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Assays to Demonstrate the Roles of Arenaviral Nucleoproteins (NPs) in Viral RNA Synthesis and in Suppressing Type I Interferon

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Abstract

Arenaviruses, such as Lassa fever virus (LASV) and Pichinde virus (PICV), are enveloped viruses with a bi-segmented ambisense RNA genome. The large (L) genomic segment encodes the Z matrix protein and the L RNA-dependent RNA polymerase, whereas the small (S) genomic segment encodes the nucleoprotein (NP) and the glycoprotein (GPC). The NP encapsidates viral genome, is required for viral transcription and replication, and acts as a type I interferon (IFN) antagonist. This article describes assays to demonstrate that NP contains 3'–5' exonuclease (RNase) activity to degrade modeled RNA of the pathogen-associated molecular pattern type and suppresses the IFN β -promoter driven luciferase reporter gene. The minigenomic (MG) assay is used to assess the role of NP in replicating and transcribing a viral promoter-driven luciferase reporter gene. These powerful assays demonstrate the versatility of NPs in mediating viral replication as well as in modulating host innate immune responses.

Keywords

Lassa virus; nucleoprotein; arenavirus

1. Introduction

Lassa virus (LASV) is responsible for an estimated 300,000–500,000 infections and ~5,000 deaths annually in several countries in West Africa (1, 2). There are currently no FDA-licensed vaccines or therapeutics to treat or protect against Lassa and other arenavirus infections. A hallmark of Lassa infection is the generalized immune suppression that leads to high levels of viremia in severe and lethally infected patients. Two of the four arenavirus-encoded proteins, namely the nucleoprotein NP and the RING-finger Z protein, have recently been described as type I interferon (IFN) antagonists that contribute to the general immunosuppression observed during the course of arenavirus infection (3–7). A better understanding of how arenaviruses evade host immune surveillance will offer important insights for the development of effective preventative and treatment options.

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Toward this goal, we have developed several new research tools and methods, including the development of the in vitro NP ribonuclease (NP RNase) assay to assess the NP 3'–5' exoribonuclease function, the interferon beta (IFN β)-promoter driven luciferase reporter assay to assess the role of NP in suppressing the IFN β promoter, and the LASV minigenome (MG) assay to evaluate LASV polymerase function in mediating transcription and replication of a model arenaviral genomic RNA template. We describe herein detailed protocols for these assays. With NP playing many crucial roles in mediating virus and host immune functions, understanding the biological function of this protein will greatly facilitate the development of therapeutic strategies against LASV and other deadly viruses in the *Arenaviridae*.

2. Materials

2.1 Protein expression and purification

1. pLOU3 vector, which is a derivative plasmid of the pMAL-C4X vector (New England Biolab), contains the LASV NP gene. In this construct, the NP gene is cloned immediately downstream of the Tobacco Etch virus (TEV) proteinase cleavage site, which is preceded by a 6His-tag at the N-terminus of the maltose binding protein (MBP). This protein expression cassette is placed under the control of an isopropyl-beta-d-thiogalactopyranoside (IPTG)- inducible (lacIq) promoter.
2. The recombinant plasmid was transformed into *E. coli* Rosetta strain.
3. IPTG.
4. Shaker-incubator.
5. Spectrophotometer.
6. J-20 ultra-centrifuge.
7. Cell disruptor.
8. Amylose resin.
9. Amylose binding buffer: 10% glycerol, 20 mM Tris pH 7.5, 0.2 M NaCl, 1 mM EDTA.
10. Amylose elution buffer: 10% glycerol, 20 mM Tris pH 7.5, 0.2 M NaCl, 1 mM EDTA, 10 mM Maltose.
11. ÄKTA purifier 10 or similar instrument to control the flow rate of the columns.
12. Desalting column (e.g. Hiprep 26/10).
13. Gel filtration column (Sephacryl S-200).
14. Gel filtration buffer: 20 mM Tris pH 7.5, 0.3 M NaCl, 10% glycerol.
15. –80°C freezer.
16. Mass spectroscopy (Q-Star MS/MS micromass MALDI-TOF).

17. Pre-cast 4–12% Bis-Tris polyacrylamide gels.
18. Power supply.
19. Unstained protein standards.
20. DNase.
21. Phenylmethylsulfonyl fluoride (PMSF) and other pretease inhibitors.
22. Binding buffer: 1/50 PBS, 50 mM Tris-HCl, 0.5 mM EDTA, 1 mM DTT, 0.3 M NaCl, 10% glycerol.
23. TEV cleavage buffer: 1/50 PBS, 50 mM Tris-HCl, 0.5 mM EDTA, 1 mM DTT, 0.3 M NaCl, 10% glycerol.

2.2. NP ribonuclease (RNase) assay

1. NP protein: see section 3.1
2. PCR thermal cycler.
3. Microcentrifuge.
4. T4 polynucleotide kinase.
5. Radio-isotope (γ -ATP).
6. RNA Oligo (5'-AGUAGAAACAAGGCC-3') to serve as RNase substrate.
7. Illustra MicroSpin G25 de-salting column.
8. 17% polyacrylamide gel solution containing urea (25 ml): 2.5 ml of 10X TBE, 10.5 g urea, 10.6 ml of 40% Acrylamide, and 11.9 ml ddH₂O. 10 μ l of TEMED and 80 μ l of 30% APS added just before pouring.
9. Ambion T7 MEGAscript Kit for T7-polymerase-driven *in vitro* transcription.
10. Water bath.
11. Electrophoresis autoradiography cassette.
12. X-ray film.
13. Vertical gel electrophoresis apparatus.
14. Power supply.
15. X-ray film developer.
16. 10x RNase buffer: 25 mM Tris-HCl, pH7.5, 10 mM NaCl, 15 mM KCl, 1 mM MnCh₂, and 0.1 mg/ml bovine serum albumin BSA.

2.3 Mini-genome (MG) assay and IFN β promoter-driven luciferase reporter assay

1. Plasmids: NP and L gene were cloned into pCAGGS mammalian vector under CMV promoter (3). IFN- β promoter-directed LUC plasmid was used for NP induced interferon suppression assay. MGLF7–5Rluc3 plasmid was used for

Mini-genome assay. β -galactosidase (β -Gal) plasmid was used for normalizing luciferase assay.

2. Cell line: 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 50 mg penicillin and streptomycin per ml.
3. Plate reader: Synergy 2 (BioTeK)
4. Lipofectamine 2000 (Invitrogen)
5. DEPC water.
6. 2X Hepes buffered saline (HBS): 50mM HEPES, 280mM NaCl, 1.5mM Na₂HPO₄, pH=7.10.
7. Beta-galactosidase, Firefly and Renilla luciferase assay kits.
8. 2.5 M calcium chloride stock solution.
9. Sendai virus, Cantell strain (ATCC, VR907).

3. Methods

3.1 Protein expression and purification

1. The recombinant plasmid was transformed into *E. coli* Rosetta strain using the conventional heat-shock transformation method (9).
2. A single colony was picked and inoculated into 500 ml LB bacterial growth medium.
3. Cells were incubated at 37°C in a shaker-incubator with constant agitation at 200 rpm overnight.
4. The overnight culture was then transferred into 10 L of the LB bacterial growth medium.
5. The culture was incubated at 37°C with shaking at 200 rpm for about 2 hours.
6. Expression of the recombinant NP protein was induced by the addition of IPTG to a final concentration of 0.03 mM when the OD₆₀₀ of the cell culture reached 0.6 (see Note 1).
7. The cell culture was kept at 20°C overnight with shaking at 200 rpm (see Note 1).
8. Cells were harvested by centrifugation at 8,000 X g for 10 min at 4 °C in the J-20 centrifuge.
9. The cell pellet collected from 10 L culture was resuspended in 100 ml of Amylose binding buffer.
10. Cells were lysed by a cell disruptor at 30 KPsi

11. The sample was centrifuged in the J-20 centrifuge at 20,000 rpm for 30 min to remove cellular debris.
12. The supernatant was applied onto the amylose resin column.
13. The column was washed using a 10-column volume of the Amylose binding buffer (see **Note 2**).
14. The fusion protein was eluted from the column with the Amylose elution buffer.
15. The protein elution buffer was changed to TEV cleavage buffer using the Hiprep desalting column.
16. Fractions containing HisMBP-NP were pooled and the fusion protein was cleaved by incubating with 1 ml His-tagged TEV protease at room temperature overnight (see **Notes 2 and 3**).
17. The sample was then applied onto the amylose resin again in order to remove the MBP.
18. The flow-through sample was concentrated into 5 ml and applied onto a Hiload 16/600 Superdex 200 gel-filtration column, which was equilibrated using the gel filtration buffer and the ÄKTA purifier 10 chromatography system.
19. The flow rate was 1 ml/min controlled by the ÄKTA purifier 10, and protein peaks were monitored at optical density of 280 nm.
20. All elution solutions were collected by the fraction collection of the ÄKTA purifier 10 using the 96-deep-well block.
21. The fractions containing purified NP protein were pooled and concentrated to 7 mg/ml and stored in a -80°C freezer.
22. The protein's identity and integrity were checked by mass spectroscopy and proteins were separated by SDS-PAGE.
23. The SDS-PAGE was carried out using pre-cast gels and the PowerEase 500 power supply according to the manufacturer's instruction.
24. The protein molecular marker was used to size the recombinant protein.
25. The SDS-PAGE gels were stained with Coomassie dye for 10 min.
26. The SDS-PAGE gels were destained by putting the gels in a beaker with water and boiling for 5 min using a conventional microwave oven.

3.2 NP ribonuclease (RNase) assay

1. The 15-nt RNA oligo (see 2.2) was 5' end labeled with $\gamma\text{-}^{32}\text{P}$ ATP in a polynucleotide kinase reaction as follow.
 - 9.5 μl DEPC-treated water
 - 2 μl RNA oligo (100pmol)
 - 5 μl $\gamma\text{-}^{32}\text{P}$ ATP (25pmol, 3uCi/pmol, 10uCi/ μl)

2µl 10x kinase buffer

1.5µl RNase-free T4 PNK

Sample was incubated at 37°C for 1 h, then at 95°C for 2 min.

Sample was collected by centrifugation at 12,000rpm for 1 min 20µl DEPC-treated water was added.

2. The un-incorporated γ -³²P ATP was eliminated by passing the radiolabeled RNA sample through the Illustra MicroSpin G25 desalting spin column.
3. The γ -³²P-labeled RNA oligo was mixed with 5-fold amount of unlabeled RNA oligos of either the same or the complementary sequence, heat denatured at 95°C for 5 min and slowly cooled to room temperature in order to form single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA) substrates.
4. The exoribonuclease reaction containing 1 pmol of radiolabeled RNA substrates in either ss or ds form and various concentrations of NP protein in an RNase buffer was incubated at 37°C for 60 min as follow.

5µl DEPC-treated water

1 µl γ -³²P-labeled RNA oligos

1µl 10x RNase buffer

1 µl recombinant LASV NP protein at various concentrations

Sample was incubated at 37°C for 1 h, 1 µl 0.5M EDTA was added, and 10 µl formamide loading buffer was also added.

The reaction was terminated by heating the sample to 95°C for 10 min and immediately chilled on ice.

Sample was collected by micro-centrifugation at 12,000 rpm for 1 min.

5. Prepare denaturing polyacrylamide gel solution.
6. Pre-electrophoresis gel at 150 V for 30 min. Use 0.5 X TBE in upper and lower reservoirs of the gel box.
7. Samples were loaded into wells and were separated at 150 V for 3 h.
8. The gel was placed onto a spent film backing and covered with plastic wrap and exposed to an X-ray film with an intensifying screen at -80°C freezer.
9. The film was developed in the film developer to visualize RNA cleavage activities (*see Note 4*).
10. As an example of results from this assay, purified LASV NP protein was able to cleave both ss and ds RNAs (Figure 1). The ladder effects are due to the different efficiencies of RNA cleavage induced by different concentrations of the LASV NP.

3.3. Mini-genome (MG) assay

This assay is meant to measure expression of viral genes from a “mini-genome” construct, and it occurs in four stages: first) the minigenome plasmid is RNA- transcribed *in vitro*, second) 293T cells are transfected with plasmids expressing the viral NP and L genes, third) 24 h later, these same 293T cells are transfected with the minigenome RNA, and fourth) the cells are assessed for luciferase expression. The first stage is covered in steps 1–8, the second stage in steps 9–14, the third stage in steps 15–21, and the fourth stage in steps 22–24.

1. Prior to RNA transcription, the plasmid MGLF7–5Rluc3 was linearized by digestion with Nhe I restriction enzyme.
2. DNA was extracted by phenol/chloroform and isopropanol-precipitated using the standard technique (9).
3. Transcription reaction was assembled at room temperature according to Ambion MEGAscript® Kit manual. Calculate the volume of nuclease-free water necessary to bring the total volume to 20 µl, and to this amount add 2 µl ATP, 2 µl CTP, 2 µl GTP, 2 µl UTP, 2 µl 10X reaction buffer, 1 µg linearized template DNA, and 2 µl enzyme mix.
4. Sample was incubated at 37°C for 4 h.
5. 115 µl nuclease-free water and 15 µl ammonium acetate stop solution were added.
6. RNA was extracted by phenol/chloroform and isopropanol precipitated using the standard technique (9).
7. Carefully discard the precipitating solution and the RNA was resuspended in 80µl of DEPC-treated water.
8. RNA was quantified and adjusted to a concentration of 1 µg/µl.
9. Prior to plasmid transfection, 293T cells were seeded into 24-well plates at 1×10^5 cells/well that contains 0.5 ml of DMEM with 10% FBS and 50 µg/ml of penicillin and streptomycin.
10. In the morning of the day of transfection, a fresh aliquot of the cell media was added to the 293T cell culture.
11. DNA and calcium phosphate mixture was prepared at room temperature as follow.

Each of the 25-µl of DNA mix (for one 24-well transfection) contains:

1µl β-gal reporter plasmid (50 ng/µl)

1µl pCAGGS-NP plasmid (250 ng/µl)

1µl pCAGGS-L plasmid (500 ng/µl)

2.5µl 2.5 M calcium chloride stock solution

19.5µl water

12. To prepare triplicates for each test condition as follow:
3µl β-Gal reporter plasmid (50 ng/µl)
3µl pCAGGS-NP plasmid (250 ng/µl)
3µl pCAGGS-L plasmid (500 ng/µl)
7.5µl 2.5 M calcium chloride stock solution
58.5µl water
13. Into each tube of DNA/calcium phosphate mixture (25×3=75 µl), add 75 µl of 2xHBS. Gently mix sample by vortexing using the lowest speed setting. Add the mixture dropwise using a pipetman into each well of cells (48 µl of sample into each well of the 24-well plate). (*see Note 5*).
14. Incubated cells in a 37°C CO₂ incubator for 24 hours.
15. 4 h before RNA transfection, an aliquot of fresh antibiotics-free DMEM medium was used to replace the overnight cell culture medium.
16. For each well, 1 µg of *in vitro*-transcribed RNA (*see Note 6*) was diluted into 25 µl OptiMEM® I Medium.
17. For each well, 1 µl of Lipofectamine™ 2000 was diluted into 25 OptiMEM® I Medium and incubated for 5 min at room temperature.
18. The diluted RNA was added into diluted Lipofectamine™ and incubated at room temperature for no more than 10 min.
19. RNA-Lipofectamine™ 2000 complexes were directly added to each well containing cells and mixed gently by rocking the plate back and forth. The cells were incubated at 37°C in a CO₂ incubator.
20. The cell medium was replaced by fresh DMEM medium (10% FBS) at 6 h post transfection.
21. 24 h post RNA transfection, cells were washed once by PBS. Then cells were lysed by lysis buffer for Renilla luciferase assay.
22. 20 µl of cell lysate was used for Renilla luciferase assay and another 50 µl of cell lysate was used for β-gal assay according to manufacturer's instructions.
23. Renilla luciferase activity is normalized by dividing the Renilla luciferase reading by the β-gal reading. The minigenome replication activity is represented by the folds of the normalized Renilla luciferase activity with respect to that of the control.
24. In MG assay (Figure 2), cotransfection of PICV NP and L expression plasmids into 293T cells was able to replicate and transcribe the transfected PICV minigenome (MG) expressing the Renilla luciferase reporter gene. P2 and P18 indicate that the NP and L genes are from different PICV strains.

3.4 IFN β promoter-driven luciferase reporter assay

1. 293T cells were seeded into 24-well plates at 1×10^5 cells/well with complete DMEM.
2. In the morning of the day of transfection, culture media was replaced by an equal aliquot of fresh media.
3. Prepare DNA and calcium phosphate mix as follow:
25 μ l of DNA mix (for one 24-well transfection) contains:
1 μ l β -Gal reporter plasmid (50 ng/ μ l)
1 μ l IFN β -promoter driven luc reporter plasmid (100 ng/ μ l)
1 μ l pCAGGS-NP plasmid (10–1000 ng/ μ l)
2.5 μ l 2.5 M calcium chloride stock solution
19.5 μ l water
To prepare triplicates for each test condition as follow:
3 μ l β -Gal reporter plasmid (50 ng/ μ l)
3 μ l IFN β -promoter driven luc reporter plasmid (100 ng/ μ l)
3 μ l pCAGGS-NP plasmid (10–1000 ng/ μ l)
7.5 μ l 2.5 M calcium chloride stock solution
58.5 μ l water
4. Into each tube of DNA/calcium phosphate mixture ($25 \times 3 = 75 \mu$ l), add 75 μ l 2xHBS, vortex using very low speed to mix, and add the mixture to 3 wells of cells (48 μ l per well) as shown in section 3.3.
5. 24 h post transfection, media was replaced by fresh complete DMEM and cells were infected with Sendai virus (SeV) at moi of 1. Cells were continued culture for 24 h at 37°C in a CO₂ incubator for 24 h.
6. Interferon promoter activity was assessed by performing the Firefly luciferase assay using a kit from Promega.
7. An example of results from this assay can be seen in Figure 3. Sendai virus (SeV) infection of 293T cells was able to stimulate the IFN β promoter activity to express a high level of the Firefly luciferase reporter gene, whereas the LASV NP expression suppresses this promoter activity. Empty vector was used as a control.

4. Notes

1. It is important to express the MBP-NP fusion protein at 20°C, and to use 0.03 mM IPTG concentration for the induction as this condition will produce more soluble MBP-NP fusion protein. Using higher IPTG or higher temperature for

protein expression will produce more MBP-NP fusion into inclusion bodies. In all the sample buffer and purification buffers, 10% glycerol is required to stabilize the protein in the buffers.

2. It is important to use TEV proteinase at 1:500 ratio of proteinase/protein to make sure the cleavage should be slow to prevent protein precipitation.
3. To reduce the protein precipitation, dilution of the protein sample to 2 mg/ml is required.
4. With new isotope, it usually took 3–6 h of exposure of the X-ray film at -80°C .
5. For calcium phosphate transfection, DNA/calcium phosphate mixture was directly added to the cells, dropwise, uniformly and gently over the surface of the monolayer of the cells. It is important to add the precipitate to the cells within 5 min of adding the 2X HBS. If waiting for more than 5 min, the precipitate will be too large and can be toxic to cells.
6. Separate 1 μg of in vitro transcribed RNA in a 1% agarose gel to check for the integrity of the RNA prior to its use in transfection.

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Figure 1. Cleavage of ssRNA and dsRNA by purified LASV NP.

5'- γ - ^{32}P -labeled ssRNA or dsRNA were used as substrates for cleavage by different concentrations of the purified LASV NP.

Figure 2. NP and L protein can replicate and transcribe the viral minigenome (MG). Cotransfection of PICV NP and L expression plasmids into 293T cells was able to replicate and transcribe the transfected PICV minigenome (MG) expressing the Renilla luciferase reporter gene. P2 and P18 indicate that the NP and L genes are from different PICV strains.

Figure 3. IFN β promoter-driven luciferase reporter assay to demonstrate the role of LASV NP in suppressing this promoter activity.

Sendai virus (SeV) infection of 293T cells was able to stimulate the IFN β promoter activity to express a high level of the Firefly luciferase reporter gene, whereas the LASV NP expression suppresses this promoter activity. Empty vector was used as a control.