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Expression and X-ray Structural Determination of the Nucleoprotein of Lassa Fever Virus

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Abstract

We describe methods to express the nucleoprotein (NP) of Lassa fever virus (LASV) in *E. coli*, to purify and crystallize it using the sitting-drop vapor diffusion method. The crystals were screened using Rigaku micro-007 X-ray generator and a dataset was collected at resolution of 2.36 Å. The crystals belong to space group P3, with the unit cell parameters $a = b = 176.35$ Å, $c = 56.40$ Å, $\alpha = \beta = 90^\circ$ and $\gamma = 120^\circ$. Using X-ray diffraction method, we constructed a three-dimensional structure of the LASV NP that should aid in the development of novel therapeutic strategies against this virus, for which vaccine and effective treatment modalities are currently unavailable.

Keywords

Lassa virus; nucleoprotein; X-ray structure; large-scale expression; arenavirus

1. Introduction

Arenaviruses are enveloped, single-stranded ambisense RNA viruses that are geographically, phylogenetically and serologically divided into two subgroups, the New World and the Old

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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.
2. In all the sample buffer and purification buffers, 10% glycerol is required to stabilize the protein in the buffers.
3. 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. To reduce the protein precipitation, dilution of the protein sample to 2 mg/ml is required.
4. The crystals were heavily twinned, and the structure as determined in the space group of P321, but the structure was refined in the space group of P3. During the structure refinement, both of the R_{factor} and R_{free} could not be reduced once reached to 0.3 and 0.35 in the space group of P321, respectively.

World viruses (1). Lassa virus (LASV) is an Old World member of the *Arenaviridae* family. It causes Lassa fever (LF), which is an endemic disease in West African countries like Nigeria, Guinea, Liberia, and Sierra Leone. Lassa fever causes up to 500,000 infections per year and 5,000 deaths annually in endemic areas (2). With the ease of modern means of traveling, Lassa cases have also been reported in America, Europe, Canada, and Japan (3,4). Due to the high mortality rate, the ease of human-to-human transmission, and the lack of effective treatments, the Lassa fever virus is categorized by the US Centers for Disease Control and Prevention (CDC) as a Category A agent, which requires its handling in proper biocontainment (BSL-4) facility and implicates it as a potential bioterror agent (5).

The LASV genome comprises a small RNA segment of 3.4 kb (S-RNA) and a large RNA segment of 7.2 kb (L-RNA). Each segment is reported to encode two viral genes separated by an intergenic region. The S-RNA encodes the nucleoprotein (NP, 64 kDa) and a glycoprotein precursor GPC, which is cleaved into two viral glycoproteins GPC1 (42 kDa) and GPC2 (38 kDa) by the cellular SKI-1/S1P subtilase (6). The L-RNA encodes the RNA-dependent RNA polymerase (L, 200 kDa) and a small RING finger protein (Z, 11 kDa).

The NP protein interacts with the viral RNA forming a ribonucleoprotein (RNP) complex, which is required for viral RNA transcription and replication. In addition, the NP protein from Lassa fever virus is reported to interact with the Z protein, and possibly also with host cell factors to regulate human immune responses to viral infection (7,8). Studies from our laboratory and others have recently shown that LASV NP contains 3'–5' exoribonuclease function that degrades potential pathogen-associated molecular pattern (PAMP) RNAs and results in the suppression of the host's innate immunity (9,10,11). With NP playing many crucial roles in mediating virus and host cellular functions, understanding the structure and function of this protein will greatly facilitate the development of therapeutic strategies against this and other deadly viruses in this family. Here, we describe detailed methods of expression, purification and structural determination of the LASV NP.

2. Materials

2.1 Plasmids and bacterial cells

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 (*lacI^H*) promoter.
2. *E. coli* Rosetta strain, expressing codons abundant in humans, was transformed with recombinant plasmids.
3. Bacterial growth medium is Luria Broth (LB) with antibiotics: LB liquid medium with 50 µg/ml Ampicillin and 34 µg/ml Chloramphenicol.

4. IPTG is a structural mimic of galactose and binds to the Lac Repressor to induce Beta-galactosidase or the protein expression cassette, MBP -- TEV cleavage -- NPV_{LASV}, described in 2.1.1.
5. Amylose binding buffer: 10% glycerol, 20 mM Tris pH 7.5, 0.2 M NaCl, 1 mM EDTA is supplemented with 2 protease inhibitor tablets, 1 µM DNase and 1 mM phenylmethylsulfonyl fluoride (PMSF).

2.2. Protein expression and purification

1. Shaker-incubator (Multitron standard, INFORS HT) for growth of bacteria.
2. Spectrophotometer (SmartSpec 3000)
3. High-performance centrifuge (Beckman J-20 Avanti™)
4. *E. coli* cell disruptor (Ts series 1.1 KW and Constant Systems LTD)
5. Amylose resin for affinity isolation of proteins bound to Maltose Binding Protein.
6. Amylose binding buffer: 1/50 PBS, 50 mM Tris-HCl, 0.5 mM EDTA, 1 mM DTT, 0.3 M NaCl, 10% glycerol.
7. Amylose elution buffer: 10% glycerol, 20 mM Tris pH 7.5, 0.2 M NaCl, 1 mM EDTA and 10 mM Maltose.
8. 3 mg/ml TEV protease for cleaving the expressed protein.
9. TEV cleavage buffer: 1/50 PBS, 50 mM Tris-HCl, 0.5 mM EDTA, 1 mM DTT, 0.3 M NaCl and 10% glycerol. This is used with the Desalting column (Hiprep 26/10).
10. ÄKTA purifier 10, a rapid protein liquid chromatography system.
11. 96 Deep well block for fraction collection.
12. Gel filtration column (Hiload 16/600 Superdex 200).
13. Gel filtration buffer: 20 mM Tris pH 7.5, 0.3 M NaCl, 10% glycerol.
14. −80°C freezer (U535 Innova)
15. Mass spectroscopy (Q-Star MS/MS micromass MALDI-TOF).
16. Pre-cast electrophoresis gels (NuPAGE 4–12% Bis-Tris gels).
17. 20X electrophoresis Running buffer (NuPAGEâMES, NP0002).
18. Electrophoresis Power supply (PowerEase 500).
19. SDS-PAGE unstained protein standards (Mark12)
20. Coomassie stain (SimplyBlue™ Safe stain).
21. Microwave oven.

2.3. Crystallization and crystal screening

1. Crystallization conditions were screened on 2-well MRC plates (SW2T-PS/1–100) with the sitting drop vapor diffusion method.
2. The crystallization drops were built using protein crystallization robot Honeybee system (model 961, Isogen Life Sciences).
3. Crystallization screen PEG/ION (# HR2–126, Hampton Research).
4. Lithium-Polyethylene glycol (Li-PEG) solutions for protein crystallization were #1Li-PEG: 0.2 M LiCl, 20% PEG3350 at 20°C. After optimization, hexagonal prism crystals were grown in #2Li-PEG: 0.2–0.3 M LiCl and 12–16% PEG3350 at 20 °C. For cryopreservation of crystals we used #3Li-PEG:15% glycerol, 0.2 M LiCl, 16% PEG3350.

2.4. X-ray diffraction

1. MicroMax-007 HFM fine focus X-ray generator and the CCD detector Saturn 944+(Rigaku)
2. ACTOR™ crystal sample changing robot (Rigaku).
3. The data were indexed and scaled using HKL2000 package (12).

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 (13).
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 liters of the LB bacterial growth medium.
5. The culture was incubated at 37°C with shaking at 200 rpm for about 2 hours.
6. The 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. Figure 1 shows whole protein lysate that contains the Lassa NP-MBP fusion protein, MBP-NP fusion protein purified through the amylose column, and the NP protein after it has been cleaved off from the MBP-NP fusion protein by the Tev proteinase.
19. 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.
20. Figure 2 shows Lassa NP protein purified by gel filtration column to homogeneity.
21. The flow rate was 1ml/min controlled by the ÄKTA purifier 10, and protein peaks were monitored at optical density 280 nm.
22. All elution solutions were collected by the fraction collection of the ÄKTA purifier 10 using the 96-deep-well block.
23. The fractions containing purified NP protein were pooled and concentrated to 7 mg/ml and stored in a -80°C freezer.
24. The protein's identity and integrity were checked by mass spectroscopy and separated by SDS-PAGE.
25. The SDS-PAGE were carried out using pre-cast gels and the PowerEase 500 power supply according to the manufacturer's instruction.
26. The protein molecular marker was used to size the recombinant protein.
27. The SDS-PAGE gels were stained with Coomassie for 10 min.
28. 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. Crystallization

1. Crystallization conditions were screened on 2-well plates with the sitting drop vapor diffusion method.
2. The drops were built with 0.15 μ l of the protein and 0.15 μ l of the mother liquid using two different protein concentrations (7 mg/ml and 3.5 mg/ml), with 100 μ l of mother liquid in each reservoir.
3. The initial crystals were formed in #1Li-PEG solution at 20°C after seven days. After optimization, hexagonal prism crystals were grown in #2Li-PEG solution at 20 °C for 5 days.
4. Figure 3 shows the purified NP forming crystals with a dimension of $0.2 \times 0.2 \times 0.6$ mm, which are suitable for X-ray diffraction. In order to confirm that the crystals contain the full-length NP protein, a single crystal was picked up and washed three times in the cryoprotectant solution, and dissolved in SDS-loading buffer for SDS-PAGE analysis. The result showed that there was a single band corresponding to the full-length NP protein, implicating its absolute purity and integrity.

3.3 X-ray data collection

1. The crystals were cryoprotected with #3Li-PEG.
2. The crystals were screened using the Rigaku X-ray generator, detector and robot.
3. One of the crystals was selected and the data generated from it were collected at oscillation angle 0.1° and 100 second exposure per frame with 60 mm of distance from crystal to detector, using the Cu radiation with the wavelength of 1.5418 Å.
4. A total of 1000 frames were reordered. The data were indexed and scaled as described (12).
5. Figure 4 shows the three-dimensional structure of the LASV NP that was built using ARP/wARP automatically and Coot by hands. (*see Note 4*)

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Figure 1. MBP-NP fusion was purified with an amylose column and the fusion protein was cleaved by Tev proteinase.

Lane 1 is protein molecular weight standard; Lane 2 shows whole protein lysate of the supernatant; Lane 3 shows MBP-NP fusion purified by the amylose column; Lane 4 shows NP protein that is cleaved from the MBP-NP fusion protein by the Tev proteinase.

Figure 2. LASV NP protein was purified by the gel filtration column.

A) photograph of the gel filtration profile. There are three major peaks: peak 1 shows aggregation of NP proteins, peak 2 shows the NP protein alone and peak 3 show NP fusion with MBP. B) protein purity was checked by SDS-PAGE electrophoresis. Lane 1, protein molecular weight standard; Lane 2–6 are elution samples from peak 1; Lane 7–11 are elution samples from peak 2; Lane 12–16 are elution samples from peak 3.

a

b

Figure 3. Photograph of crystals of Lassa NP protein and SDS-PAGE photograph of the crystals. (a) Crystals of the NP protein. (b) SDS-PAGE of the NP from the crystals. Lane 1 shows the protein molecular weight standard; Lane 2 shows the NP from the crystals.

Figure 4. Three dimensional structure of LASV NP.

(a) The protomer structure of NP presented in cartoon. (b) Electrostatic potential map of the protomer structure of NP.

Table 1.

Data collection and structure determination statistics.

Data collection	In house Data collection	Samarium peak	Samarium inflection	Samarium remote	Native Data
Wavelength (Å)	1.548	1.83	1.84	1.45	0.979
Resolution (Å)	50–2.36 (2.40–2.3 6)	57.86–2.50 (2.53–2.50)	57.95–2.50 (2.53–2.50)	58.00–2.50 (2.53–2.50)	47.63–1.80(1.81–1.80)
Space group	P3	P3	P3	P3	P3
Cell dimensions (Å, °)	a = b = 176.35, c = 56.40; $\alpha = \beta = 90$, $\gamma = 120$	a = b = 176.75, c = 56.35; $\alpha = \beta = 90$, $\gamma = 120$	a = b = 177.01, c = 56.45; $\alpha = \beta = 90$, $\gamma = 120$	a = b = 177.20, c = 56.54; $\alpha = \beta = 90$, $\gamma = 120$	a = b = 177.16, c = 56.49; $\alpha = \beta = 90$, $\gamma = 120$
Unique reflections	41405 (1998)	68131(2042)	68424(2029)	68691(2057)	184629(5543)
Redundancy	5.4 (3.9)	5.5(5.4)	5.5(5.4)	5.6(5.5)	5.6(5.5)
Completeness (%)	99.7 (97.1)	100(100)	100(100)	100(100)	99.7(99.0)
I/ σ (I)	27.6 (7.2)	15.3(4.0)	13.6(2.6)	15.5(3.3)	16.4(4.2)
R _{merge}	0.057 (0.166)	0.062(0.342)	0.066(0.541)	0.06(0.433)	0.073(0.393)
Anomalous completeness		97.1(92.6)	97.1(93.2)	99.1(97.4)	
Structure refinement					
R _{factor}					0.1781
R _{free}					0.2014
Rmsd bonds (Å) / Angles(°)					0.04/2.62
					96.83
PDB code					3MWP