HIV-1 Tat RNA silencing suppressor activity is conserved across kingdoms and counteracts translational repression of HIV-1

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The RNA silencing pathway is an intracellular innate response to virus infections and retro-transposons. Many plant viruses counter this host restriction by RNA silencing suppressor (RSS) activity of a double-stranded RNA-binding protein, e.g., tomato bushy stunt virus P19. Here, we demonstrate P19 and HIV-1 Tat function across the plant and animal kingdoms and suppress a common step in RNA silencing that is downstream of small RNA maturation. Our experiments reveal that RNA silencing in HIV-1 infected human cells severely attenuates the translational output of the unspliced HIV-1 gag mRNA, and possibly all HIV-1 transcripts. The attenuation in gag mRNA translation is exacerbated by K51A substitution in the HIV-1 gag mRNA, and possibly all HIV-1 transcripts. The attenuation in gag mRNA translation is exacerbated by K51A substitution in the HIV-1 gag mRNA, and possibly all HIV-1 transcripts. The attenuation in gag mRNA translation is exacerbated by K51A substitution in the HIV-1 gag mRNA, and possibly all HIV-1 transcripts.

Results

HIV-1 Tat Suppresses RNA Silencing in Plant Cells Downstream of the Maturation Step of dsRNA Duplexes. We used Nicotiana benthamiana protoplasts to investigate whether or not Tat RSS activity is maintained in the plant kingdom similar to the activity of influenza A virus NS1 RSS (23). Tat was expressed in plant cells downstream of the strong and constitutive 35S promoter derived from cauliflower mosaic virus. The RSS activity of Tat was compared to that of plant viral RSSs by coelectroporation with GFP reporter plasmid and 700 nt GFP-specific dsRNAs that downregulate GFP expression (24). Representative images from 5 independent triplicate transfection assays (Fig. L4) demonstrated that HIV-1 Tat (Tat), TBSV P19 (P19) and tobacco etch virus helper component-protease (HC-Pro) restored GFP fluorescence compared to the empty vector control. Quantification of GFP fluorescence in the bulk cultures revealed that the RSS activity was statistically significant (p value < 0.0001) (Fig. 1B).

Northern blot analysis using a sense strand GFP-specific probe revealed low but detectable levels of the 700 nt GFP effector RNA in the cells electroporated with the empty vector (Ve), HC-Pro, P19, or Tat. By comparison, Turnip crinkle virus coat protein (CP) caused the accumulation of effector dsRNA, consistent with its role in preventing processing of dsRNAs (24, 25).

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miRNA Maturation.

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mature siRNAs (19, 21, 22, 26, 27). The results indicated that Tat
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RSS activity is conserved across kingdoms and functions down-
stream of dsRNA duplexes.

TBSV P19 Suppresses RNA Silencing in Animal Cells Downstream of
miRNA Maturation. We next compared the RSS activity of Tat and
plant virus RSS in animal cells. The miR30-based luciferase
reporter system (28) contains 8 copies of the mir30 target
sequence (pCMV-Luc-8(x)-miR30) (Fig. 2A). Co-transfection of
HeLaT4 cells with the primary precursor mir30 (pri-premiR30)
expression plasmid pCMV-miR30 and the pCMV-Luc-8(x)-
mir30 reporter robustly silenced Luc activity (compare tan bars,
reduction by factor of 5, in Fig. 2B). Cotransfection with P19
suppressed mir30 activity by a factor of 7 (compare blue bar in
Fig. 2B). By comparison, downregulation of Dicer by Dicer-
specific siRNAs likewise eliminated miR30 activity (dicer
siRNA, blue bars, Fig. 2C) as compared to treatment with
scrambled siRNAs (sc, tan bars, Fig. 2C). Real-time RT PCR to
quantify steady state mRNA levels showed that the copy number of
Luc mRNA did not change in response to P19 expression (data
not shown).

We used the mir30 assay to compare suppressor activity of
P19 with HIV-1 Tat, vaccinia virus E3L, influenza A virus NS1,
and adenovirus VA1. miR30 reduced Luc activity from pCMV-
luc8(x)-miR30 by a factor of 5 (blue bars, treatment group 1 and
2, Fig. 2D). Statistically significant partial restoration of Luc
activity was observed for E3L, NS1, P19, and Tat and VA1
completely restored Luc activity (blue bars, Fig. 2D, P < 0.05).
Real-time RT PCR showed that the steady state level of luc
mRNA was similar among all samples (gray bars, Fig. 2D) and
indicated that the increased Luc activity was attributable to
reversal of translation repression.

Northern blotting with the antisense miR30 RNA probe on
size fractionated RNA was used to investigate the amounts of the
71 nt premiR30 and 22 nt mature miR30 (Fig. 2E). Low but
detectable endogenous miR30 was detectable (treatment group 1
and 2) and abundant premiR30 and mature miR30 was
observed upon transfection of pCMV-miR30 (treatment group 3).
Similar levels of premiR30 were detected in E3L, NS1, Tat,
and P19 (treatment groups 4–7) and a reduction was observed
in response to VA1 (treatment group 8). The ratio of premiR30
to mature miR30 was similar between empty vector control
(-suppressor) and E3L (1.2 and 1.4, respectively). The ratio was
reduced in response to NS1, Tat, and P19 (range 0.75 to 0.9,
indicating no significant block in the processing of premiR30 to
mature miR30. VA1 treatment resulted in baseline levels of
premiR30 and mature miR30 and the appearance of a smaller
species of premiR30 (treatment group 8) and supports the
observation that VA1 serves as a decoy substrate for exportin 5,
Dicer, and RISC (28, 29). The accumulation of premiR30 and
miR30 in the Tat treatment group indicated that, similar to the
plant viral RSS P19, Tat does not disrupt the maturation of
dsRNA, but reduces the efficiency of a downstream step in the
RNA silencing pathway.

RNA Silencing Restricts HIV-1 Gag Protein Synthesis in Human Cells. To
determine whether the RNA silencing pathway inhibits gag
mRNA translation, Dicer was downregulated in HeLa T4 cells
by transfection with Dicer-specific siRNAs (30). Northern and
Western blotting detected significant Dicer mRNA downregu-
lation by 24 h and 48 h posttransfection (Fig. 3A), and Dicer
protein synthesis was increased significantly by Dicer
downregulation (Fig. 3D, P < 0.05). By comparison, gapdh
translation was not affected, as determined by GAPDH
immunoprecipitation (IP). Analysis of CEMx174 lymphocytes infected with HIV-
1NL4-3 yielded a similar increase in Gag protein synthesis upon
Dicer knockdown (data not shown). Meanwhile, TCA-
precipitable counts measuring [35S]cytochrome/methionine incor-
poration were similar between the samples treated with the
Dicer-specific siRNAs (3.3 × 10^6 ± 8 × 10^5 cpm) and the sc
siRNAs (3 × 10^6 ± 6 × 10^5 cpm). The results demonstrate that
HIV-1 replication in human cells is attenuated by RNA silencing and that downregulation of the RNA silencing pathway significantly increases de novo Gag protein synthesis independently of general effects on cellular protein synthesis.

RNA Silencing-Mediated Restriction of the HIV-1 Gag Protein Synthesis Is Suppressed Equivalently by Tat and P19. We next evaluated whether Tat and P19 RSS rescue gag mRNA translation. Flag-tagged P19 was expressed in HelaT4 cells and P19 was consecutively verified by Western blotting at 12, 24, 36, and 48 h (Fig. 4 A). At the 24 h time point, the cells were transfected with HIV-1NL4–3 and virion levels were measured by Gag p24 ELISA on supernatant medium. P19 produced a significant increase in virion level at each time point (Fig. 4B) but did not alter the copy number of gag mRNA (Fig. 4C). Real-time RT PCR results revealed no change in gapdh or c-myc mRNA in response to P19 or HIV-1 expression (Fig. 4C). At the 48 h time point, the rate of Gag protein synthesis was assessed by pulse labeling and Gag IP experiments. The increase in virion level by P19 expression was attributable to increased Gag protein synthesis (Fig. 4D).

We next compared virion production between HIV-1NL4–3 and the derivative provirus HIV-1/RSS that contains the K51A mutation in the tat gene, which eliminates RSS activity but retains transcriptional transactivation activity (16). Gag ELISA results showed a significant reduction in virion production by a factor of ten in response to K51A (tan bars, Fig. 4E). Expression of P19 was sufficient to complement the defect in virion production from HIV-1/RSS and bolstered virion production from HIV-1 (blue bars, Fig. 4E). Real-time RT PCR data showed no difference in the copy number of HIV-1/RSS gag mRNA nor gapdh and c-myc RNA loading controls in response to P19 (Fig. 4F). These levels were also unchanged in relation to HIV-1 (Fig. 4C). Pulse labeling and Gag IP demonstrated that HIV-1/RSS leads to a significant reduction in the rate of Gag protein synthesis.
Similar to the Gag ELISA results, P19 expression bolstered Gag control: novo cellular protein production (by Dunnett's method determined no significant difference in de

Fig. 3. Down-regulation of Dicer enhances the production of the HIV-1 structural protein in human cells. (A) Northern blot of total cellular RNA from HeLaT4 cells transfected with dicer siRNA (dicer) or scrambled siRNA (sc) determined downregulation of dicer mRNA at both 24 and 48 h posttreatment. (B) Immunoblot with Dicer and Grp78 antisem determined downregulation of Dicer protein at 48 h. (C) Down-regulation of Dicer enhanced Gag production in HeLaT4 cells transfected with HIV-1NL4–3. Gag ELISA was performed on cell-free medium from 3 independent transfections. Gag levels were normalized to cotransfected Luciferase. (D) IP assay determined that Dicer downregulation increases rate of synthesis of Gag P55 but not GAPDH.

Conclusion

Our data indicate that the production of HIV-1 Gag protein, and thereby production of virus particles, is restricted by RNA silencing, which confirms the results of Triboulet, et al. (17) and de Vries, et al. (31). Viral strategies to counter RNA silencing include RNA protection, silencing suppression, evasion, modulation, and adaptation (4). Our results indicate that HIV-1 Tat confers RNA silencing suppression, which counters host-mediated inhibition of HIV-1 translation that is attributable to cell-encoded miRNAs (17, 18). The observation that virion production from an HIV-1 strain lacking RSS activity is severely attenuated indicates that Tat RSS promotes acute HIV-1 infection. Heterologous plant virus P19 RSS is sufficient to overcome translational RNA silencing and provides a mechanistic explanation for the observation that Ebola V35 can replace Tat RSS activity (32). HIV-1 Tat and TBSV P19 also cosegregate in their loss of activity by point mutation of the dsRNA-binding domain.

Our results demonstrate that the RSS activity of Tat and P19 is modest in comparison to adenovirus VA1 RNA. While modest in our system, P19 RSS activity is paramount to prevent host attenuation of TBSV infection and pathogenesis (reviewed in refs. 1, 2). Given our observation that P19 expression does not reduce global cellular translation and that Tat RSS activity was not recapitulated in a shRNA reporter assay (13), viral RSS activity is not a global phenomenon and is targeted to select small RNAs.

The evidence demonstrating reversal of HIV-1 translation repression by a plant RSS support the recent finding in Arabidopsis that plant miRNAs operate by translational inhibition (33). Our IP results, quantitative RNA analyses in human cells, and functional analysis in N. benthamiana cells indicate that the underlying mechanism of RNA silencing suppression by human virus Tat and plant virus P19 are related. The demonstration that Tat and P19 do not reduce maturation of dsRNA duplexes indicates a common mechanism to prevent guide strand programming of RISC, and agrees with research by Lin and Cullen (13) that Tat does not block premiRNA processing by Dicer. The results indicate that influenza A virus NS1 RSS also relies on si/miRNA-binding, whereas vaccinia virus E3L functions upstream and relies on binding to long dsRNAs, thereby preventing their processing into mature si/miRNAs.

Materials and Methods

Plasmids. HIV/RSS was constructed by PCR-based site-directed mutagenesis of pNL4–3 to introduce K51A (16). P19 eukaryotic expression plasmid pCMVp19L9 was constructed by PCR of the P19 ORF from template pRTL2p19 (34) with primers containing terminal ClaI and BamHI restriction sites [supporting information (SI) Table S1]. Plant expression plasmid pRTL2Tat was constructed by SacI and BamHI restriction of pRTL2 (35) and pCMV-Tat-1 (36) (a gift of Andrew Rice) isolation from agarose and ligation. All constructions were verified by sequencing. Previously described plasmids are pRTL2:smGFP, UC, and PC (24), VA1 (28), NS1 and E3L (23, 32). The pCMV-Luc-B(x)-miR30(p) and pCMV-miR30 was a gift of Bryan Cullen (28).

Cells Culture, Transfection and Infection. Monolayer human HEK293 embryonic kidney cells and HeLaT4 (CD4+ HeLa) cells were cultured in DMEM with 10% FBS at 37 °C and 5% CO2. Human CEMx174 lymphocytes were cultured in RPMI medium 1640/10% FBS. Transient transfections of 105 HelaT4 cells in triplicate in 6-well plates featured 1 μg of p19FL or empty pCAM vector and 10 μg of pGL3 firefly luciferase transfection control in Fugene6. After 2 days, transfected cells were lysed in 100 μl of lysis buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 2 mM EDTA, and 1% Nonidet P-40). Ten μl of cell lysate was used in 100 μl of P-40 lysing buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, and 1% Nonidet P-40). Ten μl of lysate was assayed in Luciferase reagent (Promega) and relative light units were used to standardize minor differences in transfection efficiency.

Virus stocks for infections were generated by transfection of 105 HEK293 cells with HIV-1NL4–3 or HIV-1/RSS proviral plasmid. Culture media were harvested for HIV-1 Gag P24 ELISA (Zeprometry). Cells lysates were harvested in parallel in 100 μl of lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, and 1% Nonidet P-40). Ten μl of lysate was assayed in Luciferase reagent (Promega) and relative light units were used to standardize minor differences in transfection efficiency.

Virus stocks for infections were generated by transfection of 105 HEK293 cells with HIV-1NL4–3 or HIV-1/RSS proviral plasmid into CEMx174 T cells. After 48 h, cells were harvested from Ficoll Hypaque and cultured with naïve CEMx174 cells at a 1:10 ratio. Fluorescence-activated cell sorting (FACS) analysis of intra-cellular Gag expression was performed with anti-p24 KC57-FITC antibody (Beckman-Coulter) and Fix and Perm (CALTAG).
Protein Analysis. Cells were lysed in RIPA buffer (50 mM Tris pH 8.0, 0.1% SDS, 1% Triton-X, 150 mM NaCl, 1% deoxycholic acid, 2 mM PMSF) and 50 μg protein was subjected to SDS/PAGE and transferred to nitrocellulose membrane. Immunoblotting antibodies detected Flag and β-actin (Abcam). Visualization was performed with Luminol reagent (Santa Cruz Biotechnology). The IP and TCA precipitation protocols are described previously (37).

Fig. 4. Expression of P19 enhanced production from HIV-1 and HIV/RSS. (A) Immunoblot of HeLaT4 cells transfected with indicated plasmids with Flag or beta-actin antiserum determined expression of P19-Flag fusion protein at indicated times posttransfection. (B) Gag production from HeLaT4 cells transfected with HIV-1^NL4-3 was reduced by P19 RSS. Gag ELISA was performed on cell-free medium from 3 independent cotransfections of HIV-1^NL4-3 and pCMV-p19FL (P19) or empty vector (pCAM). Gag levels were normalized to cotransfected Luciferase. (C) P19 expression did not change steady state levels of HIV-1 gag, c-myc, or gapdh transcripts. Evaluation of total cellular RNA preparations from 3 replicate transfections by reverse transcription and real-time PCR with HIV-1 gag, c-myc, and gapdh specific primers. (D) IP assay demonstrated that P19 expression increases rate of synthesis of HIV-1 Gag P55 but not GAPDH. (E) Gag production from HeLaT4 cells transfected with HIV-1^NL4-3 or HIV/RSS was increased by coexpression of P19 RSS. Gag ELISA was performed on cell-free medium from 3 independent cotransfections of indicated provirus and pCMV-p19FL (P19) or empty vector (pCAM). Gag levels were normalized to cotransfected Luciferase. (F) P19 expression did not change steady state levels of HIV/RSS gag, c-myc, or gapdh transcripts. Evaluation of total cellular RNA preparations from 3 replicate transfections by reverse transcription and real-time PCR with HIV-1 gag, c-myc, and gapdh specific primers. (G) IP assay determined that P19 expression increases rate of synthesis of HIV/RSS Gag P55 but not GAPDH.
Fig. 5. Tat is the viral RSS and the plant viral RSS P19 can replace Tat RSS activity. Quantification of Gag IP assay results of Fig. 4C and F. Introduction of K51A mutation in HIV/1 reduces Gag production and P19 expression increases rate of HIV-1 and HIVR55 Gag protein synthesis to similar levels.

Statistical Analysis. One-way analysis of variance model was applied to log base 2-transformed data. Dunnett’s method analyzed the mean difference among multiple groups.

N. benthamiana Protoplast Assays. Isolation of N. benthamiana cultured cell protoplasts and electroporation are described in detail by Qi, al et. (24). One-million protoplasts were electroporated with 5 µg of pRT2:3mGFP, in the presence or absence of 5 µg of double stranded GFP effector RNA by electroporation. The assays were performed in triplicate wells of 6-well plates with 5 µg of suppressor plasmid. At 3 days postelectroporation, GFP fluorescence intensity was measured by CytofluorTM 2350 Fluorescence Measurement System with the plate reader software (Millipore).

Northern Blot Analyses. For detection of plant GFP effector RNA, 5 µg of total RNA was separated on 5% PAGE with 8M urea and 0.5X TBE. For detection of miR30 precursor miRNA and mature miRNA, 10 µg of enriched small RNA were separated by 15% PAGE/8M urea/0.5X TBE. The small RNA preparations were isolated from total cellular RNA with differential ethanol precipitation using miRNA protocol (Ambion). The RNAs were transferred to Hybond-XL nylon membrane (Amersham Biosciences) and subjected to UV crosslinking. The membranes were hybridized in ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion) overnight, washed twice in 2X SSC/0.1% SDS for 15 min and twice in 0.2X SSC/0.1% SDS for 15 min. Hybridization and washing were performed at 65 °C and at 37 °C for detection of large RNA species and small RNA species, respectively, and visualized by PhosphorImaging. To generate 32P-UTP-labeled antisense miR30 probe, 1 µg of PCR product containing 79 promoter was in vitro transcribed by T7 RNA polymerase.

Real-Time RT PCR. The RT-PCR protocol (37) used random hexamers and Sensiscript reverse transcriptase (Qiagen) and 100 ng RNA. Ten percent of the cDNA was used for real-time PCR with primers complementary to luc mRNA or actin (Table S1) in Lightcycler (Roche).

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Supporting Information

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Table S1. Primer sequences

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