Derepression of MicroRNA-mediated Protein Translation Inhibition by Apolipoprotein B mRNA-editing Enzyme Catalytic Polypeptide-like 3G (APOBEC3G) and Its Family Members

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The apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G or A3G) and its fellow cytidine deaminase family members are potent restrictive factors for human immunodeficiency virus type 1 (HIV-1) and many other retroviruses. A3G interacts with a vast spectrum of RNA-binding proteins and is located in processing bodies and stress granules. However, its cellular function remains to be further clarified. Using a luciferase reporter gene and green fluorescent protein reporter gene, we demonstrate that A3G and other APOBEC family members can counteract the inhibition of protein synthesis by various microRNAs (miRNAs) such as mir-10b, mir-16, mir-25, and let-7a. A3G could also enhance the expression level of miRNA-targeted mRNA. Further, A3G facilitated the association of microRNA-targeted mRNA with polysomes rather than with processing bodies. Intriguingly, experiments with a C288A/C291A A3G mutant indicated that this function of A3G is separable from its cytidine deaminase activity. Our findings suggest that the major cellular function of A3G, in addition to inhibiting the mobility of retrotransposons and replication of endogenous retroviruses, is most likely to prevent the decay of miRNA-targeted mRNA in processing bodies.

MicroRNAs (miRNAs) are 20–22-nt regulatory RNAs that participate in the regulation of various biological functions in numerous eukaryotic lineages, including plants, insects, vertebrates, and mammals (1–3). More than 474 miRNAs have been identified in humans so far, and ~30% of the genes in the human genome are predicted to be subject to miRNA regulation (4). The expression of many miRNAs is usually specific to a tissue or developmental stage, and the miRNA expression pattern is altered during the development of many diseases (3). Mature miRNAs are generated from RNA polymerase II-transcribed primary miRNAs that are processed sequentially by the nucleases Drosha and Dicer. Although miRNA can guide mRNA cleavage, the basic function of miRNA is to mediate inhibition of protein translation (1, 5–8) through miRNA-induced silencing complexes (miRISCs). The guiding strand of miRNA in a miRISC interacts with a complementary sequence in the 3′-untranslated region (3′-UTR) of its target mRNA by partial sequence complementarities, resulting in translational inhibition (1). A 7-nucleotide “seed” sequence (at positions 2–8 from the 5′-end) in miRNAs seems to be essential for this action (4). The composition of the miRISC is similar to that of the RNA-induced silencing complex (RISC), which is responsible for mRNA cleavage guided by small interfering RNAs (siRNAs) (1, 3, 7). Nevertheless, some differences exist between miRISCs and siRNA RISCs. For example, the major Argonaute protein in siRNA RISC is Ago-2, whereas all four of the Ago proteins (Ago1–4) are found in miRISC (3, 8). Further, the siRNA RISC may be associated with various RNA-binding proteins such as fragile-X mental retardation protein (FMRP), TAR RNA-binding protein (TRBP), and the human homolog of the Drosophila helicase Armitage, Mov10, possibly in a cell type-specific manner (9–13).

The miRNA-mediated translational repression consistently correlates with an accumulation of miRNA-bound miRNAs at cytoplasmic foci known as processing bodies (P-bodies) (8). Several lines of evidence have indicated that P-bodies are actively involved in miRNA-mediated mRNA repression (14). The P-body-associated protein GW182 associates directly with Ago-1 (15, 16). Depletion of P-body components such as GW182 and Rck/p54 prevents translational repression of target miRNAs (8, 14–19). Furthermore, several miRISC-related components, such as miRNAs, miRNAs repressed by miRNAs, Ago-1, Ago-2, and Mov10, are found in P-bodies (14). P-body formation is a dynamic process that requires continuous accumulation of repressed miRNAs (20). However, P-bodies serve not only as sites for RNA degradation, but also for storage of repressed miRNAs (15). These miRNAs may later return to polysomes to synthesize new proteins (14). In fact, some cellular
proteins can facilitate the exit of miRNA-bound mRNAs from P-bodies. For example, a stress situation may induce the relocation of HuR, an AU-rich element-binding protein, from the nucleus to P-bodies in the cytoplasm where it binds to the 3'-UTR of its target mRNA encoding CAT-1 (21). This binding increases the stability of the miR-122-bound mRNA by assisting it to egress from the P-body and return to polysomes. However, the mechanism underlying this reverse transport of miRNA-bound mRNA out of P-bodies remains to be further clarified.

The cellular apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G protein (APOBEC3G or A3G) is a potent antiretroviral factor that belongs to the cytidine deaminase family (22, 23). A3G can be incorporated into HIV-1 particles and cause extensive C to U conversion in the viral minus-stranded DNA during reverse transcription (24–26), which can trigger its degradation by virion-associated uracil DNA glycosylase-2 (UNG2) and apurinic/apyrimidinic endonucleases (APE) or lethal hypermutation in the HIV-1 genome (26, 27). However, accumulating evidence indicates that A3G protein carrying mutations in the catalytic domain of the cytidine deaminase retains substantial anti-HIV-1 activity (24, 28–31).

Interestingly, A3G is found in P-bodies and stress granules (32, 33). It is associated with a high molecular mass structure (>700 kDa) in replicating cells, and this interaction is RNase-sensitive (34, 35). Further studies indicate that A3G interacts with many RNA-binding proteins, among which are several miRNA-related proteins, such as Ago1, Ago2, Mov10, and poly(A)-binding protein 1 (PABP1). These interactions are either partially or completely resistant to RNase A digestion (32, 35, 36).3 Aside from its inhibitory function in relation to endogenous retroviruses and other retrotransposons (37–41), the major cellular function of A3G seems to be related to P-body-related RNA processing and metabolism. As recent development has indicated that the function of P-body is closely related to miRNA activity, we therefore investigated the possibility of a connection between A3G and miRNA function.

**Experimental Procedures**

*Plasmid Constructions*—The miRNA reporter constructs used in this study (pmir16-luc, pmir10b-luc, pmir25-luc, and pEGFP-c1-let-7a) were obtained by directly inserting the annealed corresponding miRNA-binding sites into the 3'-UTR region of the luciferase gene in the pMIR-REPORT vector (Ambion Inc., Austin, TX) between the SpeI and HindIII sites or into the 3'-UTR region of the green fluorescent protein gene, *gfp*, in the pEGFP-C1 vector (BD, Mountain View, CA) between the EcoRI and XhoI sites. A3B gene with a V5 epitope tag sequence at its 3' terminus was amplified from the mRNA of H9 cells through RT-PCR, and the sequence was confirmed. Then the modified A3B was inserted into the pcDNA3 vector. Wild-type and mutant A3G-expressing plasmids were amplified by PCR using constructs described previously as templates and cloned into the pcDNA3 vector (24). Detailed information about the oligonucleotides used for cloning or PCR is provided in supplemental Table S1.

3 H. Zhang, unpublished data.

Cell Isolation, Culture, and Transfection—Primary CD4+ T lymphocytes were isolated from peripheral blood mononuclear cells using the CD4+ T cell isolation kit II (Miltenyi, Auburn, CA) and subsequently activated by treating with phytohemagglutinin (PHA) (42). The purity of CD4 T-cells can reach 98%. For isolation of monocytes, CD14+ cells were purified from PBMCs by positive selection with CD14+ microbeads (Miltenyi, Auburn, CA) using auto MACS according to the manufacturer’s instructions. The purity of the CD14+ cells is large than 98% as determined by FACS staining with CD14 antibody. Monocytes were further cultured in complete RPMI in the presence of 0.5 ng/ml recombinant human macrophage colony stimulating factor (M-CSF, R&D Systems) and 0.5 ng/ml granulocyte colony-stimulating factor (G-CSF, R&D Systems) for 7 days. The activated CD4+ T cells and H9 T cells were transfected using FuGENE 6 (Roche Applied Science, Indianapolis, IN) for plasmids or HiPerfect (Qiagen, Valencia, CA) for siRNAs or antisense miRNA inhibitors. Macrophages were transfected using jetPEI (Polyplus-Transfection Inc. New York, NY).

Synthesis of siRNAs and Antisense miRNA Inhibitors—The miRNA gene sequences were selected from the Sanger Center miRNA Registry. The siRNAs and synthetic antisense miRNA inhibitors (2’-O-methyl-oligoribonucleotides) against mir-16 and mir-28 were chemically synthesized by Integrated DNA Technologies (Coralville, IA).

*miRNA Array Analysis*—Total RNA (10 μg) from 293T cells transfected with pcDNA-A3G-HA or the pcDNA3 parent vector was isolated with TRIzol reagent (Invitrogen). The following RNA processing, microarray fabrication, array hybridization, and data acquisition were performed at LC Sciences (Houston, TX). Briefly, 2–5 μg of total RNA sample, which was size-fractionated using a YM-100 Microcon centrifugal filter (Millipore, Billerica, MA) and the small RNAs (<300 nt) isolated were 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining. Two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was performed overnight on a Paraflo microfluidic chip using a microcirculation pump (Atactic Technologies, Houston, TX). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target microRNA or other RNA (control or customer defined sequences) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. Each region in the chip comprises a miRNA probe region, which detects miRNA transcripts listed in Sanger miRBase Release 9.0. Total 469 human miRNAs were tested. The detection probes were made by in situ synthesis using PGR (photogenerated reagent) chemistry. Hybridization used 100 μl of 6× SSPE buffer (0.90 M NaCl, 60 mM Na2HPO4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34 °C. After hybridization detection used fluorescence labeling using tag-specific Cy3 and Cy5 dyes. Hybridization images were collected using a GenePix 4000B laser scanner (Molecular Device, Sunnyvale, CA) and digitized using Array-Pro image analysis software (Media

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Cybernetics, Bethesda, MD). Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression). The ratio of the two sets of detected signals (log2 transformed, balanced) and p values of the t-test were calculated; differentially detected signals were those with less than 0.01 p values.

Real-time RT-PCR Detection—To confirm the miRNA array results, "stem-loop" real-time reverse transcription (RT)-PCR was used to detect cellular miRNAs, as described, but with minor modifications (43). The primers for RT-PCR to detect miRNA were designed based on the miRNA sequences provided by the Sanger Center miRNA Registry (supplemental Table S1). The miRNAs were isolated from 293T cells with the mirVana miRNA isolation kit (Ambion). RT reactions were performed by means of the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed on the 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). U6 RNA was used as an endogenous control for miRNA detection, while β-actin or β-tubulin mRNA was measured as an endogenous control for luciferase gene expression detection. The cycle number at which the product level exceeded an arbitrarily chosen threshold (Ct) was determined for each target sequence, and the amount of each miRNA relative to U6 RNA (or luciferase to β-actin mRNA) was described using the formula 2−ΔΔCT, where ΔCT = CT(miRNA or luciferase) − CT(U6 RNA or β-actin)−T.

Flow Cytometric Analysis—Primary CD4+ T cells and H9 cells transfected with gfp-containing plasmids were subjected to flow cytometric analysis on a Beckman Coulter cytometer (Fullerton, CA) at 48 h post-nucelofection. The mean fluorescence intensity (MFI) and positive percentage rate (%) of green fluorescing cells was determined.

Luciferase Assay—A luciferase assay was performed as described (42).

Immunoprecipitation and Western Blotting—The co-immunoprecipitation analysis and Western blotting assays were performed as described (44). Rabbit polyclonal anti-A3G and anti-A3F antibodies were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

Polysome Profile Analysis—Polysome profiles were determined as described with modifications (45). Briefly, 293T cells were cultured in Dulbecco’s modified Eagle’s medium, and treated with various reagents as indicated in the figure legend, and harvested at 48-h post-transfection at 70–80% confluency by replacing the culture media with fresh media containing 10% fetal bovine serum. The nuclei and other cellular debris were then removed by centrifugation at 10,000 × g for 10 min at 4 °C. The supernatants were subsequently layered on top of 15–50% sucrose gradients. Centrifugation proceeded at 36,000 rpm for 90 min at 4 °C in a Beckmann SW41Ti rotor. The location of polysomes in the gradient was determined by measuring the absorbance at 254 nm using a spectrophotometer. RNAs in each fraction were extracted using TRIzol reagent and subjected to RT-PCR detection using primer pairs for the luciferase and β-tubulin genes.

RESULTS

APOBEC3G Counteracts miRNA-mediated Repression of Protein Translation—We first examined the effect of A3G on the expression of miRNAs. Using a miRNA microarray method, we did not find that A3G significantly changed the miRNA expression in 293T cells (supplemental Figs. S1 and S2). A3G also did not significantly change the expression of miRNA processors such as Drosha and Dicer1 or RISC components such as Ago2 and Mov10 (supplemental Fig. S3). Further, A3G also did not change the level of expression of P-body components such as GW182, Xr1 and Lsm1 (supplemental Fig. S3). Nevertheless, the microarray data did indicate that several miRNAs, such as mir-16, mir-10b, mir-25, and let-7a, are abundant in 293T cells.

To study whether A3G affects the efficiency of miRNA-mediated translational repression, various 293T cell-enriched
miRNA-binding sites with perfect or partial complementarity to their corresponding miRNAs were inserted into the 3′-UTR of luciferase (luc) or gfp (Fig. 1a). These plasmids were transfected into 293T cells, which naturally do not express A3G (22, 27), with or without an A3G-HA-expressing plasmid. Fig. 1b shows that the presence of mir-16, mir-10b, or mir-25 miRNA-binding sites in the 3′-UTR of luc gene remarkably inhibited the expression of luciferase. Interestingly, A3G significantly counteracted this inhibition. Similar phenomenon can be observed in HeLa cells (Fig. 1c). To verify this derepression, a dose dependence experiment was performed and derepression was found to correlate with the A3G expression level (Fig. 1d). Real-time PCR data showed that the expression level of luciferase mRNA also substantially increased concomitantly with the expression level of A3G (Fig. 1e). This derepression of miRNA-mediated translational inhibition still occurred when the reporter gene was changed to gfp (Fig. 1f).
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**FIGURE 2.** A3G/F-specific siRNA restores miRNA-mediated repression of protein translation in A3G/F-rich T-lymphocytes and macrophages. PHA-activated CD4+ T cells (a) and H9 cells (b) were first transfected with A3G- and A3F-specific siRNAs via Nucleofector (AMAXA). A siRNA for luciferase was used as a control for transfection. After 48 h, the cells were transfected with pEGFP-c1 or pEGFP-c1-let-7a. 48 h later, luciferase activity was measured. The means ± S.D. are shown.

**FIGURE 3.** APOBEC3 family members inhibit miRNA-mediated repression of protein translation. 293T cells were co-transfected with plasmids expressing APOBEC3 family members and pmir16-luc (a) or with plasmids expressing various A3G mutants and pmir16-luc (b). At 48-h post-transfection, luciferase activity was measured. The 4C mutant represents an A3G mutant that has four point mutations: C97A/C100A/C288A/C291A. The means ± S.D. are shown.

Furthermore, to confirm this effect, H9 T-cells, PHA-activated primary CD4+ T-lymphocytes and macrophages, which naturally harbor significant amounts of A3G and another APOBEC3 protein, A3F, were treated with A3G- and A3F-specific siRNAs. Western blotting showed that expression of A3G and A3F could be effectively decreased by these siRNAs (Fig. 2, a–c). The depletion of A3G and A3F enhanced the efficiency of let-7a miRNA-mediated translational repression in these A3G/F-enriched cells (Fig. 2, a–c). Conversely, overexpression of A3G/F in macrophages can substantially enhance the derepression of miRNA-mediated translational inhibition (Fig. 2c, lane 1).

**Other APOBEC3 Family Members Also Inhibit miRNA-mediated Repression of Protein Translation—**To test whether other APOBEC3 family members also regulate miRNA repression, vectors expressing the APOBEC3 family members A3B, A3C, and A3F were transfected into 293T cells. All the tested APOBEC3 family members were able to inhibit the miRNA-repression of protein translation with pEGFP-c1-let-7a or pEGFP-c1. pcDNA3-A3G-HA (2 μg) was also cotransfected for overexpression experiment. At 48-h post-transfection, GFP expression was analyzed by Western blotting analysis via anti-GFP antibody. The expression of A3G and A3F were also examined by Western blotting.
mediated translational repression (Fig. 3a). Interestingly, a synergistic effect was found between various APOBEC3 family members (Fig. 3a).

Given that A3G has cytidine deaminase activity, we examined whether this activity is responsible for the A3G inhibitory effect on miRNA translational repression. Mutation in the N-terminal zinc-binding domain of A3G important for virion incorporation and mutation in the C-terminal zinc-binding domain important for cytidine deaminase activity were examined for their possible influence on miRNA-mediated translational repression (28–31). The mutations that inactivate the N-terminal domain, C97A and C100A, had a modest effect on miRNA-mediated translational repression (28–31). The mutations that inactivate the C-terminal domain C288A and C291A mutations had no significant effect on miRNA-mediated translational repression, whereas the C-terminal domain C288A and C291A mutations had no significant influence on the inhibitory effect of A3G (Fig. 3b), suggesting that the cytidine deaminase activity is unlikely involved in this effect.

A3G Enhances the Association of miRNA-targeted mRNA with Polysomes—To examine whether the A3G inhibitory effect on mir-16-mediated repression was at the level of translation, a polysome profile analysis was performed (Fig. 4). As shown in Fig. 4c, mir-16 decreased the association of its target mRNA with polysomes, which is consistent with previous reports (45, 46). However, A3G, as well as an antisense anti-mir-16 inhibitor, significantly enhanced the association of the target mRNA with polysomes (Fig. 4, d and e). Puromycin treatment can disrupt this association, further confirming the complex that luciferase mRNA bound with is polysome (Fig. 4g).

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We then examined whether A3G had any effect on the interaction between miRNA-targeted mRNA and P-bodies by performing in situ hybridization with confocal microscopy, as described (21). The location of luciferase mRNA was detected with a Cy3-conjugated oligonucleotide probe, and the location of P-bodies was visualized with GFP-GW182 (19). The mRNA without miRNA-binding sites did not associate with GW182 (Fig. 6, a and b). In the absence of A3G, mir-16-targeted luciferase mRNA was found associated with GW182 and in P-bodies (Fig. 6c), indicating that miRNAs such as mir-16 mediate the association of mRNA with P-bodies. However, in the presence of A3G, mir-16-targeted luciferase mRNA was not found in the P-body (Fig. 6d), suggesting that A3G either facilitates the exit of miRNA-bound mRNA from P-bodies or prevents miRNA-bound mRNA from entering P-bodies. As a control, an anti-mir-16 antisense inhibitor, which can specifically block the function of mir-16, but not an anti-mir28 inhibitor, also prevented the miRNA-targeted luciferase mRNA from associating with GW182 and P-bodies (Fig. 6, e and f).

DISCUSSION

In this report, we have found that the APOBEC3 family protein A3G significantly counteracts the translational inhibition of several miRNAs: mir-10b, mir-16, mir-25, and let-7a in 293 T and HeLa cells. This effect can be found when
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A3G (wildtype)
A3G (C97A/C100A)

anti-GW182
mouse serum ctrl
RNase A

A3G
GW182
Merge

alpha A3G
alpha GW182
beta-actin

A3G facilitates the dissociation of mir-16-targeted mRNA from P-bodies.

FIGURE 5. Interaction between A3G and GW182. a, 293T cells were co-transfected with pcDNA-A3G-HA or pcDNA3-A3G-C97A/C100A-HA. At 48-h post-transfection, cells were collected and lysed. Lysates were treated with and without RNase A, followed by immunoprecipitation with mouse anti-GW182 antibody. b, HeLa cells were co-transfected with pcDNA-A3G-HA and pGFP-GW182delta1 (19). At 48-h post-transfection, the localization of GW182 was visualized with GFP-GW182 fluorescence and A3G was detected with rabbit anti-A3G antibody. c, 293T cells were co-transfected with pcDNA-A3G-HA and pmir16-luc. After another 48 h, a luciferase assay was performed. The means ± S.D. are shown.

A3G is expressed at a low level (0.1 μg of pcDNA3-A3G-HA) (Fig. 1, d and e). As such, it is unlikely that this phenomenon is a consequence of over-expression of A3G. Other APOBEC3 family members, such as A3B, A3C, and A3F, also had a similar function, and the family members had a synergistic inhibitory effect with A3G, indicating that it is a conserved function of APOBEC3 family proteins. Given that A3G localizes to P-bodies and stress granules and interacts with many RNA-binding proteins, such as Ago1, Ago2, Mov10, GW182, and PABP1 (32, 35, 36), we suggest that A3G and other

APOBEC3 family members participate in regulating miRNA-mediated translational repression.

Endogenous A3G can be found in various cells such as H9 T-cells, primary CD4 T-cells, macrophages, and many other normal tissues/organs such as spleen, thymus, testis, ovary, small intestine, mucosal lining of colon (22, 47). They can effectively inhibit the replication of vif-defective HIV-1 (22, 48, 49). Although miRNAs are still able to mediate translational inhibition in H9 T-cells, primary CD4 T-cells at a moderate level and in macrophage at a significant level, we believe that their activity has been restricted by endogenous A3G/A3F. As shown in Fig. 2, a−c, A3G/F-specific siRNAs, which effectively deplete A3G/F in these cells, can significantly further enhance the miRNA-mediated translational inhibition, indicating endogenous A3G or A3F are functional to prevent the activity of miRNA. Furthermore, overexpression of A3G/F can effectively counteract the miRNA-mediated inhibitory effect on translation, supporting this argument (Fig. 2c). Nevertheless, the result from overexpression of exogenous A3G/F also suggests that the either quantity or quality of endogenous A3G/F could need to be improved for an efficient counteraction to miRNA activity. Recently, we and others have found that interferon-(IFN)-α/β can significantly enhance the expression of A3G/F in various primary cells such as resting CD4 T-lymphocytes, mac-
The function of A3G in protein translation regulation is related to the translation repression and P-bodies. It remains to be determined whether this cellular function of A3G is related to the cellular activity of miRNA-mediated inhibition of protein translation by targeting the cytidine deaminase activity of A3G, but A3G was still able to enhance the expression of luciferase when P-bodies and stress granules were not engaged by A3G. However, as the mechanism of the regulation of mRNA degradation remains to be clarified, further experiments are required to demonstrate the exact mechanism underlying this cellular function of A3G.

Interestingly, the mutations C228A and C291A inactivated the cytidine deaminase activity of A3G, but A3G was still able to enhance the expression of luciferase when A3G was engaged by miRNA targeted miRNA in P-bodies and stress granules. Subsequently, more of the mRNA could associate with polysomes, and the translation efficiency would therefore be enhanced. However, as the mechanism of the regulation of mRNA degradation and storage in P-bodies or stress granules remains to be clarified, the relationship between miRNA-mediated translational repression and P-bodies is still under intensive investigation, further experiments are required to demonstrate the exact mechanism underlying this cellular function of A3G.

It is interesting to further investigate the correlation of IFN regulatory system and the miRNA activity in these primary cells.

Acknowledgments—We obtained pcdNA3.1-A3C-V5–6×His, pcdNA3.1-A3F-V5–6×His, and rabbit polyclonal anti-A3G and anti-A3F antibodies from the National Institutes of Health AIDS Research and Reference Reagent Program, pGFP-GW182delta1 from Dr. E. K. Chan in University of Florida.

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