Association of RNA Helicase A with Human Immunodeficiency Virus Type 1 Particles*

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RNA helicase A (RHA) belongs to the DEAH family of proteins that are capable of unwinding double-stranded RNA structure. In addition to its involvement in the metabolism of cellular RNA, RHA has been shown to stimulate RNA transcription from the long terminal repeat promoter of human immunodeficiency virus type 1 (HIV-1) as well as to enhance Rev/Rev response element-mediated gene expression. In this study, we provide evidence that RHA associates with HIV-1 Gag in an RNA-dependent manner. This interaction results in specific incorporation of RHA into HIV-1 particles. Knockdown of endogenous RHA in virus producer cells leads to generation of HIV-1 particles that are less infectious in part as a result of restricted reverse transcription. Therefore, RHA represents the first example of cellular RNA helicases that participate in HIV-1 particle production and promote viral reverse transcription.

RHA in the germ line proliferation and development of Caenorhabditis elegans (8) and also account for the early embryonic lethality observed with RHA knock-out mice (9).

The regulation activity of RHA in RNA transcription is implicated by its presence within the RNA polymerase II holoenzyme complex. For example, RHA has been shown to bridge the interactions between RNA polymerase II and transcription co-activators such as CREB-binding protein and BRAC1 (breast cancer-specific tumor suppressor protein 1) (10, 11). RHA also directly interacts with the p65 subunit of NF-κB and stimulates NF-κB-mediated reporter gene expression (12). Involvement of RHA in transcription is further indicated by the function of its homolog in Drosophila, named the maleless (MLE) gene, that increases gene expression from the male X chromosome (13, 14). RHA has been found as a component of presplicosomes (15), although it is unclear at which step of splicing RHA functions. In regard to RNA nuclear export, RHA directly binds to the constitutive transport element (CTE) RNA of type D retroviruses, such as Mason-Pfizer monkey virus. Together with cellular factors Tap (Tip-associated protein) and HAP95 (helicase A-binding protein 95), RHA promotes export of CTE-containing RNA molecules (16–18).

In light of its multiple functions in cellular RNA metabolism, it is not surprising that RHA also regulates the activity of human immunodeficiency virus type 1 (HIV-1) RNA. For instance, RHA stimulates transcription of HIV-1 RNA (19). The underlying mechanism involves binding of RHA to the transactivation response element (TAR) that is located at the very 5′ end of HIV-1 RNA. This interaction is mediated by the second double-stranded RNA binding domain of RHA, reminiscent of that taking place between TAR and TAR-binding protein (20). It is perceived that, through binding to TAR, RHA either modulates the transactivation activity of Tat or facilitates the transcription activity of RNA polymerase II. RHA also enhances Rev/Rev response element (RRE)-mediated gene expression (21). Since RHA exhibits weak interaction with RRE and thus unlikely plays a direct role in the export of HIV-1 full-length RNA, RHA may promote the release of unspliced HIV-1 RNA from splicesome. In addition to its involvement in HIV-1 gene expression, we now show that RHA associates with the Gag protein and is recruited into virus particles. These results suggest a novel activity of RHA in HIV-1 assembly.

EXPERIMENTAL PROCEDURES

Plasmid DNA Construction—The Gag-TAP (tandem affinity protein purification) plasmid DNA was generated by replacing the GFP DNA segment with the TAP sequence in the context of Gag-GFP DNA that was described previously (22). The TAP sequence was amplified using primers 5′-GCAACCTGCGAAGCGACGATGGAAAAAGAATTCC-3′/5′-GCGAGATATCTTATTCTTTTGGTAATTTGTTATC-3′ (underlined nucleotides represent the PstI and EcoRV restriction sites) and was inserted into Gag-GFP following digestion with PstI and EcoRV to generate Gag-TAP. Construction of Gag-LZ-TAP involved substitu-

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1 The abbreviations used are: RHA, RNA helicase A; CREB, cAMP-response element-binding protein; CTE, constitutive transport element; HIV-1, human immunodeficiency virus; type 1; TAR, transactivation response element; RRE, Rev response element; LZ, leucine zipper; TAP, tandem affinity protein purification; nt, nucleotide(s); siRNA, small interfering RNA; RT, reverse transcription; CA, capsid; MA, matrix; GFP, green fluorescent protein; NC, nucleocapsid; MAGI, multinuclear activation of a galactosidase indicator.
Cellular factors associated with HIV-1 Gag. A, results of mass spectrometry revealing the cellular proteins that were co-purified with HIV-1 Gag protein. Domain structure of the Gag-TAP fusion protein is shown at the top. The calmodulin binding peptide (CBD), TEV protease cleavage site, and protein A (ProtA) sequences are linked to the C terminus of Gag in the context of BH10 (protease-negative) HIV-1 cDNA clone. Following transfection of 293T cells with Gag-TAP, cell lysates were subjected to a two-step affinity purification procedure (available on the World Wide Web at www.mc.vanderbilt.edu/vumcdept/cellbio/gould/). The purified proteins were fractionated on SDS-polyacrylamide gels and visualized by Coomassie Blue staining. The protein bands were analyzed with trypsin in-gel digestion and mass spectrometry. Identity of proteins within each band is shown on the right side of the gels. B, control experiments performed with the N-TAP vector. N-TAP contains the protein A and calmodulin binding peptide sequences.
To generate the hGag-TAP DNA construct, the hGag sequence was amplified from vector pVRC3900 (kindly provided by Dr. Gary Nabel) with primers 5'-GCATGTGAACGGTAACTGCAG-3' and 5'-TCACCTCCAGACGAGCATGTCGAGACGAG-3'. The final PCR products were digested with HpaI and BglII, followed by insertion into BH10 to generate BH-D337. The 293T cells were transfected with DNA constructs expressing Gag-TAP fusion proteins. Subsequent to washing with ice-cold phosphate-buffered saline, cells were lysed in a buffer containing 50 mM Hepes (pH 8.0), 100 mM KCl, 2 mM EDTA, 0.1% Nonidet P-40, 2 mM dithiothreitol, 10 mM NaOAc, 0.25 mM NaVO_3, 5 mM okadaic acid, 5 mM calyculin A, 50 mM glycerolphosphate, 10% glycerol, and protease inhibitors (Roche Diagnostics). Following centrifugation at 10,000 g, the supernatants were incubated with IgG beads (Amersham Biosciences) at 4 °C overnight. After washing with lysis buffer and TEV buffer (10 mM Hepes, 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, and 1 mM dithiothreitol), the Gag-bound IgG beads were incubated with TEV protease (Invitrogen) in TEV buffer at 16 °C for 2 h. The released Gag-TAP proteins were further incubated with calmodulin beads (Amersham Biosciences) in calmodulin-binding buffer (10 mM Hepes, 150 mM NaCl, 1 mM MgOAc, 1 mM imidazole, 0.1% Nonidet P-40, 2 mM CaCl_2, and 10 mM β-mercaptoethanol). After washing with calmodulin-binding buffer and calmodulin rinsing buffer (100 mM Hepes, 80 mM NaCl, 1 mM MgOAc, 1 mM imidazole, and 2 mM CaCl_2), the Gag proteins were eluted with a buffer containing 200 mM Hepes, 80 mM NaCl, 1 mM MgOAc, 1 mM imidazole, and 25 mM EGTA. The eluted proteins were separated on SDS-polyacrylamide gels followed by Coomassie Blue staining. The visible protein bands were excised, in-gel-digested with trypsin, and subject to liquid chromatography/mass spectrometry analysis performed at McGill University and Genome Quebec Innovation Centre.

**Purification of Gag Protein with the TAP Procedure**—The following protocol is adapted from the TAP method (24). The 293T cells were transfected with DNA constructs expressing Gag-TAP fusion proteins. Subsequent to washing with ice-cold phosphate-buffered saline, cells were lysed in a buffer containing 50 mM Hepes (pH 8.0), 100 mM KCl, 2 mM EDTA, 0.1% Nonidet P-40, 2 mM dithiothreitol, 10 mM NaOAc, 0.25 mM NaVO_3, 5 mM okadaic acid, 5 mM calyculin A, 50 mM glycerolphosphate, 10% glycerol, and protease inhibitors (Roche Diagnostics). Following centrifugation at 10,000 g, the supernatants were incubated with IgG beads (Amersham Biosciences) at 4 °C overnight. After washing with lysis buffer and TEV buffer (10 mM Hepes, 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, and 1 mM dithiothreitol), the Gag-bound IgG beads were incubated with TEV protease (Invitrogen) in TEV buffer at 16 °C for 2 h. The released Gag-TAP proteins were further incubated with calmodulin beads (Amersham Biosciences) in calmodulin-binding buffer (10 mM Hepes, 150 mM NaCl, 1 mM MgOAc, 1 mM imidazole, 0.1% Nonidet P-40, 2 mM CaCl_2, and 10 mM β-mercaptoethanol). After washing with calmodulin-binding buffer and calmodulin rinsing buffer (100 mM Hepes, 80 mM NaCl, 1 mM MgOAc, 1 mM imidazole, and 25 mM EGTA), the eluted proteins were separated on SDS-polyacrylamide gels followed by Coomassie Blue staining. The visible protein bands were excised, in-gel-digested with trypsin, and subject to liquid chromatography/mass spectrometry analysis performed at McGill University and Genome Quebec Innovation Centre.

**Viral RNA Analysis**—RNA was prepared from purified virus particles and characterized either in RNAse protection assays or in native Northern blots (26). In RNAse protection assays, viral RNA was incubated with excessive amounts of 32P-labeled viral RNA probes (nt positions −164 to 321) at 42 °C overnight followed by digestion with single-strand RNA-specific RNases (Ambion Inc., Austin, TX). The protected RNA fragments were separated on 5% polyacrylamide, 8 M urea gels and visualized through exposure to x-ray films. As for native Northern blots,
viral RNA first underwent electrophoresis on 0.9% native agarose gels followed by transfer onto nylon membranes (Amersham Biosciences). Hybridization was performed with 32P-labeled HIV-1 DNA probes (nt positions 1–2000). Viral RNA signals were visualized through exposure to x-ray films. To assess expression of viral RNA within 293T cells that had been transfected with small interfering RNA (siRNA) oligonucleotides and the BH10 viral DNA construct, total cellular RNA was extracted with Trizol reagent (Invitrogen) and examined in Northern blots.

Immunofluorescence Staining—293T cells were transfected with the Gag-GFP DNA construct and then fixed with 4% paraformaldehyde at room temperature for 20 min. Following treatment with 0.2% Triton X-100 at room temperature for 10 min, the cells were incubated with anti-RHA rabbit antibodies (kindly provided by Dr. Jeffery D. Parvin) at a dilution of 1:200 at 7 °C. After washing with phosphate-buffered saline, a second incubation with goat anti-rabbit IgG Alexa-fluor 647-conjugated secondary antibodies (Invitrogen) was performed for 1 h at 37 °C. The stained cells were examined using the LSM 5 PASCAL confocal microscope. The images were recorded from layers of 0.9 μm in thickness.

Knockdown of RHA with siRNA Oligonucleotides—siRNA oligonucleotides 5′-GAAUGACCUGGGAAGCCAAdTdT-3′ (sense)/5′-UUUGGCUCUCCCCAAGUCAUUCdTdT-3′ (antisense) were designed to target RHA mRNA sequence at nt 2929–2947. Control siRNA oligonucleotides (catalogue number 4611 or 4613) were purchased from Ambion Inc. Lipofectamine 2000 (Invitrogen) was used to transfect siRNA oligonucleotides into 293T cells. Levels of endogenous RHA were measured in Western blots using anti-RHA antibodies. The same membranes were probed with anti-β-actin antibodies (Sigma) to measure levels of β-actin as an internal control.

Primer Extension (27)—The tRNA Lys-viral RNA template was extracted from purified virus particles, and the relative amount of viral RHA and HIV-1 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 281 • NUMBER 18 • MAY 5, 2006
RNA template was determined in slot blot experiments. The rRNA
primer was extended for 6 bases (CTGCTA) from the same amounts of
viral RNA templates by HIV-1 reverse transcriptase (RT) in a cell-free
reaction containing 50 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 2 mM MgOAc, 0.4 mM
NTPs, 1 mM n-octyl glycoside, 60 mM KCl, 10 mM NaCl, and 0.04%
Triton X-100. Reactions were terminated with buffer containing 1%
phenol/chloroform extraction, newly reverse transcribed viral cDNA
was precipitated with 2.5 volumes of 95% ethanol and further purified
in PCR and real time PCR performed with primer pairs 5′- GTACTTCA-
AGAAGCTGTGCATCGAG-3′/5′-GTCGAGGGATCTCTAGT-
AACAGAG-3′ or 5′-CTGGTTAGACAGATCAGCTG-3′/5′-
GTCGAGGGATCTCTAGTACAGAG-3′ Real time PCR was con-
ducted with the LightCycler FastStart DNA Master SYBR Green 1
kit (Roche Diagnostics) in the light cycler machine (Roche Diagnostics).

**Measurement of Virus Infectivity**—An amount of viruses equivalent
to 2 ng of viral CA protein was used to infect 5 × 10^5 MT-2 cells. Virus
growth was monitored through determining levels of RT activity in the
culture fluids at various time points. Virus infectivity was also deter-
mined by performing the MAGI assays (28). HeLa cells, that express
cD4 and are stably transfected with HIV-1 long terminal repeat-β-
galactosidase reporter DNA, were infected with viruses equivalent to 10
ng of viral CA protein. Forty-eight hours after infection, cells were fixed
and stained using the β-GAL staining kit (Invitrogen). The number of
blue cells was scored to determine the infectivity of HIV-1.

**RESULTS**

**HIV-1 Gag-associated Cellular Factor Candidates Revealed by the
Results of Mass Spectrometry**—The TAP method has been widely used
in yeast proteomic studies and was recently adapted to study protein-
protein interactions in mammalian cells (24). To identify cellular factors
that associate with HIV-1 Gag protein, we have appended the IgG-
binding units of protein A from *Staphylococcus aureus* and the calmod-
ulin-binding peptide to the C terminus of Gag and generated an HIV-1
cDNA clone named Gag-TAP (Fig. 1A) (24). These two tags allow isolation
of Gag protein from transfected cells to high purity using the IgG
beads and the calmodulin beads in a two-step affinity purification pro-
cedure. The purified protein samples were subject to mass spectrometry
analysis (Fig. 1). When N-TAP protein alone was purified from trans-
fected 293T cells and the eluted samples were separated on SDS-PAGE,
two protein bands were visualized with Coomassie Blue staining that
contained tubulins and ribosomal protein S4, respectively (Fig. 1B). This
finding supports the conclusion that the majority of the proteins shown
in Fig. 1A were purified as a result of interaction with Gag either in a
direct or indirect manner. Among these protein candidates are a group
of cellular RNA helicases, including RHA, DEAD-box protein 18
DDX18, DEAd-box protein 24 (DDX24) and RNA helicase Gu(α).
Results of immunoprecipitation and Western blots showed that both
RHA and RNA helicase Gu(α) interacted with Gag, yet only RHA was
detected to associate with virus particles (data not shown). DDX18 and
DDX24 were not tested in these latter experiments due to the lack of
antibodies. In this study, we further investigated the potential role of
RHA in production of infectious virus particles.

**RHA Associates with HIV-1 Gag Protein in an RNA-dependent
Manner**—To characterize Gag-RHA interaction, we first examined the
Gag complex isolated from transfected 293T cells in Western blots
using anti-RHA antibodies. The results showed that RHA was co-puri-
RHA and HIV-1

figured with Gag but not in the control experiments using the TAP sequence alone (Fig. 2A). When Gag was immunoprecipitated from the lysates of transfected cells using anti-CA antibodies, RHA was also detected in the immunoprecipitated materials in Western blots using anti-RHA antibodies (data not shown). Furthermore, pull-down of the FLAG-RHA protein using anti-FLAG antibody-coated agarose beads led to detection in the purified materials of Gag protein that was expressed from the BH10-PR-DNA co-transfected into 293T cells with the FLAG-RHA DNA construct (Fig. 2B). Interestingly, when the NC domain was replaced with the leucine zipper motif from yeast transcription factor GCN4 (29), the resultant Gag mutant named Gag-LZ-TAP lost the interaction with RHA (Fig. 2A). To validate the essence of NC in Gag-RHA interaction, we tested several Gag mutants that lack various lengths of peptide sequences from the C terminus (Fig. 2C). These Gag mutants were generated using the hGag sequence that contains modified RNA sequence but the same amino acid sequence as the Gag from HIV-1 cDNA clone HXB2 (30). When the isolated Gag mutants were examined in Western blots using anti-RHA antibodies, similar levels of RHA were observed to associate with the full-length hGag and the hGag mutant D449 that lacked the p6 sequence (Fig. 2C). Further deletion of the zinc finger sequence in mutants D408 and D390 led to substantially decreased amounts of RHA that could be co-purified. In the case of hGag mutant D378 that was missing the entire NC region, interaction with RHA was completely lost.

The essence of NC for Gag and RHA association suggests an RNA-dependent nature of this interaction event. To test this, RNase A was added to cell lysates during Gag purification. The efficiency of RNase A treatment was monitored by the extent of ribosomal rRNA degradation (Fig. 2D). The results of Western blots revealed that RNase A treatment drastically diminished the levels of RHA that could be co-purified with Gag. This indicates that Gag-RHA interaction is RNA-dependent.

Colocalization of RHA and Gag within Cells—RHA is a nuclear protein and shuttles between the nucleus and the cytoplasm (5). HIV-1 Gag is generally believed to locate within the cytoplasm. It is thus intriguing to determine to which extent RHA and Gag co-localize within cells such that they can interact with each other. Toward this end, 293T cells were transfected with the Gag-GFP construct followed by immunofluorescence staining using anti-RHA antibodies, and images of a few cells are shown in Fig. 2E. The results revealed that RHA was mainly seen within the nucleus with a small detectable amount within the cytoplasm. Notably, a substantial portion of RHA was readily observed at the nuclear envelope region. This observation is consistent with the identification of RHA as a component of the isolated nuclear pore complex (NPC) (31). When the fluorescence signals for RHA and Gag were merged, we observed that Gag and RHA did co-localize at the nuclear rim region (Fig. 2F). Due to the faint fluorescence signal of RHA within the cytoplasm, we were unable to reach a conclusion concerning the potential co-localization between RHA and Gag within this cell compartment.

RHA Is Incorporated into HIV-1 Particles—One implication of Gag-RHA interaction is the incorporation of RHA into virus particles. To test this, we transfected wild-type HIV-1 cDNA clone BH10 into 293T cells, and virus particles thus generated were purified through sucrose step-gradient ultracentrifugation following by Western blot analysis using anti-RHA antibodies. RHA was indeed detected to associate with purified virus particles (Fig. 3A, lane 2). To address whether RHA is encapsidated into virus particles, 1% Triton X-100 was used to deplete the virus envelope. After extensive washing, the pelleted materials were assessed for retention of viral proteins as well as the presence of RHA. The results of Western blots showed that the majority of the matrix (MA) and CA proteins were removed following treatment with 1% Triton X-100, whereas substantial amounts of reverse transcriptase and NC proteins, together with viral genomic RNA, were recovered with the pellets. Since MA is located underneath the virus envelope and CA forms the shell of the core, these data indicate that treatment with 1% Triton X-100 depleted virus envelope and disrupted virus cores as well but largely preserved the viral RNP complex. Notably, RHA was detected in the pelleted materials (Fig. 3A). These results implicate the association of RHA with viral RNP complex within virus particles.

To further rule out the possibility that association of RHA with virus particles is a result of contamination by extracellular microvesicles that can be pelleted together with virions, we first studied a mutated HIV-1 named BH10-LZ that contained the leucine zipper sequence in the place of the NC region. The BH10-LZ DNA contains a protease negative mutation (D25A) such as to avoid abnormal Gag processing due to the insertion of the leucine zipper sequence. Since this Gag mutant did not interact with RHA (Fig. 2A), RHA are not expected to be incorporated into the BH10-LZ virus particles. Indeed, only a minute amount of RHA was detected for BH10-LZ as compared with the substantial quantities of RHA that were co-purified with the wild-type BH10 (Fig. 3B). This residual amount of RHA could either reflect a weak interaction of RHA with the BH10-LZ Gag or a limited contamination by microvesicles. Second, the purified wild-type virus particles were fractionated through a 20–70% continuous sucrose gradient by ultracentrifugation at 100,000 × g for 16 h. The results of Western blots showed that the RHA

FIGURE 4. RHA binds HIV-1 RNA. A, RHA interacts with HIV-1 RNA within cells. 293T cells were co-transfected with FLAG-RHA cDNA and the BHΔGag DNA that contains stop codons within MA and CA regions and thus do not produce Gag protein (data not shown) (33). The FLAG-RHA protein was immunoprecipitated using the anti-FLAG antibody. The precipitated materials were subjected to RNA extraction, followed by RT-PCR that amplified viral RNA sequence of nt 201–506. RNA samples were prepared from transfected cells and also assessed in RT-PCR to determine the expression levels of viral RNA in both transfection experiments. Dilutions of viral cDNA BH10 were used in PCR to indicate the linear range of the reactions. B, incorporation of RHA into the Δ(306–325), ΔDIS, and BH10 virus particles. Viruses equivalent to 1 μg of viral CA protein were used in Western blots to measure levels of associated RHA protein. Viruses equivalent to 20 ng of viral CA protein were also assessed in Western blots using anti-CA antibody to show the equivalent levels of the wild-type and mutated virus particles. Levels of viral RNA associated with the same amounts of mutated or wild-type virus particles were measured in RT-PCR that amplified the viral RNA sequence of nt 201–506. The DNA products are 286, 271, and 306 bp for Δ(306–325), ΔDIS, and BH10, respectively.
protein migrated with virus particles to the same sucrose fractions. This adds further evidence for an association of RHA with virus particles (Fig. 3C).

**RHA Binds HIV-1 RNA**—Gag protein selects the full-length viral RNA from the abundant cellular RNA pool for packaging into virus particles (32). The RNA-dependent nature of Gag-RHA interaction suggests that RHA employs viral RNA as the bridge to associate with virus assembly. To test this hypothesis, the FLAG-RHA DNA was transfected into 293T cells together with the BH10-DIS and DIS(306–325) virus mutants that lack viral RNA packaging signals SL1 and SL3, respectively (26, 34, 35). Consistent with previous data, the DIS and DIS(306–325) virus mutants incorporated lower levels of viral genomic RNA than did wild-type BH10 (Fig. 4B). The results of Western blots revealed markedly lower amounts of RHA that were measured for the DIS and DIS(306–325) mutants as compared with the wild-type virus BH10. Thus, interaction with HIV-1 RNA represents one important means for RHA to be incorporated into virus particles.

**Knockdown of Endogenous RHA Leads to Generation of Virus Particles That Are Less Infectious**—Association with Gag suggests a role for RHA in production of infectious virus particles. To test this notion, we treated cells with siRNA that targets RHA mRNA. The results of Western blots revealed markedly lower amounts of RHA that were measured for the DIS and DIS(306–325) mutants as compared with the wild-type virus BH10. Thus, interaction with HIV-1 RNA represents one important means for RHA to be incorporated into virus particles.

One predication of the specific interaction between RHA and HIV-1 RNA is the reduced RHA levels in mutated virus particles that package less viral RNA. To test this, we studied two HIV-1 mutants named DIS and DIS(306–325) that lack viral RNA packaging signals SL1 and SL3, respectively (26, 34, 35). Consistent with previous data, the DIS and DIS(306–325) virus mutants incorporated lower levels of viral genomic RNA than did wild-type BH10 (Fig. 4B). The results of Western blots revealed markedly lower amounts of RHA that were measured for the DIS and DIS(306–325) mutants as compared with the wild-type virus BH10. Thus, interaction with HIV-1 RNA represents one important means for RHA to be incorporated into virus particles.
ern blots showed a 70–80% reduction of RHA in cells following RHA siRNA transfection (Fig. 5A). The RHA siRNA treatment did not markedly affect cell growth as compared with experiments performed with control siRNA (Fig. 5B). Expression of HIV-1 Gag protein was slightly affected with moderate accumulation of the p25 protein that represents the CA-SP1 intermediate product (Fig. 5A). This observation is reminiscent of that seen for the HIV-1 p15 protein whose processing is affected by RNA binding (36) and may have an impact on virus infectivity. The yield of virus particles was moderately reduced as a result of RHA siRNA transfection at the 20 nM siRNA concentration as compared with that observed for control siRNA (Fig. 5A). Notably, viruses that experienced RHA siRNA treatment exhibited markedly delayed replication kinetics in MT-2 cells as compared with viruses from the control experiments (Fig. 5C). This defect in virus replication was verified by the results of MAGI assays showing a 2–3-fold reduction of virus infectivity (Fig. 5D). This reduction is more pronounced when infection was performed in MAGI cells that had been treated with RHA siRNA (Fig. 5D). Therefore, RHA is required for generation of infectious virus particles.

Reverse Transcription Is Restricted for Viruses That Are Produced from RHA Knockdown Cells—RHA is able to unwind RNA and DNA duplex (1). This activity endows RHA with a potential role in restructuring HIV-1 RNA such that viral genomic RNA are packaged into virus particles in a properly folded conformation and are functionally competent for the subsequent reverse transcription reaction. We thus speculated that knockdown of RHA might have decreased the infectivity of virus particles through impeding either viral RNA packaging or reverse transcription. To study these two possibilities, we first performed RNase protection assays to measure levels of viral RNA associated with virus particles. The results showed that RHA siRNA treatment did not affect the amounts of virion-associated viral RNA (Fig. 6A). When levels of viral RNA expression within transfected 293T cells were assessed in either an RNase protection assay or native Northern blots, it was observed that RHA siRNA transfection did not lead to significant alteration in terms of the amounts of full-length and spliced viral RNA molecules as compared with experiments performed with control siRNA (Fig. 6A and B).

HIV-1 particle packages two copies of full-length viral RNA that are dimerized via noncovalent linkage (37). To test whether RHA knockdown affects HIV-1 RNA dimerization, we performed native Northern blots to assess viral RNA that were prepared from virus particles. The results showed that viral RNA was properly dimerized regardless of whether cells had been transfected with RHA siRNA or control siRNA (Fig. 6C). Therefore, RHA knock-down neither affects viral RNA packaging nor dimerization. This conclusion does not exclude the possibility that RHA regulates viral RNA folding in a way that cannot be reflected by the results of native Northern blots.

To study whether RHA has a role in HIV-1 reverse transcription, we performed 6-base primer extension experiments to assess the amounts of the tRNA<sub>Lys</sub> primer that were annealed to viral RNA template within virus particles (27). A fairly modest decrease in the amounts of extended tRNA<sub>Lys</sub> signals was seen for virus particles that had experienced RHA siRNA treatment (Fig. 7A), which indicates that RHA knock-down exerts limited effect on tRNA<sub>Lys</sub> annealing to viral RNA. We next measured the reverse transcription activity of this tRNA<sub>Lys</sub>-viral RNA complex through performing endogenous reverse transcription assays. Levels of minus-strand strong stop DNA were quantified in real time PCR.
using primer pair 462–485/604–579. The results showed that the RHA siRNA virus particles generated an amount of minus-strand strong stop DNA that was 44% of that produced by the control siRNA virus particles (Fig. 7B). Notably, a 6-fold reduction in (−) cDNA production, as measured with primer pair 314–339/604–579, was seen for the RHA siRNA virus particles compared with the control siRNA particles (Fig. 7B). These results implicate an important role for RHA in promoting the strand transfer step during reverse transcription.
**RHA and HIV-1**

**DISCUSSION**

In this study, we provide evidence that RHA associates with HIV-1 Gag protein. Since Gag drives the assembly of HIV-1 particles and represents the major structural protein of the virion, our finding suggests that RHA is a component of the HIV-1 assembly machinery and may thus exert an effect on virus production through modeling the secondary structures of viral RNA and rearranging RNA-protein interactions.

RHA is one of the few RNA helicases that have been reported to play a role in HIV-1 replication. For instance, the DDX3 DEAD-box protein binds CRM1 and regulates Rev/RRE-dependent nuclear export of HIV-1 RNA (38). DDX1 was also shown to affect Rev/RRE-mediated gene expression through direct interactions with Rev and RRE (39). When the RNA helicase named RH116 was overexpressed, levels of full-length and single spliced HIV-1 RNA transcripts were increased through an undefined mechanism (40). The expression levels of a number of DEAD-box proteins, such as DDX10, DDX18, DDX21, DDX23, DDX39, and DDX52, in HIV-1 latently infected ACH-2 cells changed upon treatment with phosphorib 12-myristate 13-acetate (41). However, the functions of these latter DEAD-box proteins in HIV-1 replication have not been investigated. Among the aforementioned RNA helicases, RHA is so far the only one that is shown hereby to associate with HIV-1 Gag. In light of the RNA-binding ability associated with all RNA helicases, it is reasonable to speculate that any of these proteins may bind to HIV-1 RNA equally well. Interestingly, the results of our mass spectrometry analysis revealed a limited number of RNA helicases that were co-purified with Gag protein (Fig. 1A). This suggests that some RNA helicases are either not accessible to HIV-1 RNA within cells or exhibit an affinity for HIV-1 RNA that does not suffice to compete with the other RNA binding proteins. Considering the sophisticated protein-protein and protein-RNA interactions that take place during generation of virus particles, it is not surprising that the virus utilizes RNA helicases, such as RHA, to regulate these complex molecular interactions in an energy-dependent manner. Dependence of viral replication on RNA helicases renders these molecules of either viral or cellular origin to be exploited as targets for development of antiviral agents (42).

Both Gag and RHA are RNA-binding proteins. Our results showed that Gag-RHA interaction was sensitive to RNase A treatment (Fig. 2D). At this stage, we are unable to rule out the potential direct interaction between Gag and RHA, an issue that can be resolved using purified Gag and RHA proteins. Nonetheless, were Gag and RHA engaged in direct interaction, it would be expected to be rather weak considering the sensitivity of Gag-RNA association to RNase treatment. Although any RNA can link RHA to Gag, we show that within cells RHA is able to bind HIV-1 RNA in the absence of Gag protein (Fig. 4A). This binding of RHA to HIV-1 RNA is further supported by the observation of reduced levels of RNA within virions bearing mutated RNA packaging signals (Fig. 4B). Together with the findings that RHA interacts with the TAR element of HIV-1 RNA and regulates HIV-1 gene expression within the nucleus (19, 21), our results suggest that RHA initiates interaction with HIV-1 RNA within the nucleus and that this interaction persists throughout the subsequent events that HIV-1 RNA undergoes until incorporation into virus particles. We propose that, through binding to HIV-1 RNA, RHA gains access to the virus assembly machinery within cells as well as the reverse transcription complex within virus particles and thereby regulates the activities of these molecular complexes by virtue of its helicase function.

RHA is not the only cellular protein that has been shown to associate with virus particle through binding to RNA. One example is the incorporation of APOBEC3G into HIV-1 particles. Results of mutagenesis studies revealed the NC sequence, in particular the N-terminal residues, as the region of Gag that is crucial for APOBEC3G incorporation (43–45). Consistent with this observation, it was further shown that Gag-APOBEC3G association was bridged by RNA molecules (46–48). Therefore, viral RNA is not only packaged into virus particles as viral genome but also serves as an important means for virus to recruit cellular factors.

The functional relevance of RHA association with HIV-1 Gag and virus particles is indicated by the decreased infectivity of viruses that were generated from RHA knock-down cells (Fig. 5). It is conceivable that RHA actively participates in the formation of infectious virus particles either by rearranging the Gag-RNA interaction during virus morphogenesis on plasma membranes or by restructuring the viral RNP complex during virus maturation. Unfortunately, the results of RNase protection assays and native Northern blot experiments failed to reveal any defect in viral RNA packaging and dimerization that could be associated with down-regulated RHA levels (Fig. 6). This is probably due to the limitation of these biochemical approaches in revealing conformation changes of RNA structures. Nonetheless, analysis of viral cDNA synthesis indicates that the reverse transcription activity of virus particles was negatively impacted by RHA knock-down (Fig. 7B). Therefore, RHA does have a role in the function of viral RNP, although we could not provide direct evidence on the speculated involvement of RHA in the conformation rearrangement of viral RNA.

RHA may contribute to viral reverse transcription in three possible ways. First, it may directly assist viral reverse transcriptase to copy RNA through unwinding RNA secondary structures. Second, RHA may function during virus maturation through restructuring viral RNA as well as regulating the interactions of viral RNA with NC, RT, and other factors such as to form a functional reverse transcription complex. It is also possible that RHA neutralizes the anti-HIV activity elicited by cellular restriction factors.

In summary, the results of this study demonstrate the association of RHA with HIV-1 Gag and virus particles. We propose that access to viral RNP complex during virus assembly allows RHA to regulate the folding of viral RNA as well as to model protein-RNA interactions.

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