HIV-1 TAR RNA Subverts RNA Interference in Transfected Cells through Sequestration of TAR RNA-binding Protein, TRBP

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Cells mount several defenses against viral infection. Classically, cells react to infection by RNA viruses by triggering an interferon response (1). More recently, evidence suggests that a newly characterized activity, RNAi, provides an additional cellular defense against invading viral nucleic acids (2). Relevant to the mechanism of RNAi, long double-stranded RNA (dsRNA) originating from viruses are first recognized inside cells by an RNA helicase, Dicer, which cleaves the RNA into smaller strands called hairpins (21). These hairpins are then loaded onto the Argonaute 2 (Ago2) protein (2), Ago2, and short interfering RNA sequences. The resulting Ago2-RISC complex is then guided by a guide RNA to a complementary target RNA, where it induces degradation (3). Accordingly, viral RNA interference is used, in part, by cells to defend against infection by viruses. Here, we report that transfected TAR RNA can attenuate the RNAi machinery in human cells. Our data suggest that TAR RNA sequesters TRBP rendering it unavailable for downstream Dicer-RISC complexes. This inhibition of Dicer-RISC activity in transfected cells was partially relieved by exogenous expression of TRBP.

TRBP was originally characterized and cloned 15 years ago (4). TRBP is thought to shuttle the siRNA into a multiprotein RNA-protein-protein complex formed with a cellular RNA-binding protein known as Dicer (5). For this activity, Dicer is assisted by a 21- to 23-nucleotide duplex, commonly termed small interfering RNase III enzyme, Dicer, which cleaves the RNA into smaller strands making them unavailable for PKR activation (6). Hence, miRNAs primarily trigger translational repression, while siRNAs induce mRNA degradation (6).

While the complete protein composition of RISC still remains to be fully elucidated (9), the preceding year, human TRBP protein has emerged as a newly discovered protein partner of Dicer and a contributing component (with Dicer, Ago2, and siRNA) (10–12) to RISC (5). The importance of TRBP to Dicer-RISC activities was revealed by findings that intracellular depletion of TRBP led to a loss in the RNA silencing function of the cell (5). As yet, the exact details of how loss of TRBP leads to suppression of RNAi remain incompletely understood and somewhat contested (13, 14); however, consistent with TRBP's currently inferred physiological role of TRBP in the defense against foreign nucleic acids of the cell, this protein was first cloned and identified by us based on its avid binding to a small RNA structure called TAR RNA (21) and Tat- (22) based attenuation of PKR function. These include vaccinia virus E3L protein binding to dsRNAs making them unavailable for PKR activation (19), HCV envelope protein 2 (E2), and nonstructural 5A (NS5A)-mediated direct suppression of the kinase activity of PKR (20) and HIV TAR- (21) and Tat- (22) based attenuation of PKR function.
TRBP from Dicer-RISC and reduce RNA silencing in human cells.

EXPERIMENTAL PROCEDURES

Plasmids and Reporters—Plasmids were pGL2-luciferase (Invitrogen), pEGFP-C1, pCMV-β-galactosidase (Clontech), and TAR (15). pcDNA-Dicer-myc vector was from Dr. Patrick Provost (31). Luciferase activity and GFP fluorimetry were measured 48 h after transfection (29).

In vitro Dicer Assay—293T cells were transfected with pcDNA-Dicer-myc with or without RNA using Lipofectamine 2000. 48 h later, cells were lysed (50 mM Hepes, pH 7.3, 50 mM NaCl, 1 mM EDTA, 0.4% Nonidet P-40) and incubated with anti-myc beads (Sigma) (31). 1 μg of 700-bp [32P]UTP dsRNA was incubated for 4 h with immunoprecipitated Dicer-myc. The RNA products were purified and analyzed on a 15% native polyacrylamide gel.

Quantitative Real-time RT-PCR—Small RNAs (<200 bp) were isolated using mirVana miRNA kit (Ambion). miRNA quantitation was as described previously (32, 33). RNA was polyadenylated with ATP by poly(A) polymerase at 37 °C for 1 h using the RNA tailing kit (Ambion) and reverse transcribed using 0.5 μg of poly(T) adapter primer (Invitrogen). For each PCR, equal amounts of cDNA (first normalized using the snU6 RNA) were mixed with SYBR Green PCR mix (Applied Biosystems) and 5 pmol of forward primer (designed on the entire tested miRNA sequence) and reverse primer (based on the adapter sequence). Amplification was for 15 s at 95 °C and 1 min at 60 °C for 55 cycles in an Opticon real-time PCR detection system (Bio-Rad) (32).

RESULTS AND DISCUSSION

TAR Inhibits Processing of RNA Hairpins in Cells—TRBP binds TAR RNA with high affinity (15). We reasoned that a surfeit of TAR RNA may act to sequester TRBP, shunting the latter away from Dicer-siRNA-RISC complexes. Others have found that intracellular depletion of TRBP diminishes RISC-mediated gene silencing (12); and our reasoning predicts that TAR overexpression could similarly suppress Dicer-RISC-function.

To test the above, we assessed the effect of increased TAR RNA on TRBP-dependent activity. To model the situation seen in an HIV-infected cell, we transcribed in vitro wild type TAR RNA (wtTAR) and a control mutant (mutTAR) RNA with a disrupted duplexed-hairpin (Fig. 1A). The transcribed RNAs were purified and transfected into cells. To verify that transfected RNAs functioned inside the cell, we first assayed them for the well described ability of TAR to decoy Tat from its transactivation of the HIV-1 LTR (Fig. 1B). Using a HeLa cell line stably integrated with an HIV-LTR-luciferase reporter, which was transiently transfected with a Tat-plasmid, the level of luciferase was measured. We found that Tat transactivation of the LTR reporter was reduced in a dose-dependent manner (up to 60%) by the provision of transfected TAR RNA in trans. As control, mutTAR RNA, which was engineered to be incapable of forming a stable stem-bulge-loop hairpin, did not reduce the transactivation of the LTR-luciferase reporter of Tat. These results support that transfected TAR RNA is intact and functionally active inside cells. We also checked that the amounts of TAR RNA that we transfected into cells are approximate to the

Perhaps not unexpected, there is now increasing evidence that plant and animal viruses can also manipulate the RNAi defense of the cell (2, 6, 23). Toward the latter goal, viruses can mutate both their primary nucleic acid sequences and secondary RNA structures to evade complementarity-based targeting (24); and many viruses also encode suppressor factors (25–30) that interfere with discrete steps in the RNA silencing machinery of the cell.

Based on the recent finding that TRBP plays an essential role in the Dicer-RISC activities of the cell (12), and because TAR is the highly abundant HIV-1-encoded RNA originally characterized as a tight TRBP-substrate (15), we wondered if TAR RNA could be a means used by HIV-1 to defeat the RNAi defense of the cell. Here, we show that intracellular TAR RNA can decay
level of viral RNAs expressed from an integrated HIV-1 provirus. In Fig. 1C, we demonstrated by Northern blotting that the intracellular level of transfected TAR RNA is in the same range of expression as HIV-1 RNAs transcribed from an intact latent proviral genome in the ACH2 cell line after induction with phorbol 12-myristate 13-ace tate.

We next asked how transfected TAR RNA might impact Dicer activity. For this aim, 293T cells were transfected with a myc-tagged Dicer construct with or without wtTAR RNA. 48 h later, using anti-myc beads, we immunoprecipitated myc-Dicer, and Dicer activity was tested in vitro using a 32P-labeled long GFP dsRNA as substrate (Fig. 2A). We observed that myc-Dicer immunoprecipitated from control myc-Dicer-transfected cells efficiently processed long GFP-dsRNA (700 bp) to short (22 nucleotides) GFP-siRNA. However, parallel cells, which were co-transfected with myc-Dicer plus wtTAR RNA, yielded immunoprecipitated myc-Dicer that exhibited a wtTAR-dependent attenuation in dsRNA processing activity (Fig. 2A, lanes 3–5). That the reduction in Dicer activity is due to a TAR–TRBP effect rather than a direct TAR–Dicer effect was supported by findings from co-transfection of a TRBP expression vector into wtTAR+myc-Dicer cells. Supplying TRBP in this way partially restored the RNA processing activity to myc-Dicer (Fig. 2A, lane 6), which was originally suppressed by wtTAR. We performed two control experiments, which demonstrated that transfection into cells of either mutant TAR RNA or TRBP alone showed any effect on the processing activity of immunoprecipitated myc-Dicer (Fig. 2A, lanes 7–10). Consistent with a published report (35), none of our manipulations destabilized Dicer since the amounts of immunoprecipitated Dicer appeared to be uniform in all samples (see Fig. 2A, bottom Western blottings, Dicer). While other interpretations are possible, these results are consistent with wtTAR RNA decoying or occluding TRBP from Dicer leading to reduced dsRNA-processing activity of immunoprecipitated myc-Dicer. That TAR RNA might sequester TRBP to make it less accessible to Dicer was checked by monitoring the amount of TRBP bound to Dicer when TAR was transfected into cells. Indeed, we observed a dose-dependent reduction in the amounts of TRBP that co-immunoprecipitated with Dicer from cells transfected with TAR RNA (Fig. 2B, see lanes 1–5).

TAR Interferes with Intracellular Luc- and GFP-shRNA Activity—We next asked if the ability to reduce in vitro Dicer activity correlated with a capacity to lower intracellular RNA silencing function. To address this point, two previously characterized reporter systems were used to evaluate the effect of TAR RNA on RNAi function. We employed the pGL2 luciferase (Luc-shRNA (short hairpin RNA)) (Fig. 3A) and the CMV GFP (GFP-shRNA) (Fig. 3C) assays (29). When co-expressed, Luc-shRNA inhibited luciferase (luc) mRNA expressed from the pGL2 plasmid by 83%. Intriguingly, when wtTAR was titrated into cells, the ability of Luc-shRNA to silence Luc-mRNA was blunted. Hence, when we introduced 500 ng of wtTAR into cells, the silencing effect of Luc-shRNA dropped from 83 to 44% (Fig. 3A, middle bars). This reduction is specific to wtTAR since the sequence altered TAR mutant did not perturb the silencing activity of Luc-shRNA (Fig. 3A, +mutant TAR lanes).

The effect of TAR on the silencing function of Luc-shRNA might be explained by the sequestration of the former of the limiting amount of TRBP of the cell. If such were to occur, we reasoned that TRBP-dependent Dicer-RISC function should be affected. To verify this reasoning, we transfected increasing amounts of TRBP-plasmid into wtTAR+Luc-shRNA cells. Interestingly, whereas wtTAR suppressed the action of Luc-shRNA, we found that the ability of Luc-shRNA to silence Luc-mRNA became progressively restored with increasing doses of transfected TRBP (Fig. 3B). Since TRBP has no effect on the transcription from the SV40 promoter in pGL2 (data not shown), these results support the interpretation that exogenous TRBP complemented the function lost due to endogenous TRBP being sequestered by TAR.

We next checked the pGL2 luciferase (Luc-shRNA) findings using the CMV EGFP (EGFP-shRNA) pairing. We found that EGFP-shRNA effectively inhibited EGFP fluorescence up to 79% as observed by microscopy and quantified by fluorimetry (Fig. 3C). Here, wtTAR RNA (Fig. 3C, panels 3 and 4), but not mutant TAR RNA (Fig. 3C, panel 7), when co-expressed led to a 3-fold reduction in the silencing activity of EGFP-shRNA. Co-transfection of exogenous TRBP countermanded the RNAi suppressive activity of wtTAR RNA (compare Fig. 3C, panel 3 versus panel 5, and panel 4 versus panel 6), but transfected TRBP in the context of mutTAR RNA did not affect the activity of EGFP-shRNA (Fig. 3C, panel 8). As additional controls, nei-
ther TAR alone nor TRBP alone significantly affected EGFP fluorescence (Fig. 3C, panels 9 and 10). Collectively, the luciferase and EGFP findings are consistent with TAR RNA targeting TRBP to interfere with the RNAi of the cell.

TAR Affects Cellular miRNA Expression—HIV-1 encodes a viral RNA-binding protein, Tat, which partially suppresses the Dicer function of the cell (29). The above reporter assays (Figs. 1 and 3) suggest that HIV-1 may additionally use a viral RNA, TAR, to target TRBP. Because reporter assays may occasionally yield unrepresentative findings, we wished to employ a more physiological readout to assess the consequence of TAR-TRBP interaction. In this regard, in addition to processing artificially constructed shRNA, TRBP-Dicer is involved in authentic processing of the miRNAs of the cell. Hence, we asked whether TAR has an effect on TRBP-Dicer function when assessed in the context of the miRNA maturation of the cell.

Earlier, we reported that transfection of an infectious HIV molecular clone into cells reduced the abundance of many mature human miRNAs (33). Retrospectively, such findings could be explained by TAR RNA expressed from transfected HIV-1 molecular clone modulating the TRBP function of the cell, which is required for miRNA maturation. Previously (33), the three human miRNAs most notably suppressed by HIV-1 were miR-16, miR-93, and miR-221 (21). To ask if TAR RNA alone can explain all or part of the effect on miRNA processing of HIV-1, we used these three miRNAs as readouts in cells transfected singly with wtTAR or mTAR RNA. Mature miR-16, miR-93, and miR-221 were measured using quantitative real-time RT-PCR (32) with internal normalization to an invariant U6 snRNA control (Fig. 4). Provocatively, while the level of the U6 snRNA was unchanged, miR-16, miR-93, and miR-221 were quantitatively reduced in the presence of wtTAR RNA (Fig. 4). This reduction in miRNA abundance was not due to decreased transcription, as assayed by northern blot analysis (33).

**FIGURE 3.** TAR overexpression suppresses Luc- and GFP-shRNA mediated interference activity. A, cells were co-transfected with pGL2-luciferase + CMV β-galactosidase and two discrete Luc-shRNAs (1.5 µg each) with increasing wtTAR or mTAR (50 and 500 ng) RNA. B, 0.5–2.0 µg of pCMV-TRBP were co-transfected in the presence of wtTAR + Luc-shRNA. C, HeLa cells were transfected with pEGFP (panel 1) and EGFP-shRNA (panel 2), as indicated, with increasing wtTAR (panels 3 and 4), mutTAR (panel 7), wtTAR + TRBP (panels 5 and 6), or mutTAR + TRBP (panel 8). As additional controls, TRBP alone (panel 10) or TAR alone (panel 9) was transfected with pEGFP without EGFP-shRNA into cells. Fluorescence was visualized and quantified by fluorimetry, normalized to β-galactosidase. Results are represented as percentage of control luciferase activity in the absence of Luc-shRNA (in A), in the presence or wtTAR + Luc siRNA (in B) or EGFP fluorescence inhibition in the presence of EGFP-shRNA (in C). Values are averages ± standard deviation (where indicated) from at least three independent transfections.

**FIGURE 4.** wtTAR RNA reduces the abundance of mature cellular miRNAs. miR-16, miR-93, or miR-221 abundance was assessed by quantitative real-time RT-PCR in HeLa cells transfected with wtTAR or mutTAR RNA with U6 snRNA included for normalization of the samples. Signals for each of the PCR cycles (x axis) were collected and converted into log10 values (y values). Ct (threshold cycle) determines the minimum PCR cycles required for the reaction to give a threshold signal. Samples with more templates require fewer PCR cycles to reach the threshold. Relative miRNA expression ratios were quantified using MiniOpticon™ system software. Essentially, the expression ratio between two samples is equal to $2^{-\Delta C_t}$, where $\Delta C_t$ is the difference in $C_t$ between the two samples. After checking that the reaction efficiencies are equivalent, relative expression analysis are performed by comparing the expression in the sample to mock transfected cells (fixed as the calibrator). Results are expressed in the text as fold differences.
miR-93, and miR-221 were all down-regulated in the presence of wtTAR, but not mutant TAR, by 3.5-, 3.6-, and 38.5-fold, respectively. Viewed collectively with its effect on shRNA maturation, the effect of TAR RNA on miRNA processing suggests that it acts at a step shared by both pathways (i.e. TRBP-Dicer).

For survival, viruses apparently have evolved varied and complex stratagems to neutralize the RNAi-antiviral defense of the cell (26, 29). Previously, it was described that viruses can escape base pair complementarity-mediated attack through mutations in viral genomes, which alter primary sequences and secondary structures (24, 36, 37). Additionally, viruses can also encode proteins that interfere negatively with various steps of the si/miRNA processing machinery of the cell (2). Here we provide the first evidence that HIV-1 could use an abundantly expressed RNA to attenuate antiviral RNAi by targeting TRBP, a key component of Dicer-RISC complexes. It makes sense that HIV-1 might use TAR, since this RNA sequence is found in all viral transcripts, twice. However, we should point out that our current study employed transfected TAR RNA, and the true biological role of virally transcribed TAR in the miRNA pathway in HIV-1 infected cells requires further study. We anticipate that with further investigation many other viruses may also be found to use decoy RNAs to quell the RNAi defenses of the cell. Indeed, the adenovirus RNA polymerase III transcribed VA RNA has been proposed to quench Dicer function through binding (30), (38). Unlike TAR, VA acts not via TRBP but by direct contact to Dicer (30).

We note with interest that PACT, a human dsRNA-binding protein related to TRBP, has been identified recently to provide a si/miRNA processing function partially redundant to TRBP (39). Because PACT and TRBP conserve RNA-binding domains, in principle, excess TAR RNA can also sequester dsRNA-binding proteins (40), and cellular dsRNA-binding proteins (41). Because TAR, VA acts not via TRBP but by virtue of its effect on shRNA maturation, the effect of TAR RNA on miRNA processing suggests that it acts at a step shared by both pathways (i.e. TRBP-Dicer).

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