Small Interfering RNAs against the TAR RNA Binding Protein, TRBP, a Dicer Cofactor, Inhibit Human Immunodeficiency Virus Type 1 Long Terminal Repeat Expression and Viral Production[†]

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RNA interference (RNAi) is now widely used for gene silencing in mammalian cells. The mechanism uses the RNA-induced silencing complex, in which Dicer, Ago2, and the human immunodeficiency virus type 1 (HIV-1) TAR RNA binding protein (TRBP) are the main components. TRBP is a protein that increases HIV-1 expression and replication by inhibition of the interferon-induced protein kinase PKR and by increasing translation of viral mRNA. After HIV infection, TRBP could restrict the viral RNA through its activity in RNAi or could contribute more to the enhancement of viral replication. To determine which function will be predominant in the virological context, we analyzed whether the inhibition of its expression could enhance or decrease HIV replication. We have generated small interfering RNAs (siRNAs) against TRBP and found that they decrease HIV-1 long terminal repeat (LTR) basal expression 2-fold, and the LTR Tat transactivated level up to 10-fold. In the context of HIV replication, siRNAs against TRBP decrease the expression of viral genes and inhibit viral production up to fivefold. The moderate increase in PKR expression and activation indicates that it contributes partially to viral gene inhibition. The moderate decrease in micro-RNA (miRNA) biogenesis by TRBP siRNAs suggests that in the context of HIV replication, TRBP functions other than RNAi are predominant. In addition, siRNAs against Dicer decrease viral production twofold and impede miRNA biogenesis. These results suggest that, in the context of HIV replication, TRBP contributes mainly to the enhancement of virus production and that Dicer does not mediate HIV restriction by RNAi.

RNA interference (RNAi) is a natural mechanism used by eukaryotes for gene silencing (27, 38, 80). While invertebrates use 23-bp double-stranded RNAs (dsRNAs), called small interfering RNAs (siRNAs), with perfect complementarity to degrade targeted mRNA in the RNA-induced silencing complex (RISC), vertebrates predominantly use imperfectly paired 23-bp micro-RNAs (miRNAs) to control mRNA translation with the RISC. In the RISC, the Dicer protein has RNase III domains and generates the siRNAs, whereas Argonaute, an enzyme with RNase H-like domains, mediates gene silencing. The ability of 21- to 23-nucleotide siRNAs, short hairpin RNAs (shRNAs), and miRNAs to inactivate gene expression, while avoiding dsRNA-activated protein kinase (PKR) activa-

tion (5, 25), has allowed the use of RNAi as a tool for specifically decreasing the expression of both cellular (22, 51) and viral (17, 37) genes in mammalian cells.

While inactivating human immunodeficiency virus (HIV) genes with siRNA, shRNA, and miRNAs decreases viral replication (9, 11, 16, 41, 50, 73), the high mutation rate in the HIV genome allows the virus to readily escape this sequence-specific mechanism (8, 77), highlighting the advantages of targeting cellular genes necessary for viral replication. Cellular factors required for the HIV replication cycle that have been downregulated by RNAi include cell surface receptors CD4, CCR5, and CXCR4 (55, 58, 65); expression factors NFκB, cyclin T1, CDK9, SPT5, and PARP1 (15, 43, 62, 74); and proteins involved in intracellular trafficking and viral packaging including Staufen, tRNA synthetase, Arp2/3, cyclophilin A, and Rab9 GTPase (13, 35, 45, 53, 57). In each case, HIV replication was significantly decreased with minimal cell death, indicating that it is a very promising approach.

Human transactivation response (TAR) RNA binding protein 1 (TRBP1) and TRBP2 were initially identified as proteins that bind the HIV type 1 (HIV-1) TAR RNA and activate long terminal repeat (LTR) expression in the absence and in the presence of the viral transactivator Tat (24, 29, 30). The two proteins differ by 21 additional amino acids in the N-terminal end of TRBP2 (4). TRBPs have two double-stranded RNA binding domains, the second one containing a KR-helix motif that mediates dsRNA binding (21, 26, 28, 44). A third basic

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domain in the C-terminal end of TRBP mediates protein-protein interactions (36, 47). TRBPs have a physiological role in spermatogenesis and growth control during development (49, 81). They also bind the interferon-induced dsRNA-activated protein kinase PKR (19). TRBPs are oncogenic upon overexpression, likely because of their association with PKR (7), with the PKR activator PACT (60; G. Laraki, A. Daher, and A. Gatignol, unpublished data), and with the tumor suppressor Merlin (47, 48).

In the context of HIV-1 replication, TRBP1 and TRBP2 increase viral expression similarly by blocking the inhibitory effect of PKR on viral translation. TRBPs also restore the translation of TAR-containing RNAs by a PKR-independent pathway (2, 7, 19, 23, 24). In the glioblastoma/astrocytoma cell line U251MG, an enhanced PKR response blocks the translation of HIV structural proteins and inhibits viral production (59). Increasing levels of TRBP rescued the expression of HIV-1 proteins and virion production. This ability can be explained by the low endogenous TRBP1 and TRBP2 expression in primary astrocytes and U251MG cells, which are unable to modulate PKR activation (4, 59). The specific low expression of TRBP1 in astrocytes is due, at least in part, to a lack of the NF-Y transcription factor in these cells (3). All available data indicate that TRBPs contribute to the high level of HIV-1 expression and replication in permissive cells and suggest that reducing TRBP expression could decrease HIV replication (2).

Recent data obtained in the elucidation of the RNAi mechanism in mammalian cells have shown that the Dicer protein is associated both with Ago2, a protein from the Argonaute family, and with TRBP (14, 36, 52, 75). The use of siRNAs directed against TRBP in functional assays has shown that TRBP is involved in the RNAi mechanism as a Dicer partner (14, 36, 67). This activity may contribute to the role of TRBP during development. This recent discovery that TRBP is involved in both the RNAi mechanism and HIV replication raises the question of its role during the early steps of HIV infection (31). In this study, we investigated if a decrease in TRBP or Dicer expression could decrease HIV-1 production. We targeted TRBP and Dicer mRNAs by RNAi with siRNAs and show that inhibiting their expression induces a decrease in HIV-1 expression and production in permissive cells to different extents.

MATERIALS AND METHODS

siRNA synthesis. Target sequences for TRBP and EGFP were chosen with the Ambion software program, and their specificity was verified by a BLAST search (www.ncbi.nlm.nih.gov/BLAST). The nonsilencing (NS) siRNA sequence was designed by QIAGEN to have no homology to any mammalian gene. The siRNA sequences targeting HIV-1 Tat (SF2) protein and TAR RNA were from references 16 and 41, respectively. siRNA sequences are indicated in Table 1. All siRNAs transfected alongside TRBP siRNAs were synthesized *in vitro* with the Silencer siRNA Construction Kit (Ambion Inc.). Their concentration and integrity were verified by optical density and gel analysis. The NS and Dicer siRNAs used to assess Dicer decrease were purchased from QIAGEN.

Semiquantitative reverse transcriptase PCR (RT-PCR). Total cell RNA was extracted with TRIzol reagent 48 h after transfection (Invitrogen). cDNA was made from 3 µg of total RNA in a 20-µl reaction mixture containing 3 µM annealed random hexamer, 100 mM dithiothreitol, 8 U avian myeloblastosis virus RT (Promega), 1.25 mM deoxynucleoside triphosphate, and 40 U RNasin (Promega) at 42°C for 1 h. One microliter of the cDNA template was used for PCR amplification in a 20-µl reaction mixture containing 0.2 µl phusion polymerase (Finnzymes), 200 µM deoxynucleoside triphosphate, 0.5 µM each forward and reverse primer, and an MgCl₂ concentration of either 1.5 mM for Dicer or 2.5 mM for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification, respec-

TABLE 1. Sequences of the siRNAs used in this study

Target	Sequence	Reference
NS	5' AAUUCUCCGAACGUGUCACGU 3'	QIAGEN
EGFP	5' AAGGGCAUCGACUUAAGGAG 3'	This report
Tat (SF2)	5' AACUGCUUGUAACAAUUGCUA 3'	16
Tat1c (NL4-3)	5' AAGAGCUAUCAGAACAGUCA 3'	This report
TAR	5' AGACCAGAUUCUGAGCCUGGUU 3'	41
TRBP567	5' AAAGAAUUCACCAUGACCUGU 3'	This report
TRBP571	5' AAUUCACCAUGACCUGUCGAG 3'	This report
TRBP657	5' AAAUGCUGCUUCGAGUGCACA 3'	This report
Dicer	5' ACTGCTTGAAGCAGCTCTGGA 3'	QIAGEN; 40

tively. The primers used to amplify GAPDH were 5' TGAAGGTCGGAGTCAA CGGATTTGGT 3' and 5' CATGTGGGCCATGAGGTCCACCAC 3', while the primers for Dicer were 5' CTGAGCTTAGGATCTGAG 3' and 5' GGAA CCTGAGGTTGATTAGC 3'. Conditions for amplification were 98°C for 30 s and either 23 or 27 cycles of 98°C for 10 s, 50°C for 20 s, and 72°C for 30 s for GAPDH or Dicer, respectively. The products were resolved on a 1.5% agarose gel and quantified with Fuji Film Image Gauge software.

Plasmids. pLTR-Luc, pCMV1-Tat (from HIV-1 SF2 strain) (19), and pEGFP-C₁-TRBP2 (59) expression plasmids were previously described. The enhanced green fluorescent protein (EGFP) pre-miRNA sequence was derived by incorporation of the EGFP siRNA sequence into the stem of the miR-30 miRNA as previously described (9). To generate a pEGFP pre-miRNA vector, a cassette expressing EGFP pre-miRNA from the U6 + 27 promoter (32) was made by two-step PCR as previously described (12) and ligated into the pCRII-TOPO cloning vector (Invitrogen). The pNL4-3 (1) and pAD8 (76) proviral plasmids were obtained from M. Martin (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). Proviral plasmids pELI-1, pMAL-2 (61), and pROD-10 (68) were obtained from K. Peden (Center for Biological Evaluation and Research, Food and Drug Administration, Rockville, MD). Proviral plasmid p89.6 (18) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, from R. J. Collman.

Cells and transfections. HeLa cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine, and 1% penicillin-streptomycin (Invitrogen). For transfections followed by fluorescence-activated cell sorter (FACS) analysis, 7.5×10^4 HeLa cells were seeded in 24-well plates 16 h prior to cotransfection with pEGFP-C₁-TRBP2 and siRNA by using Lipofectamine 2000 (Invitrogen). For transfections followed by luciferase expression assay or immunoblot analysis, 1.6×10^5 HeLa cells were plated in six-well plates 16 h prior to transfections with siRNAs and the LTR-Luc plasmid or siRNAs alone by using FuGENE 6 Reagent (Roche) at a 1:3 DNA/FuGENE or RNA/FuGENE ratio. Luciferase expression was measured 48 h posttransfection and normalized to the same amount of protein as previously described (19). Transfections of cells with HIV proviral constructs were either in T25 flasks seeded with 7.5×10^5 cells or on six-well plates seeded with 3.0×10^5 cells 24 h prior to cotransfection with siRNA by using Lipofectamine 2000 (Invitrogen). To control for transfection efficiency, either pEGFP-N1 (Clontech) was cotransfected as a transfection efficiency reporter and assessed by FACS or experiments were performed at least three times to account for variations. Large variations in siRNA concentrations among the experiments are due to different experimental conditions when using FuGENE 6 or Lipofectamine 2000. Transfection efficiency was verified by PCR with 4 µl of cell lysate and 250 ng of luciferase primers to amplify a 456-nucleotide DNA. The Luc sense primer was 5'-CTAT CCTCTAGAGGATGGAACC-3', and the antisense primer was 5'-CGTCTAC ATCGACTGAAATCCC-3'. Amplification was performed at 94°C for 2 min; 94°C for 45 s, 55°C for 45 s, and 72°C for 2 min for 30 cycles; and 72°C 2 min. Ten percent of the reaction mixture was loaded onto an agarose gel. Only transfections in which the efficiencies vary by less than 5% were considered for the average-value calculation.

FACS analysis. Cells were analyzed for EGFP expression on a FACSort (Becton Dickinson), with the Cellquest control software (Becton Dickinson). Transfected cells were gated by green fluorescence greater than cells in a mock transfection. Relative fluorescence values were calculated as the product of the percentage of EGFP fluorescent cells gated and the mean fluorescence of cells gated positive.

For cell viability, 2×10^5 HeLa cells were seeded in six-well plates 24 h prior to transfection with 14 nM siRNA or 1.8 µg of poly(I) · poly(C) (Sigma) with 1 µl Lipofectamine 2000 (Invitrogen). At 48 h later, cells were harvested with

trypsin and stained with 7-amino-actinomycin D (7-AAD) solution (Pharmingen) by following the manufacturer's protocol. The nonfluorescent population representing viable cells was gated and calculated as a percentage of the total population.

Fluorescence. HeLa cells were plated in 12-well plates on coverslips (Fischer Scientific) and were 70% confluent at the time of transfection. siRNAs at 100 nM were cotransfected with 0.5 μ g pEGFP-C1-TRBP₂ by using FuGENE (Roche). At 48 h posttransfection, the cells were washed twice in phosphate-buffered saline (PBS). The cells were fixed in a 4% paraformaldehyde solution for 10 min at room temperature, followed by two washes in PBS. Fixed cells were mounted in Airvol (Air Products and Chemicals, Allentown, PA), and fluorescence was detected on an Olympus BX-51 microscope.

Measurement of mRNA stability. Cells were grown in serum-free medium in the presence of either the transcriptional inhibitor actinomycin D (ActD; Sigma) at 5 μ g/ml or the same volume of ethanol (control). After various times of ActD treatment, cells were harvested and total RNA was isolated by the TRIzol isolation treatment (Invitrogen). RT-PCR was performed as previously described (4). cDNA was synthesized from 5 μ g of total RNA with 5 pmol of TRBP antisense primer (5'-CTCAATGAAACGCTCCAC-3') or *c-myc* antisense primer (5'-GGGGCTGGTGCATTTTCGGTTGTTGC-3'). PCR amplifications were performed with a 100- μ l reaction mixture containing 250 ng each of TRBP primer (5'-CGGGTCACCGTTGGCGAC-3') or *c-myc* primer (5'-GCTCCTG GCAAAAGTCAAGAGTCTGG-3'). Antisense primers were as described above for the reverse reaction. To respect the PCR exponential phase, the PCR amplifications for TRBP mRNA were performed with 23 cycles. The products were resolved on a 1.5% agarose gel.

HIV RT assay. The RT assay was performed as previously described (39). Each reaction mixture contained 6 μ l viral supernatant in a 30- μ l RT cocktail {60 mM Tris-HCl (pH 7.8), 75 mM KCl, 5 mM MgCl₂, 0.1% (wt/vol) NP-40 (Fisons), 1 mM EDTA, 5 μ g/ml poly(A), 162.5 ng/ml oligo(dT), 4 μ M dithiothreitol, 1 μ Ci/ml [α -³²P]dTTP (Perkin-Elmer)} and was incubated at 37°C for 2 h. Six microliters of each reaction mixture was spotted onto DEAE cellulose (DEAE) filter paper (Whatman International), which was subsequently washed four times in 2 $\times$ SSC (3 M NaCl, 0.3 M trisodium citrate [pH 7.0]) and twice in 100% ethanol (10 min per wash) before being air dried and exposed to a Fuji Film BAS-MS-IP 2340 imaging plate and read on a phosphorimager (FLA3000 Fuji Photofilm Co.). Quantitation of samples was performed with Fuji Film Image Gauge software.

Immunoblotting. At 48 h posttransfection, cells were washed twice with PBS and lysed in cold lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA [pH 8], 10% glycerol, 1% NP-40) with the protease inhibitor cocktail (Roche) and with phosphatase inhibitors (30 mM sodium fluoride, 10 mM *p*-nitrophenylphosphate, 40 mM β -glycerophosphate, and 1 mM sodium orthovanadate) when a phosphospecific antibody was used. The lysates were chilled on ice and centrifuged for 15 min. Equivalent amounts of whole cell extract, measured by Bradford assay (Bio-Rad), were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to a Hybond ECL nitrocellulose membrane (Amersham) as previously described (6). The membrane was blocked for 1 h in 5% nonfat milk and Tris-buffered saline-0.1% Tween 20 (TBST) (69) or 5% BSA and 0.1% TBST for anti-PKR and anti-P-PKR or in 5% milk/PBST for HIV serum. The membranes were incubated overnight at 4°C with anti-TRBP672 (21) at a 1/500 dilution, with anti-Dicer 349 (36) at a 1/1,000 dilution, or with serum from a HIV-1 subtype B patient at a 1/5,000 dilution in the corresponding buffers. They were incubated for 1 h at room temperature with an anti-actin monoclonal antibody (Chemicon) at a 1/10,000 dilution. For probing PKR and phosphorylated PKR, they were incubated overnight at 4°C with monoclonal anti-PKR 71/10 (46, 56) or polyclonal anti-P-PKR (Biosource) at a 1/1,000 dilution in 3% BSA-TBST. After five washes in TBST or PBST, membranes were incubated with peroxidase-conjugated secondary goat anti-rabbit antibody (Amersham) for TRBP, P-PKR, Dicer, goat anti-mouse (Amersham) for PKR and actin and with rabbit anti-human antibody (DacoCytomation) for HIV-1 at a 1/10,000 dilution. The bands were visualized as previously described (6).

Northern blot analysis. Total RNA was harvested with TRIzol reagent (Invitrogen), of which 10 μ g was resolved in a 15% denaturing polyacrylamide-7 M urea gel and then transferred by electroblotting onto a GeneScreen Plus nylon membrane (NEN Life Sciences). An EGFP (5'-GGGCATCGACTTCAAGGA G-3') radiolabeled oligonucleotide probe was hybridized in buffer (0.5 M sodium phosphate [pH 7.2], 7% [wt/vol] SDS, 1 mM EDTA) to the membrane overnight at 42°C. Membranes were washed twice in wash buffer 1 (1 $\times$ SSC, 1% [wt/vol] SDS) for 20 min and twice in wash buffer 2 (0.5 $\times$ SSC, 0.1% [wt/vol] SDS) for 40 min at 50°C and then exposed to imaging plates for 2 days. Blots were quantified with Fuji Film Image Gauge software.

FIG. 1. siRNAs against TRBP decrease exogenous TRBP expression. (A) Schematic representation of TRBP2 mRNA and location of the siRNAs. One microgram of each siRNA was run on a 2% agarose gel and visualized by ethidium bromide staining. MW indicates DNA molecular weight markers. (B) siRNAs against TRBP reduce the fluorescence of EGFP-TRBP. HeLa cells were cotransfected with 0.5 μ g pEGFP-C1-TRBP₂ and with 100 nM siRNA NS, siRNA567, siRNA571, and siRNA657, as indicated, by using FuGENE. At 48 h posttransfection, cells were fixed, mounted, and assayed for GFP expression by fluorescence detection. (C) Titration of EGFP-TRBP by FACS after cotransfection with siRNAs. HeLa cells were cotransfected with 0.5 μ g pEGFP-C1-TRBP₂ and with 3 (white bars), 7 (light gray bars), 14 (dark gray bars), or 18 (black bars) nM siRNA NS, siRNAEGFP, siRNA567, siRNA571, or siRNA657, as indicated, by using Lipofectamine. Reporter expression is calculated as the percentage of EGFP-TRBP2 expression in the absence of siRNA. This result is the average of six independent experiments $\pm$ the standard error of the mean.

RESULTS

siRNAs against TRBP decrease exogenous TRBPs. To analyze the effect of decreasing TRBP on HIV-1 expression and replication, six potential siRNAs against TRBP were synthesized and transfected in HeLa cells. The total RNA was extracted and assayed by semiquantitative RT-PCR for the amount of TRBP mRNA (data not shown). Three sequences that decreased TRBP mRNA significantly were chosen (Fig. 1A) and numbered according to the nucleotide sequence starting at

the initiating AUG of TRBP2. All three sequences target both TRBP1 and TRBP2.

To assess the activity of the selected siRNAs in decreasing the expression of TRBP, HeLa cells were transfected with a reporter plasmid expressing the EGFP-TRBP2 fusion protein and the different siRNAs (Fig. 1B). Compared to the NS siRNA control, all TRBP siRNAs decreased the expression of transfected EGFP-TRBP2 with increasing activities from siRNA657 to siRNA567 and siRNA571. To quantify these results and assess the activity of the siRNAs at different concentrations, reporter expression was measured by FACS analysis (Fig. 1C). All three TRBP siRNAs decreased the expression of EGFP-TRBP from two- to fourfold compared to the siRNA-NS control. siRNA571 had the most potent silencing activities at the highest concentration.

siRNAs against TRBP decrease endogenous TRBPs. Because newly synthesized mRNA from transfected plasmid may have different accessibility and stability compared with the endogenous mRNA, we next assessed TRBP mRNA stability and the activity of the siRNAs to decrease endogenous TRBPs (Fig. 2). We first determined TRBP mRNA half-life in the Jurkat lymphocytic cell line, which supports HIV replication (Fig. 2A). TRBP mRNA stability was measured after treating the cells with the transcriptional inhibitor ActD for 1 to 10 h. The amount of mRNA was then analyzed by RT-PCR and compared to control cells without ActD (Fig. 2A, top); *c-myc* mRNA, which is known to be very unstable in lymphoblastoid, as well as HeLa, cells (20, 66), was used as a reference. The decay of TRBP and *c-myc* mRNAs was plotted as the percentage of the original amount of mRNA at time zero (Fig. 2A, bottom). The half-life of TRBP mRNA was about 3 h, whereas the half-life of *c-myc* mRNA was less than 1 h, indicating that TRBP mRNA is about four times more stable than *c-myc* mRNA.

The ability of siRNAs to decrease endogenous TRBP1 and TRBP2 proteins was then evaluated in HeLa cells (Fig. 2B). We used an antibody which recognizes both proteins as shown previously (3). The siRNA-NS did not decrease the concentration of the endogenous proteins, but all three TRBP siRNAs did. siRNA567 and siRNA657 caused a partial decrease in endogenous TRBP, whereas siRNA571 induced almost complete inhibition of the protein. Because some siRNAs can activate the interferon pathway (10, 72), we verified if the TRBP decrease caused by siRNA571 could be due to a translational shutdown by activated PKR. PKR phosphorylation remained at the same weak endogenous level whether the cells were transfected by NS or siRNA571, indicating that the low TRBP amount is not due to PKR-induced translation shutdown (Fig. 2C). Previous reports with different siRNAs targeting TRBP showed an incomplete loss of TRBP (14, 36). Therefore, siRNA571 gives rise to a higher level of expression inhibition.

siRNAs against TRBP decrease expression from the HIV-1 LTR. TRBP1 and TRBP2 were previously shown to influence HIV-1 gene expression and to act in concert with Tat, although at different levels (2). Therefore, we expected that a decrease in TRBPs would decrease HIV-1 basal LTR expression and Tat-mediated transactivation. The siRNAs directed against TRBPs were assayed on HIV-1 LTR expression in the absence and in the presence of Tat (Fig. 3). To better evaluate their

FIG. 2. siRNAs against TRBP decrease endogenous protein expression. (A) Determination of TRBP mRNA stability. Jurkat cells were incubated with ActD (5 μ g/ml) for 1, 2, 4, 6, 8, or 10 h. Five micrograms of total RNA were reverse transcribed and subjected to PCR amplification with specific primers for TRBP and *c-myc*. PCR products were quantified by densitometric scanning of the gel (Typhoon scanner). TRBP (open squares) and *c-myc* (solid circles) mRNA levels were expressed as percentages of the initial value and plotted against time after ActD treatment. The results are the means of two separate experiments. (B) siRNAs against TRBP decrease endogenous TRBPs. HeLa cells were transfected with 0 (lane 4), 20 (lanes 1 and 5), 40 (lanes 2 and 6), or 80 (lanes 3 and 7) nM siRNA-NS, siRNA567, siRNA571, or siRNA657, as indicated, by using FuGENE. Cells were transfected with 0.5 μ g pCDNA3-TRBP1 (lane 8) or pCDNA3-TRBP2 (lane 9). Two hundred (lanes 1 to 7) or 20 (lanes 8 and 9) μ g of cell extract was resolved by SDS-PAGE, analyzed by immunoblotting with an antibody against TRBP and exposed for 1 h or analyzed by immunoblotting with an antibody against actin and exposed for 1 min. The TRBP1 start codon is included within TRBP2 reading frame. This is a representative experiment of three that gave similar results. (C) siRNAs do not activate PKR. HeLa cells were transfected with 0 (lane 1) or 80 nM siRNA-NS (lane 2) or siRNA571 (lane 3) by using FuGENE. Two hundred micrograms of cell extract was resolved by SDS-PAGE; analyzed by immunoblotting with antibodies against phosphorylated PKR (top), PKR (middle), and actin (lower); and exposed for 1 min. This is a representative experiment of three that gave similar results.

FIG. 3. siRNAs against TRBP decrease the expression of the HIV-1 LTR. (A) siRNAs against TAR and TRBP reduce HIV-1 LTR basal expression. HeLa cells were mock transfected (lane 1) or cotransfected with 0.05 μ g of LTR-Luc and the indicated siRNAs at 80 nM (lanes 2 to 6) by using FuGENE. (B) siRNAs against Tat, TAR, and TRBP reduce HIV-1 LTR transactivated expression. HeLa cells were mock transfected (lane 1) or cotransfected with 0.05 μ g of LTR-Luc (lanes 2 to 8), 0.01 μ g of pCMV1-Tat, and the indicated siRNAs at 80 nM (lanes 3 to 8) by using FuGENE. Luciferase activity is the ratio of the luciferase level in the presence of the siRNA versus NS normalized to 100% for cells transfected with NS. Each value represents the average of five independent experiments $\pm$ the standard error of the mean. A representative quantitative PCR on the luciferase gene is shown at the bottom of each graph as a transfection control. nt, nucleotides.

activity, they were compared to the previously described siRNAs directed against TAR or Tat RNAs (16, 41). Whereas siRNA657 had little activity, siRNA-TAR, siRNA567, and siRNA571 showed a twofold inhibition of LTR basal expression (Fig. 3A). These results indicate that TRBP or TAR inactivation has the same effect on LTR basal expression. This is consistent with siRNAs-TAR that have a maximum of 50% reduction in a comparable luciferase reporter gene assay due to the tight TAR RNA structure (79). In the context of Tat transactivation, siRNA657 had the same activity as siRNA-TAR with an approximately twofold reduction in LTR expression, whereas siRNA567 showed a threefold reduction (Fig. 3B). The activity of siRNA571 was close to that of siRNA-Tat with a 10-fold reduction over the NS control. Overall, the decrease in TRBP expression by siRNAs results in a 50 to 90% reduction of HIV-1 LTR expression in the presence of the Tat transactivator.

siRNAs against TRBP decrease HIV-1 production in transfected cells. The ability of siRNAs against TRBP to decrease

HIV-1 production was evaluated next. HeLa cells were transfected with the previous TRBP siRNAs or another siRNA (Tat1c) targeting a region of Tat present in the HIV molecular clone pNL4-3. Virus production was monitored by RT assay, and the expression of intracellular viral proteins evaluated by Western blotting (Fig. 4). In agreement with previous results (16), the Tat siRNA was very effective at inhibiting HIV-1 production with a 20-fold reduction of RT activity (Fig. 4A). siRNAs against TRBP also showed high activity, with siRNA567 and siRNA571 inhibiting RT activity by four- to fivefold (lanes 4 and 5) and siRNA657 inhibiting RT activity by twofold (lane 6). Intracellular viral proteins, particularly the viral capsid p24, were mildly decreased with siRNA657 but highly decreased with siRNA567 and siRNA571 compared to the negative control (Fig. 4B). Unsurprisingly, targeting virtually all viral spliced mRNAs, except Nef1, one of five Nef mRNAs (63), the Tat1c siRNA abolished all viral protein production.

To determine if the reduced expression of viral proteins and

FIG. 4. siRNAs against TRBP decrease HIV-1 production in transfected HeLa cells. (A) HIV-1 RT activity in cell supernatants. HeLa cells were mock transfected or cotransfected with 0.5 μ g of pNL4-3 and 14 nM siRNA-NS, siRNA-Tat1c, siRNA567, siRNA571, or siRNA657, as indicated, by using Lipofectamine in a T25 flask format. RT activity was calculated by densitometry with the software referred to in Materials and Methods. Each value represents the average of four independent experiments normalized as a percentage of the siRNA-NS RT value $\pm$ the standard error of the mean. (B) HIV protein expression in cell lysates. HeLa cells were transfected as described above. One hundred twenty micrograms of cell lysate was resolved by SDS-PAGE, analyzed by immunoblotting with an antibody against HIV-1, and exposed for 15 min. The values on the left are molecular sizes in kilodaltons. (C) Cellular protein expression in cell lysates. Two hundred micrograms of the above-described cell lysate was resolved by SDS-PAGE and analyzed by immunoblotting with antibodies against P-PKR, PKR, TRBP, and actin successively. The blots were exposed for 10 min for P-PKR, 1 min for PKR and TRBP, and 10 s for actin. The blots shown in panels B and C are representative data among four independent experiments.

TABLE 2. HIV proviral plasmids targeted by siRNA571

Plasmid	Clade	Reference
pNL4-3	B (X4)	1
pAD8	B (R5)	76
p89.6	B (X4/R5)	18
pELI-1	D	61
pMAL2	A+D	61
pROD-10	HIV-2	68

virion production could be ascribed to increased PKR activation, we measured PKR expression and PKR activation in the same extracts (Fig. 4C). In assays with siRNA Tat, siRNA567, and siRNA571, PKR expression was increased twofold compared to the siRNA-NS, suggesting a partial activation of the interferon pathway, likely due to the concomitant presence of viral RNA and active siRNAs. In parallel, phosphorylated PKR increased in similar proportion, suggesting that activated PKR is only a moderate component of the reduced HIV production. These results demonstrate that decreased levels of endogenous TRBP induce a significant reduction in viral protein synthesis. Subsequently, viral production is inhibited to levels comparable to those achieved by directly reducing Tat expression.

siRNAs against TRBP decrease the HIV production of various strains. One advantage of targeting cellular factors required for HIV replication is the ability to target many viral strains with the same sequence. We therefore assessed if decreasing the TRBP concentration will also affect the production of other lymphotropic (X4) or macrophage-tropic (R5) HIV-1 strains, as well as an HIV-2 strain. The HIV strains that were assayed are summarized in Table 2. All HIV strains showed decreased virus production in the presence of siRNA571 compared to siRNA-NS (Fig. 5). The HIV-2 pROD-10 strain showed a 40% decrease, indicating that TRBP is also required, to some extent, for this virus. pELI-1 (HIV-1 D clade) was the most affected, with a 90% reduction in virus production, indi-

FIG. 5. siRNAs against TRBP decrease the production of various HIV strains in transfected HeLa cells. HeLa cells were cotransfected with 0.5 μ g of pNL4-3; 1 μ g each of pAD8, p89.6, pELI-1, pMAL-2, or pROD-10, as indicated; and 14 nM siRNA-NS (black bars) or TRBP 571 (gray bars), by using Lipofectamine in a six-well format. RT activity was calculated as described in the legend to Fig. 4. Each value represents the average of three independent experiments normalized as a percentage of the NS siRNA RT value $\pm$ the standard error of the mean.

FIG. 6. siRNAs against Dicer decrease HIV-1 production in transfected HeLa cells. (A) siRNAs against Dicer decrease mRNA and protein levels. HeLa cells were mock transfected or transfected with 14 nM siRNA against NS or 4, 8, or 14 nM siRNA against Dicer, as indicated, by using Lipofectamine. (Top) Semiquantitative RT-PCR with primers that amplify Dicer or GAPDH mRNA as indicated. (Bottom) Two hundred micrograms of cell lysates was resolved by SDS-PAGE and analyzed by immunoblotting with antibodies against Dicer or actin, as indicated. The blots were exposed for 10 min for Dicer and 10 s for actin. (B) HIV-1 RT activity in cell supernatants. HeLa cells were mock transfected or cotransfected with 0.5 μ g of pNL4-3; 14 nM siRNA against NS or Tat1c; or 4, 8, or 14 nM siRNA against Dicer, as indicated, by using Lipofectamine in a six-well format. RT activity was calculated as described in the legend to Fig. 4 and corrected for transfection efficiency. Each value represents the average of four independent experiments normalized as a percentage of the NS siRNA RT value $\pm$ the standard error of the mean.

cating a strong TRBP requirement. Overall, HIV-1 virion production was reduced 60 to 90% by a decrease in TRBP.

siRNAs against Dicer decrease HIV-1 production in transfected cells. To determine if the activity of siRNAs against TRBP could be ascribed to its activity with HIV RNA or to its function in the RISC, we targeted another component of the RISC. It has previously been shown that reduced Dicer expression diminishes the activity of the RISC (40). We evaluated the activity of siRNA against Dicer by RT-PCR and Western blotting (Fig. 6A) and found that transfection of cells with 14 nM siRNA against Dicer by using Lipofectamine effectively reduced both Dicer mRNA and protein levels. To assess the effects this decreased Dicer expression had on viral expression, we cotransfected the HIV molecular clone pNL4-3 with siRNA against Dicer (Fig. 6B). Surprisingly, HIV production was re-

duced up to 40%, suggesting that RISC activity contributes to, rather than inhibits, viral production.

siRNAs against TRBP or Dicer do not affect cell viability. To determine if some or all of the activity of siRNA against TRBP or Dicer on HIV expression and production could be attributed to a nonspecific cytotoxic effect, we measured cell viability of transfected cells. Using 7-AAD, a fluorescent dye taken up by nonviable cells, we were able to discriminate between viable and nonviable transfected cell populations on the basis of non-fluorescence (71). The results show that the siRNAs do not reduce cell viability at the concentrations used in this study, while the poly(I) $\cdot$ poly(C) control significantly reduced cell viability (Fig. 7A).

siRNAs against TRBP or Dicer decrease the processing of miRNAs against GFP. Because siRNA-TRBP and siRNA-Dicer affect HIV-1 production, this activity could be due to their function in RNAi or to another function. To discriminate between these, we assessed the extent to which the respective siRNAs affect the RNAi pathway. With a system reliant on the processing of a vector-delivered EGFP pre-miRNA into mature miRNA, we directly assessed the effects various siRNAs had on the production of EGFP miRNA species by Northern blotting. siRNA567 and siRNA571 decreased the miRNA processing by 23 and 41%, respectively, whereas siRNA-Dicer decreased it by 73% compared to the siRNA-NS. Therefore, TRBP siRNAs disrupt the RNAi pathway partially while Dicer siRNA largely impedes it.

DISCUSSION

RNAi has been widely used within the last 5 years to inhibit gene expression in mammalian cells. Applications range from fundamental knowledge about genomics to therapeutics in disease-associated genes (51). In parallel, the intricate RNAi mechanism is being elucidated to understand how siRNA or miRNA functions in cells and recent progress has been made to elucidate the differences between lower eukaryotes and mammalian cells (14, 34, 36, 52, 54). It has become clear within the last year that Dicer, Ago2, and TRBP are the main components of the mammalian RISC that trigger either RNA cleavage or translation inhibition, although other proteins have been identified in this complex (14, 36, 52). Both TRBP1 and TRBP2 bind to Dicer in several assays, and the two isoforms may play a similar role in the RISC (36).

Because of the high efficiency of siRNAs, they have been used to target viruses and combat viral diseases. Viruses whose replication has been silenced include respiratory syncytial virus, influenza virus, poliovirus, herpesvirus, hepatitis B virus, hepatitis C virus, human papillomavirus, HIV, and a growing list of other viruses (17, 37). In the choice of targets against a virus, the best one seems to be the virus itself because a specific target will less likely damage the cell. However, one concern with HIV is its high mutation rate that induces drug resistance. Indeed, a single mismatch in the sequence can decrease or inhibit the activity of siRNAs (41, 64). In support of these concerns, HIV was shown to escape RNAi by mutating the targeted sequence (8, 77). The use of a highly conserved sequence and the use of multiple targets are ways to overcome this inconvenience but may also have some limitations. Targeting cellular factors required for HIV replication is another

FIG. 7. Effects of siRNA against TRBP or Dicer on cell viability and RNAi. (A) siRNAs do not affect cell viability. HeLa cells were transfected with no siRNA (lane 1), 1.8 μ g poly(I) $\cdot$ poly(C) (lane 2), or 14 nM siRNA against NS (lanes 3 and 4), siRNA against EGFP (lane 5) siRNA against Tat (lane 6), siRNA567 (lane 7), siRNA571 (lane 8), siRNA657 (lane 9), or siRNA against Dicer (lane 10). All siRNAs were generated from the Ambion siRNA construction kit, except siRNA against NS2 and siRNA against Dicer, which were purchased from QIAGEN. Cell viability was assessed by FACS analysis of 7-AAD-stained cells. The viable cell population of the mock-transfected cells was set at 100%. Each value represents the average of four independent experiments $\pm$ the standard error of the mean. (B) miRNA biogenesis is impaired in HeLa cells transfected with siRNA567, siRNA571, or siRNA against Dicer. (Top) Schematic representation of the vector-delivered EGFP pre-miRNA and the predicted structure of the fully processed EGFP miRNA. The EGFP sense sequence is highlighted. (Bottom) HeLa cells were cotransfected with 0 (lane 1) or 2 μ g EGFP pre-miRNA vector (lanes 2 to 5) and 14 nM siRNA against NS (lane 2) siRNA567 (lane 3), siRNA571 (lane 4), or siRNA against Dicer (lane 5) in six-well plates. Expression of EGFP miRNA was determined by Northern blotting and quantified by densitometry. The results represent the average of three independent experiments $\pm$ the standard error of the mean.

way to circumvent this problem. This strategy has already been used against HIV by inhibiting cellular receptors, expression factors, and factors required for viral trafficking and packaging. In each case, the inhibition of the targeted cellular factor decreased HIV production and did not damage the cell. TRBP is a cellular factor that enhances HIV replication by its activity on HIV-1 gene expression and more specifically on translation (7, 19, 23, 24, 29). We recently showed that astrocytes that have a low level of TRBP expression have a concomitant low level of HIV mRNA translation and poor HIV replication (3, 4, 33, 59). Astrocytes and live *Tarbp2*^{-/-} mice (81) indicate that cells can live with little or no TRBP. There-

fore, it seemed likely that inactivating TRBP should be an effective way to decrease HIV replication. The recent elucidation of components of the RISC required for RNAi in mammalian cells showed that TRBP is a Dicer partner and a necessary component of the RNAi mechanism (14, 36, 67). Because RNAi may be part of the cell reaction against viruses in mammalian cells, it became less obvious that decreasing TRBP may result in reduced HIV replication (31, 70, 78). The results shown in this paper indicate that transfected and endogenous TRBPs can be effectively decreased by RNAi, with different transfection protocols (Fig. 1 and 2). In previous cellular settings (14, 36), the inhibition was good but not com-

plete, likely because TRBP is part of the RISC and because the mRNA and the protein are quite stable. Here, siRNA571 gave a high decrease in protein concentration, indicating that effective inhibition can be achieved (Fig. 2). Because TRBP overexpression can increase HIV-1 LTR basal expression (19, 24, 29), we expected that its inhibition may lower this level. The results show that inhibiting TRBP mRNA by siRNAs decreases HIV LTR basal expression (Fig. 3A) to a mild extent similar to that induced by siRNA-TAR in the best case. This twofold effect of siRNA571 may be mainly the result of an unrepressed translation control mediated by TAR (23), rather than by increased PKR phosphorylation, as siRNA571 does not activate PKR (Fig. 2C). In contrast, in the presence of Tat, the activity of siRNA571 on HIV-1 LTR transactivated expression was close to the activity of siRNA-Tat and the weakest siRNA657 had an activity similar to that of siRNA-TAR (Fig. 3B). These results suggest that TRBP contributes to the transactivated level of HIV-1 expression to a greater extent than to the basal level, which is compatible with the observed synergistic effect of TRBP and Tat (29). The comparison of the efficiencies of siRNA-TAR and siRNA571 on the basal and transactivated levels suggests that siRNA-TAR has a consistent twofold decrease. Indeed, various TAR siRNAs have been extensively studied and were shown to have a maximum efficiency of 50% in a comparable luciferase gene reporter assay because the highly structured TAR RNA prevents access to the siRNA-TAR (79). Because TRBP acts mainly to increase translation of mRNAs, it is likely that by reducing its concentration in cells, the remaining amount is almost sufficient to translate the LTR basal level, but in limiting amount to translate the large amount of mRNA present after transactivation (compare Fig. 3A, lane 5, with B, lane 6).

These results suggest that TRBP contributes largely to HIV expression, and we assayed TRBP siRNAs on HIV-1 production. In this context, the decreased expression of TRBP reduced expression of HIV-1 proteins and formation of viral particles (Fig. 4). This restriction was also observed with different HIV-1 strains and HIV-2, indicating that TRBP is a protein required for all strains (Fig. 5). Together with studies that show that overexpression of TRBP can overcome the translation and replication block caused by activated PKR in lymphocytes (7) and astrocytes (59), our results show that TRBP is important to HIV replication. This requirement is likely due to all TRBP activities on PKR inhibition (7, 19), increased translation of TAR-containing RNAs (23), PACT inhibition (Laraki et al., unpublished), and possibly its function in RNAi (14, 36). Results in Fig. 4C show a moderate increase in PKR and activated PKR with both siRNA Tat and siRNA TRBP, demonstrating that increased PKR activation only partly contributes to reduced HIV production. Furthermore, the activity of TRBP siRNAs cannot be ascribed to a loss of cell viability (Fig. 7A) and their modest impediment of the miRNA biogenesis pathway (Fig. 7B) cannot explain the entire activity. The results obtained in the context of Tat transactivation and HIV production show comparable activity (Fig. 3B and 4A). Therefore, the overall results suggest that the activity of TRBP siRNAs on HIV expression and production can be mainly ascribed to TRBP functions other than RNAi. This study strongly suggests that, in the context of HIV replication, TRBP is more important to help HIV replication than to

restrict viral RNA by RNAi, as suggested by other experiments (31, 59). To determine more precisely if RNAi function contributes to HIV replication, we also decreased Dicer. Surprisingly, we did not obtain an increase in viral production that would have supported a role for RNAi in HIV restriction. Instead, as with the TRBP siRNAs, we obtained a decrease in HIV-1 production, although to a lesser extent (Fig. 6). Because Dicer siRNA largely impedes miRNA processing (Fig. 7), we conclude that, in this cellular and viral context, RNAi does not restrict HIV replication (31, 70, 78); rather, it contributes moderately to virus production, suggesting that, similar to hepatitis C virus, HIV uses RNAi for its own benefit rather than being restricted by RNA cleavage (42). The mechanism by which this function is accomplished and if it can also be observed in lymphocytes and macrophages, which are the natural targets of HIV, remain to be determined.

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