,12).

To assay the effect of A3G on minus-strand transfer, we first measured NC-mediated annealing using DNA 128 and acceptor RNA 148 (see schematic diagram in Figure 5C) in the absence or presence of A3G (Figure 5B). NC is required to transiently destabilize the complementary TAR RNA and DNA structures within the repeat region, before hybrid formation can occur (43).
Interestingly, A3G had only a minimal effect on annealing in the presence of NC. The rates were fairly similar with and without A3G and there was only a small reduction (11%) in the extent of the reaction when A3G was added. In a control reaction, we found that the extent of annealing minus NC, plus A3G was 10% (data not shown), in accord with the value obtained in the absence of NC and A3G (63).

We also assayed minus-strand transfer with the complete system, which depends upon both annealing and RT-catalyzed elongation reactions (Figure 5C). The percent of total DNA present as the 182-nt strand transfer product (% Transfer Product) was quantified and plotted versus Time (Figure 5D). As we showed previously (49,64,65), NC significantly enhanced minus-strand transfer. However, when A3G was added, NC-mediated strand transfer was dramatically reduced (Figure 5D). Note that in the absence of NC, the amount of transfer product made was extremely small and little effect of A3G was observed.

Viewed collectively, the results of Figure 5 strongly suggest that A3G inhibited minus-strand transfer by blocking RT-catalyzed DNA elongation. In contrast, A3G did not significantly interfere with NC-facilitated annealing of (−) SSDNA to acceptor RNA. These findings parallel the results obtained for primer placement (Figure 2A and B) and tRNA-primed synthesis of (−) SSDNA (Figure 2C).
plus-strand 32P-labeled 80-nt DNA is detected. In the (Figure 1, steps 6–8) (43). In our assay, only the DNAs to yield a double-stranded DNA product of the complementary PBS sequences in (+) SSDNA and transfer, consists of two steps: (i) NC-mediated annealing by RT. [42x198]RNA-primed DNA-dependent DNA polymerization clear from the data that A3G was also able to inhibit region in the template (12,66). Nevertheless, it is two molecules of A3G can bind to the available 20-nt ss long templates were used for those assays. By contrast, in the experiment shown in Figure 6A, only one or at most two molecules of A3G can bind to the available 20-nt ss region in the template (12,66). Nevertheless, it is clear from the data that A3G was also able to inhibit RNA-primed DNA-dependent DNA polymerization by RT. The plus-strand transfer reaction, like minus-strand transfer, consists of two steps: (i) NC-mediated annealing of the complementary PBS sequences in (+) SSDNA and minus-strand acceptor DNA; and (ii) RT-catalyzed elongation of both the plus- and minus-strand DNAs to yield a double-stranded DNA product (Figure 1, steps 6–8) (43). In our assay, only the plus-strand 32P-labeled 80-nt DNA is detected. In the absence of A3G, NC stimulated plus-strand transfer by 3-fold (Figure 6B), as reported previously (50). When A3G was added, reduction of synthesis was observed beginning at 10–15 min (e.g. at 15 min, by ~4-fold), whereas the extent of the reaction at 120 min was decreased by 2-fold. These results showed that A3G inhibited DNA-dependent DNA polymerization. Taken together, the in vitro data demonstrate that A3G interfered with all of the elongation reactions catalyzed by RT. In contrast, A3G was shown to have virtually no inhibitory effect on NC-mediated annealing reactions.

**Effect of A3G on (+) SSDNA synthesis and plus-strand transfer**

While elongation of minus-strand DNA is being completed, the PPT RNA primer initiates synthesis of a short DNA termed (+) SSDNA (Figure 1, steps 4 and 5) (43). To determine whether A3G interferes with this step, we used a simple oligonucleotide assay that was previously developed in our laboratory (48,57). The 15-nt PPT was heat-annealed to a 35-nt minus-strand DNA template and the hybrid was then incubated with RT, which catalyzed the extension and subsequent removal of the PPT. The amount of 20-nt DNA product formed in reactions with and without A3G was quantified and plotted (Figure 6A).

The results demonstrated that A3G reduced both the rate and extent of (+) SSDNA synthesis by 2- to 3-fold. The effect of A3G on this reaction was lower than what we observed for (−) SSDNA synthesis (Figure 3A and B) or minus-strand transfer (Figure 5D). This is expected since long templates were used for those assays. By contrast, in the experiment shown in Figure 6A, only one or at most two molecules of A3G can bind to the available 20-nt ss region in the template (12,66). Nevertheless, it is clear from the data that A3G was also able to inhibit RNA-primed DNA-dependent DNA polymerization by RT. The plus-strand transfer reaction, like minus-strand transfer, consists of two steps: (i) NC-mediated annealing of the complementary PBS sequences in (+) SSDNA and minus-strand acceptor DNA; and (ii) RT-catalyzed elongation of both the plus- and minus-strand DNAs to yield a double-stranded DNA product (Figure 1, steps 6–8) (43). In our assay, only the plus-strand 32P-labeled 80-nt DNA is detected. In the absence of A3G, NC stimulated plus-strand transfer by 3-fold (Figure 6B), as reported previously (50). When A3G was added, reduction of synthesis was observed beginning at 10–15 min (e.g. at 15 min, by ~4-fold), whereas the extent of the reaction at 120 min was decreased by 2-fold. These results showed that A3G inhibited DNA-dependent DNA polymerization. Taken together, the in vitro data demonstrate that A3G interfered with all of the elongation reactions catalyzed by RT. In contrast, A3G was shown to have virtually no inhibitory effect on NC-mediated annealing reactions.

**Single molecule DNA stretching and FA-binding measurements**

In an effort to understand these results, the nucleic acid binding properties of A3G, NC and RT were investigated using single molecule DNA stretching and FA.

When optical tweezers are used to stretch single DNA molecules by applying forces approaching 60 pN (Figure 7), a force-induced melting transition occurs, in which dsDNA is converted to ssDNA (59). In the absence of protein, this transition occurs over a very narrow force range due to the cooperative melting of DNA. Saturating levels of HIV-1 NC result in a significant increase in the width of the force-induced melting transition (46), which correlates with NC’s relatively efficient chaperone activity. In addition, the reversibility of DNA stretch/relax curves in the presence of NC (i.e., DNA stretch curves show very little hysteresis), suggests that NC has a fast nucleic acid binding on/off rate and is therefore capable of rapidly switching between dsDNA and ssDNA bound states (60).

To probe the nucleic acid binding properties of A3G, we stretched single lambda DNA molecules in the presence of varying amounts of A3G (Figure 7A; data not shown) and characterized the transition width and hysteresis. The transition width increased from 3.7 ± 0.2 pN in the absence of protein to 18.3 ± 2.4 pN at protein saturation (~90 nM A3G and above). Examination of the stretching and relaxation curves showed that there was significant hysteresis (Figure 7A), which increased with A3G concentration, reaching a value of \( \Delta G_{\text{hysteresis}} = 0.56 \pm 0.02 \text{ kcal/mol per bp at 150 nM A3G} \), as compared to \( \Delta G_{\text{hysteresis}} = 0.22 \pm 0.06 \text{ kcal/mol per bp for saturated HIV-1 NC binding (Figure 7B)} \) (60). The greater hysteresis in the presence of A3G reflects the inability of DNA to reanneal due to the presence of bound protein, which dissociates more slowly than the relaxation step time of ~1 s. In other experiments, RT was found not to have any measurable effect on lambda DNA stretching (Wang, F. and Williams, M.C., unpublished data).

The apparent binding affinities of HIV-1 NC, RT and human A3G to a 20-mer ssDNA oligonucleotide, 5’-FAM-JL587D, are given in Table 1 (also see Figure S2). HIV-1 NC binds to the 20-mer DNA with an approximately 3-fold greater affinity than human A3G. Both A3G and HIV-1 NC bind ssDNA with a significantly higher affinity (8- and 22-fold, respectively) than HIV-1 RT. These studies suggested that A3G should compete very effectively with RT for binding to ssDNA, but would not readily displace NC. Taken together with the stretching data, these results help to explain...
Figure 5. Effect of A3G on minus-strand transfer reactions. (A) Effect of A3G on the time course of RNase H cleavage in the absence or presence of NC. 32P-labeled TAR RNA (0.1 pmol) and TAR DNA (0.2 pmol) were heat annealed and the hybrid was incubated at 37°C in reaction buffer (see above) with 0.4 pmol HIV-1 RT with or without NC (7 nt/NC, 0.1 μM), with or without A3G (80 nM). Samples were loaded on a 15% denaturing gel. Positions of the major cleavage products are indicated on the right. (B) Time course of annealing of 32P-labeled DNA 128 to RNA 148 incubated in the absence or presence of A3G (80 nM) with or without NC (3.5 nt/NC, 0.4 μM). Symbols: no NC/+A3G (filled circles); +NC/no A3G (open squares); and +NC/+A3G (open circles). (C) Schematic diagram illustrating the minus-strand transfer assay system. The R homology is 94 nt; U5 and U3 are 34 and 54 nt, respectively. (D) Graph of percent transfer product plotted versus incubation time. To quantify the percentage of strand transfer, the amount of transfer product was divided by the total amount of DNA, multiplied by 100. Symbols: no NC/no A3G (filled circles); no NC/+A3G (open squares); +NC/no A3G (open circles); and +NC/+A3G (open triangles).
why A3G inhibits RT-catalyzed elongation reactions, but
fails to impact NC-mediated annealing.

**DISCUSSION**

Previous studies demonstrated that A3G inhibits reverse
transcription during infection with Δvif HIV-1 (28–31),
with effects on synthesis of both early and late products
(5,10,13,15,35–37). In the present study, for the first time,
we show how A3G affects each individual step in reverse
transcription (Figure 1), using a series of well-defined,
reconstituted assay systems and highly purified A3G,
RT and NC proteins. The results demonstrate that A3G
inhibits elongation by HIV-1 RT directly, and not by
blocking NC’s nucleic acid chaperone activity (43). These
findings are consistent with our previous result showing
that NC and A3G do not compete for binding to RNA
(12) and taken together, represent strong evidence that

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**Table 1.** Apparent binding affinities of HIV-1 NC, HIV-1 RT and A3G to 20-mer DNA oligonucleotide (5′-FAM-JL587D)

|                | $K_d$ (nM)$^a$ |
|----------------|---------------|
| HIV-1 NC       | 84.1 ± 7.8    |
| HIV-1 RT       | 1840 ± 390    |
| A3G            | 238 ± 95      |

$^aK_d$ values were determined from three independent experiments. The error determinations represent the standard deviation.
at a physical and functional level, NC and A3G do not interfere with each other’s activities.

More specifically, we report that A3G inhibits (−) SSDNA and (+) SSDNA synthesis, minus- and plus-strand DNA transfer, and elongation of minus- and plus-strand DNAs. Using a deaminase-deficient A3G mutant, we also show that (−) SSDNA synthesis was inhibited in the absence of A3G’s enzymatic activity (Figure 4). Additionally, during (−) SSDNA synthesis in the presence of A3G, we find that RT pauses at unique sites, which map to bases in or near ss regions in the RNA template structure (Figure 3C; also see Figure S1), consistent with A3G’s preference for binding to ss nucleic acids (6,12). Since a direct interaction between A3G and RT could not be detected in pull-down assays (data not shown), the results further suggest that A3G binding to the template physically blocks RT movement along the template. It should be noted that A3G has a more profound effect on DNA-primed DNA polymerase activity than the ss-binding protein T4 gene 32. Thus, in a primer extension assay with a DNA template, gene 32 protein inhibited minus-strand DNA synthesis at a concentration of 400 nM. In contrast, an A3G effect was already observed at a concentration of 12.5 nM and by 50 nM, the DNA product was no longer detectable (data not shown).

Systematic study of A3G inhibition of individual reverse transcription reactions is not amenable to investigation with more complex cell-based assays. However, PCR analysis has indicated that during infection, A3G inhibits synthesis of late DNA products to a greater extent than early products (15,35,37). Conceivably, A3G inhibits synthesis of late DNA products to a greater extent than early products (15,35,37). Conceivably, A3G inhibition of late DNA synthesis may be due to its binding to the RNA template, thereby interfering with synthesis of plus-strand DNA (Figure 6A) and formation of a proper substrate for tRNA removal. Based on their results, Mbisa et al. (37) have made a similar proposal.

It was of interest to determine whether the A3G concentrations in the present study were within the physiological range. A recent report showed that only 7 (±4) molecules of A3G per virion are incorporated into vif-deficient HIV-1 produced from human PBMCs, indicating that only a few molecules of A3G are sufficient to inhibit HIV-1 replication (68). Interestingly, even the highest concentrations of A3G used in our experiments (up to 80 nM) are well below the estimated concentration of A3G in the virion (13 ± 8 μM). This value was obtained by assuming that the virus is a sphere of radius 60 nm (69).

Despite the high estimated concentration of A3G in the virus, the expected ratio of A3G:total nt of genomic RNA in vivo is much lower than the ratio that is typically used in vitro. However, RNA is highly folded and rarely ss (39). Indeed, the vast majority of vRNA is likely to be involved in secondary and tertiary interactions (70–72). Since A3G binds poorly to dsRNA (6,12), the effective ratio of A3G:ss nt in vivo is higher than predicted on the basis of total nt of RNA. Thus, the preferential binding of A3G to ssRNA (6,12) and the slow rate of A3G dissociation (Figure 7) could still result in A3G-induced inhibition of reverse transcription during infection.

The interpretation of the results presented here follows from our studies on the nucleic acid binding properties of NC, A3G and RT. Thus, the DNA stretching data in the presence of A3G show an increase in the force-induced melting transition width, suggesting that it is capable of binding to both ss and dsDNA. However, relative to NC, considerably higher concentrations of A3G are required to observe changes in the shape of the DNA stretching curve. Moreover, in sharp contrast to the small hysteresis observed with NC (Figure 7B), which decreases with increasing protein concentration (60), significant hysteresis is observed with A3G (Figure 7A). In this case, the amount of hysteresis increases when the protein concentration is elevated (data not shown). Similar behavior is observed with the ssDNA-binding protein T7 gene 2.5 (73), whose primary role in DNA replication is to stabilize ssDNA.

The DNA stretching results indicate that A3G binds preferentially to ssDNA and is unable to rapidly switch between binding to ssDNA and dsDNA. This finding can help to explain A3G’s inhibition of reverse transcription, which requires rapid access of RT to the ssDNA or RNA template. Similarly, the fact that HIV-1 NC does not interfere with DNA synthesis is consistent with its ability to rapidly adjust to different binding states. The stretching data complement the FA–binding measurements, which show that the ssDNA binding order of the three proteins is NC>A3G>RT (Table 1; Figure S2).
Taken together, these data explain why NC is not readily displaced by A3G.

A major issue in research on A3G's antiviral effect is the question of whether this activity is deaminase-dependent or -independent (or both), but there is still no clear consensus. There are data favoring the dependent mechanism, as shown in several studies of G to A hypermutation (3,4,6) and in some reports on reverse transcription in Δvif HIV-1-infected cells (15,37). However, there is also accumulating evidence indicating that both types of mechanisms might be involved in A3G's effect on reverse transcription in vivo (13,15). Our studies do not exclude a requirement for editing, but based on biochemical and biophysical analysis, provide support for a novel molecular mechanism that could account for deaminase-independent inhibition of reverse transcription and virus replication that is observed in vivo.

In summary, our findings demonstrate for the first time that A3G can inhibit RT-catalyzed elongation in a deaminase-independent manner without interfering with NC-mediated chaperone activity. These results suggest that A3G has an intrinsic effect on viral DNA synthesis, which is independent of the replication steps following reverse transcription. Interplay of the equilibrium and kinetic differences between HIV-1 NC, A3G and RT with respect to their nucleic acid binding interactions is likely to be a major determinant of deaminase-independent A3G inhibition of RT-catalyzed DNA extension.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR online.

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