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A method to study the role of DDX3 RNA helicase in HIV-1

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Abstract

Viral replication requires the use of host cell proteins and enzymes. Many viruses utilize viral helicases at various stages of their life cycle; these viruses have evolved to encode directly helicase or helicase-like proteins. In contrast, the genomes of retroviruses are devoid of viral helicases. Human immunodeficiency virus (HIV-1) has adopted the ability to use one or more cellular RNA helicases for its replicative life cycle. In this chapter, we briefly summarize the approach for assaying the RNA unwinding activity of RNA helicases and measurement of the effect helicase inhibitors on HIV-1 replication.

Keywords

RNA helicases; Human immunodeficiency virus type 1 (HIV-1); DEAD-Box domain; DDX3

1. Introduction

Helicases, including DNA and RNA helicases, are enzymes that separate stretches of duplexed DNA and/or RNA into single-stranded components in an energy-dependent manner. Based on motifs and sequences, RNA helicases are grouped into three superfamilies (SF1 to SF3) and two smaller families (F4 and F5) ¹. Most of the RNA helicases are in the SF2 superfamily and are conserved among different species. They can be found in organisms ranging from bacteria to humans to viruses.

Viral replication requires the use of host cell proteins and enzymes. Many viruses utilize viral helicases at various stages of their life cycle; these viruses have evolved to encode directly helicase or helicase-like proteins. However, viruses that synthesize their genome within the cell nucleus tend to exploit cellular helicases and thus do not encode for any RNA helicase. Human immunodeficiency virus type 1 (HIV-1) is one example of this latter class of virus.

HIV-1 is a human retrovirus that packages two copies of positive strand full-length viral RNA per virion. HIV-1 infects human cells using a major receptor (CD4) with one of several co-receptors (CCR5, CXCR4, DC-SIGN), and it infects mostly T helper (TH) cells, macrophages, and some microglial and dendritic cells ². The viral genome is > 9 kilobases and encodes nine proteins. HIV-1 structural proteins include Gag (group-specific antigen), Pol (polymerase), and Env (envelope). In addition, HIV-1 encodes two regulatory proteins, the transcriptional transactivator (Tat) and the regulator of post-transcriptional gene expression (Rev). The virus also has four genes that encode accessory proteins: Nef, Vif,

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4. Notes: For using Radioactive isotopes in RNA unwinding assay and RT assays users should follow their Institutional Guidelines for safe use of Radioactive compounds in research. The users must be certified and properly trained in use of radioactivity.

Vpr, and Vpu. Replication of HIV-1 RNA starts inside an infected cell with the reverse transcription of its RNA into a cDNA form followed by its integration into the host chromosomal DNA to form a provirus. HIV-1 RNA is then transcribed from the proviral DNA by the cellular RNA polymerase II, processed like cellular mRNAs including 5' - and 3' -end modifications as well as splicing, and is then exported into the cytoplasm for translation. There are three processes pertaining to HIV-1 RNA that do not normally occur for cellular RNAs: nuclear export of intron-containing HIV-1 RNAs, packaging of viral RNAs into the space-limited interior of virions, and completion of the reverse transcription of HIV-1 genomic RNA in the cytoplasm. In these regards, it is important to appreciate that HIV-1 encodes the nucleocapsid (NC) protein which bears RNA chaperone activity and has been shown to regulate HIV-1 RNA packaging and viral reverse transcription ³

A single HIV-1 transcript in its unspliced and spliced forms directs the synthesis of all viral proteins. Although export of intron-containing cellular transcripts from the nucleus into the cytoplasm is restricted in mammalian cells, HIV-1 must use unspliced RNA for the packaging of its genome into new virions and for the translation of Gag protein. Similarly, HIV-1 has to use a partially spliced transcript for translation of its Env protein. Thus, the virus must overcome the cell's normal constraint of retaining unspliced/partially spliced RNAs in the nucleus preventing the nuclear-cytoplasmic export of such entities. To overcome this constraint, HIV-1 has evolved the Rev protein. Rev utilizes CRM1 as a cellular cofactor for Rev-dependent export of unspliced and partially spliced HIV-1 RNA. There is evidence that for export of HIV-1 RNAs, Rev/CRM1 activity also needs an ATP-dependent co-factor, RNA helicase DDX3 ⁴. DDX3 is a nucleocytoplasmic shuttling protein that binds CRM1 and localizes to nuclear membrane pores. Experimentally, abolition of the cell's DDX3 activity suppressed Rev-RRE (Rev-responsive element) function for unspliced and partially spliced HIV-1 RNAs ⁴, supporting that DDX3 is a human RNA helicase that functions in the CRM1 RNA export pathway.

2. Materials

2.1. Purification of DDX3 protein

1. LB broth
2. Chitin beads (NEB)
3. Column Buffer: 20 mM Tris-HCl, 1000 mM NaCl, 0.5% Triton X-100, 0.1 mM EDTA and 20 μ M PMSF.
4. Cleavage Buffer: 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA and 50 mM DTT

2.2. RNA Unwinding assay

1. Helicase buffer: 20 mM Tris-HCl, pH 8.0, 70 mM KCl, 2 mM MgCl₂, 2 mM dithiothreitol, 15 units RNasin, and 2 mM ATP
2. Maxiscript T7 in vitro transcription kit (Ambion)
3. MaxiScript T3 in vitro transcription kit (Ambion)
4. Sample loading Buffer: 10 mM EDTA, 40% glycerol, bromphenol blue, xylene cyanol.
5. EasyTides Uridine 5'-triphosphate,[α -³²P] (Perkin Elmer).
6. 5X RNA Annealing Buffer: 30 mM HEPES (pH 7.4), 100 mM Potassium Acetate, 2 mM Magnesium Acetate

7. 10X Tris Boric acid EDTA (TBE) buffer
8. Accugel 29:1, (National Diagnostics), TEMED, 10% Ammonium persulfate

2.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Separating buffer (4X): 1.5 M Tris-HCl, pH 8.7, 0.4% SDS (Protogel Resolving Buffer, National Diagnostics). Store at room temperature.
2. Stacking buffer (4X): 0.5 M Tris-HCl, pH 6.8, 0.4% SDS (Protogel Resolving Buffer, National Diagnostics). Store at room temperature.
3. 30% acrylamide/bis solution (37.5:1 w/v) (ProtoGel (30%), National Diagnostics)
4. N,N,N',N'-Tetramethyl-ethylenediamine (TEMED, Bio-Rad, Hercules, CA)
5. 10% Ammonium persulfate: prepare 10% solution in water.
6. Running buffer (10X): 125 mM Tris, 960 mM glycine, 0.5% (w/v) SDS (Tris-Glycine-SDS PAGE Buffer (10X), National Diagnostics). Store at room temperature.
7. Prestained Protein Marker, Broad Range (7–175 kDa), (NEB)
8. Hoeffer S600 electrophoresis unit (GEBiosciences)
9. Sample buffer (SDS reducing buffer) (store at room temperature) Deionized water 3.8 ml, 0.5 M Tris-HCl, pH 6.8 1.0 ml, Glycerol 0.8 ml, 10% (w/v) SDS 1.6 ml, 2-mercaptoethanol 0.4 ml, 1% (w/v) bromophenol blue 0.4 ml, add water to 8.0 ml.

2.4. Western Blotting

1. Tris-Glycine transfer buffer: 25 mM Tris (do not adjust pH), 190 mM glycine, 20% (v/v) methanol.
2. Casein Hydrolysate (USB)
3. CSPD (Applied Biosystems)
4. Nitro-Block (Applied Biosystems)
5. PVDF membrane (Millipore)
6. Semi-dry Blotting apparatus
7. 10X Assay Buffer: 200 mM Tris (pH 9.8), 10 mM MgCl₂.
8. Blocking Buffer: 1X PBS containing 0.2% casein hydrolysate, 0.1% Tween 20 detergent

2.5. HIV-1 RT assay

1. RT assay buffer: 60mM Tris HCl pH 7.8, 75mM KCl, 5mM MgCl₂, 0.1 % Nonidet-P40, 1.04 mM EDTA, 5μg/ml poly rA, 0.16 μg/ml Oligo dT₍₁₈₎, 4mM DTT.
2. alpha ³²p dTTP (Perkin Elmer)
3. DE81 paper (Whatman)
4. Phosphorimaging plate/Phosphorimager (Fuji Medical, FLA7000)
5. 20X SSC buffer (Invitrogen)
6. Plasmid pNL4-3 (proviral HIV-1 molecular clone)

3. Methods

3.1. Purification of DDX3 protein

1. DDX3 cloned into pTYB11 vector, fused at its N-terminus with Chitin binding domain was used to transform *E. coli* BL21-DE3 cells. Cells were grown at 25° C overnight.
2. A single colony was selected and grown at 25°C in LB medium containing 100 µg/ml ampicillin. When the OD 600 of the culture reaches 0.8, protein expression was induced at 15°C with IPTG at a final concentration of 0.5 mM.
3. Cell extracts were prepared by lysing the cells by sonication in column buffer. Extracts were clarified by centrifugation and supernatant was used for protein purification.
4. Chitin column (20 ml for 1 liter culture) was equilibrated with 10 volumes of Column Buffer and slowly load with the clarified lysate.
5. The columns were then washed with at least 20 bed volumes of Column Buffer to thoroughly remove the unbound proteins.
6. Column was then washed 2 times with 3 bed volumes of Cleavage Buffer [20 mM HEPES or Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA] and then once with Cleavage Buffer containing 50 mM DTT. The column flow was stopped without draining the cleavage buffer completely. The column then left at 8°C for days to allow for cleavage.
7. Cleaved protein was eluted by adding 0.5 bed volumes of cleavage buffer and continuing the column flow with Cleavage Buffer.
8. Eluted protein was dialyzed and concentrated before use. Purification was confirmed by SDS-PAGE electrophoresis and coomassie blue staining of gel.

3.2. RNA unwinding assay (Fig. 1)

A protocol for an RNA unwinding assay is as follows.

1. pBluescript plasmid was used to generate partially double stranded RNA for the RNA unwinding assay. The pBluescript vector was digested to completion with *Kpn* I and transcribed with T3 polymerase to generate a 120 base long transcript. The vector was also digested with *Eco*RI and transcribed with T7 polymerase in the presence of (α³²p) UTP.
2. The two complementary transcripts were purified and suspended in 1X annealing buffer. Samples were heated for 1 minute to 95° C and gently cooled to RT in the heating block.
3. Double stranded RNA was then incubated with purified DDX3 in ATPase/helicase (20 mM Tris-HCl, pH 8.0, 70 mM KCl, 2 mM MgCl₂, 2mM dithiothreitol, 15 units RNasin, and 2 mM ATP).
4. After incubation for 30 mins at 37 °C, the reactions were stopped by the addition of a solution containing 10 mM EDTA, 40% glycerol, bromphenol blue, xylene cyanol.
5. The reaction was resolved in a 10% polyacrylamide gel (30 ml of Accugel 29:1, 10 ml 10X TBE and 60 ml of H₂O, 1 for 2 hours. The gel was dried and exposed to a phosphorimaging plate.

3.3. SDS-PAGE Electrophoresis (Fig. 2)

1. Prepare a 0.75 -mm thick, 10% gel by mixing 3.75 ml of 4X separating buffer, with 5.0 ml acrylamide/bis solution, 6.25 mL water, 150 μ L ammonium persulfate solution, and 10 μ L TEMED. Pour the gel, leaving space of 3–4 cms for a stacking gel, and overlay with water. Wait until the gel polymerizes (approx 20 mins).
2. Pour off water and prepare the stacking gel by mixing 0.65 ml of 30% acrylamide/0.8% bisacrylamide, 1.25 ml of 4' Tris·Cl/SDS, pH 6.8 (see reagents, below), and 3.05 ml H₂O. Add 100 μ l of 10% ammonium persulfate and 5 μ l TEMED. Swirl gently to mix. Insert the comb and pour the stacking gel avoiding air bubbles. Once the stacking gel polymerizes remove the comb gently and rinse the well with deionized water.
3. Prepare the running buffer by diluting 100 mL of the 10X Tris-Glycine SDS running buffer with 900 ml of deionized water.
4. Add diluted Tris-Glycine SDS running buffer to the upper and lower chambers of the gel unit and load the 25 μ L of each sample in a well. Include one well for prestained protein molecular weight markers.
5. Complete the assembly of the gel unit and connect to a power supply. The gel can be run either overnight at 45 V.

3.4. Western Blotting (Fig. 2)

1. The samples that have been separated by SDS-PAGE are transferred to PVDF membrane electrophoretically. The gel unit is disconnected from the power supply and disassembled. The stacking gel is removed and discarded.
2. Five 3M sheets wetted with transfer buffer are laid over the electrode (anode). The PVDF membrane activated in methanol and after rinsing in transfer buffer laid on top of the 3M paper. The separating gel is then laid on top of the PVDF membrane. Five more sheets of 3MM paper are wetted in the transfer buffer and carefully laid on top of the gel, ensuring that no bubbles are trapped in the resulting sandwich. The lid (electrode/cathode) is put on the gel sandwich and transfer is performed at a constant current of 250 milliAmp for 2 hrs.
3. Following transfer, incubate the blot in Blocking Buffer (at least 30 mL) for 60–120 min
4. Dilute primary antibody in Blocking Buffer (30 ml). Incubate with blot 30–60 min.
5. Wash the membrane at least twice for 5 min in Blocking Buffer. Use at least 30 ml for all washes.
6. Dilute secondary antibody-AP (alkaline phosphatase) conjugate 1:5,000 in Blocking Buffer (30 ml). Incubate with blot for 30–60 min.
7. Wash 3 times for 5 min each as in Step 3, then rinse 2 times for 2 mins with 1X Assay Buffer.
8. Drain blots by touching a corner on a paper towel, then place on plastic wrap on a flat surface (do not let blots dry).
9. Pipette a thin layer of CSPD solution containing 5% nitro-block onto the blot and incubate 5 min.
10. Drain excess substrate solution and wrap the blot in plastic sheets. Smooth out bubbles or wrinkles.

11. Blots may be imaged by placing them in contact with standard X-ray film.

3.5. Reverse Transcriptase assay (RT assay) to measure inhibition of HIV (Fig. 2)

RT assays provide an inexpensive approach to quantify the amount of virus present in the sample. RT assay is an indirect measure of virus particles present in sample; it measures the amount of viral protein reverse transcriptase which is incorporated into the virions⁵.

1. Culture supernatants from pNL4-3 transfected HeLa cells or PBMC infected with HIV-1 were used in the assay. In case of HIV-1 infected peripheral blood mononuclear cells, PBMC, cell culture supernatants were collected every 3rd day. HeLa cells were transfected with 2 μ g of pNL4-3 (HIV-1 molecular clone) using Lipofectamine - Lipofectamine plus reagent. 48 hrs post transfection the culture supernatant was collected and assayed for RT activity.
2. 10 μ l of culture supernatant was mixed with 50 μ l of RT assay buffer containing ³²P dTTP (2 μ l/ml of RT assay buffer).
3. The reaction mix was incubated for 2 hrs at 37°C. Subsequently 10 μ l of the reaction mix was spotted on DE81 paper and allowed to dry.
4. The paper was washed three times with 2X SSC buffer, dried and used to expose a phosphorimaging plate.
5. The RT activity was quantified by phosphorimager FLA-7000 (Fuji Medical) and also using scintillation counter (Beckman).

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Fig. 1.

A schematic illustration of a helicase assay A) Preparation of partial RNA duplex for RNA unwinding assay. B) RNA unwinding assay.

Fig. 2.
An illustration of an approach for assaying the effect of Helicase inhibitors on HIV-1 replication.