Biochemical and Biophysical Research Communications 451 (2014) 603–608

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Structure based modification of Bluetongue virus helicase protein VP6 to produce a viable VP6-truncated BTV

Eiko Matsuo [a,b], Esther Leon [c], Steve J. Matthews [c], Polly Roy [b,*]

[a] Microbiology & Immunology, Division of Animal Science, Department of Bioresource Science, Graduate School of Agricultural Science, Kobe University, 1-1, Rokkodai, Nada-ku, Kobe-City 657-8501, Japan
[b] Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK
[c] Division of Molecular Biosciences, Centre for Structural Biology, Imperial College London, South Kensington, London SW7 2AZ, UK

ARTICLE INFO

Article history:
Received 15 July 2014
Available online 14 August 2014

Keywords:
Bluetongue virus
VP6
Multinuclear nuclear magnetic resonance
Reverse genetics

ABSTRACT

Bluetongue virus core protein VP6 is an ATP hydrolysis dependent RNA helicase. However, despite much study, the precise role of VP6 within the viral capsid and its structure remain unclear. To investigate the requirement of VP6 in BTV replication, we initiated a structural and biological study. Multinuclear nuclear magnetic resonance spectra were assigned on his-tagged full-length VP6 (329 amino acid residues) as well as several truncated VP6 variants. The analysis revealed a large structured domain with two large loop regions that exhibit significant conformational exchange. One of the loops (amino acid position 34–130) could be removed without affecting the overall fold of the protein. Moreover, using a BTV reverse genetics system, it was possible to demonstrate that the VP6-truncated BTV was viable in BHK cells in the absence of any helper VP6 protein, suggesting that a large portion of this loop region is not absolutely required for BTV replication.

1. Introduction

Bluetongue virus (BTV), the etiological agent of Bluetongue disease of livestock, is a member of the *Orbivirus* genus of the *Reoviridae* family. BTV particles have three consecutive proteins layers that are organized into two capsids; an outer capsid comprising VP2 and VP5 and an inner icosahedral capsid (core) composed of two major proteins, VP7 and VP3, which encloses the three minor proteins, VP1, VP4 and VP6, in addition to the viral genome. The viral genome consists of 10 linear dsRNA molecules, segment 1 to segment 10 (S1-S10). In addition to 7 structural proteins, BTV genome also encodes 3 or 4 nonstructural proteins (NS1, NS2, NS3 and NS4) in infected host cells [1–3].

The catalytic activities of both VP1 and VP4 have been confirmed by a range of *in vitro* studies [4–8]. Furthermore, structural studies have revealed their close association in a complex located at the 5-fold vertices of the VP3 subcore [9,10]. In contrast, despite considerable information regarding its enzymatic function of VP6 *in vitro* [11,12], little is known regarding its structure or location in the BTV core. While VP6, like VP1 and VP4, is co-purified with core from virus-infected cells, VP6 is not readily taken up into core-like particles (CLPs) when co-expressed with major core structural proteins VP3 and VP7 [10,13]. Furthermore, although VP6 is likely to unwind dsRNA either ahead or behind the transcribing polymerase [11,12], it is also possible that the helicase activity could play an entirely different role in virus assembly.

Recently, we constructed VP6-deficient BTV strains using a reverse genetics (RG) system and a complementary BSR-VP6 cell line, demonstrating that VP6-deficient mutant viruses were replication-deficient in non-complementary cells (normal BSR cells) [14,15]. Moreover, we demonstrated that the BTV core particle, purified from normal BSR cells infected with VP6-deficient mutant virus, contained neither the genomic dsRNA nor the two proteins of polymerase complex, VP1 and VP4 [16]. Thus, it is reasonable to hypothesize that VP6 plays a role in RNA packaging in addition to its role as a helicase in BTV mRNA transcription [16].

In this study, to further understand the importance of VP6, we assigned Multinuclear nuclear magnetic resonance (NMR) spectra of VP6 to provide insight into the VP6 structure. Based on NMR-derived secondary structure, it was possible to design a series of truncated proteins removing dynamic loop regions. Most importantly, in spite of the essential role of VP6 in the primary replication cycle of BTV, we show that the region between aa 34 and aa 92 of this large loop is not required for viral replication. However the second half of this loop (aa 93–130) plays an essential role in virus replication.

* Corresponding author. Fax: +44 (0)20 79272842.
E-mail address: polly.roy@lshtm.ac.uk (P. Roy).

http://dx.doi.org/10.1016/j.bbrc.2014.08.028
0006-291X/

2. Materials and methods

2.1. Cell lines

BSR cells (BHK-21 subclone) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 4% (vol/vol) fetal bovine serum (FBS) (Life Technologies). The stable cell line, BSR-VP6 [15], were grown in DMEM-4% FBS supplemented with 7.5 μg/ml of puromycin (Sigma).

Preparation of dsRNA was as described previously [17–20].

2.2. Plasmids and BTV T7 transcripts

A bacterial expression plasmid for the fusion of BTV-10 VP6 protein incorporating N-terminal hexahistidine tag (his-tag) was produced by inserting the coding region of S9 into the modified bacteria expression vector, pRSETA-imperial. A region, which includes transcript stabilizing sequence from gene 10 of phage T7, the Xpress™ epitope, and the enterokinase cleavage recognition sequence, was replaced with the thrombin cleavage recognition sequence.

Mammalian expression plasmids for the RG system, pCAG-PBTV1VP1, pCAG-PBTV1VP3, pCAG-PBTV1VP4, pCAG-PBTV10VP6, pCAG-PBTV1VP7 and pCAG-PBTV1NS2, were as described previously [15,16]. T7 plasmids for BTV transcripts used in the RG system were as described previously [15,18].

Modification of VP6 and S9 was generated by site-directed mutagenesis, using the method as described previously [21]. The sequence of each modified VP6 and S9 plasmid was confirmed.

Synthesis of uncapped BTV transcripts was performed as described previously [15]. For synthesis of uncapped T7 transcripts, RiboMAX Large Scale RNA Production System-T7 (Promega) was used according to the manufacturer's protocols.

2.3. Expression and purification of a series of BTV VP6 proteins in an Escherichia coli strain

An *E. coli strain*, BL21, was transformed with each VP6 expression plasmid. The transformed cells were incubated in 10 ml of LB broth containing 100 μg/ml of ampicillin (ABPC) at 37 °C for 16 h. After incubation, 1 ml of culture was inoculated to 10 ml of the fresh LB broth containing 100 μg/ml of ABPC and was incubated at 37 °C for 1 h. The expression of his-tagged VP6 was then induced for 5 h with the presence of 0.5 mM of IPTG.

For the production of labelled NMR, sample BL21 (DE3) was used. After the transformation, a clone for each protein was selected and amplified in 1 L of LB broth upon reaching $OD_{600} \sim 0.3–0.5$. The cell pellets were transferred from the culture to 1 L of minimal media containing $^{15}NH_4Cl$ and $^{13}C_6$-glucose in D_2O for efficient ^{15}N-^{13}C-labelling and deuteration of VP6. Expression of the his-tagged protein was induced after 5 h by adding 0.5 mM IPTG, and then harvested after an overnight induction period at 28 °C. Note that for the expression of mutated VP6 proteins, an optimized expression protocol based on the double-colony selection method described previously was used [22].

The cells were collected by centrifugation and lysed by sonication. The his-tagged protein was purified by bench top chromatography using a nickel–nitrilotriacetic acid (Ni–NTA) resin. A second step of size exclusion chromatography was carried out on the previous protein enriched fractions. Protein samples were concentrated to 0.75 mM in 50 mM sodium phosphate pH7.5, 50 mM NaCl, 50 mM L-arginine, 50 mM L-glutamic acid, 10 mM DTT and 10% D_2O.

2.4. Acquisition of NMR spectra

NMR spectra were collected at 298 K on Bruker DRX600 and DRX800 spectrometers equipped with Z-shielded gradient triple resonance cryoprobes. The chemical shifts of ^1HN, ^{15}N, $^{13}C_\alpha$, $^{13}C_\beta$ and ^{13}CO cross peaks were assigned using the double- and triple-resonance heteronuclear three-dimensional NMR spectra and in-house assignment algorithms [23].

2.5. Transfection of cells

Confluent monolayers of BSR-VP6 cells were transfected, first with six mammalian expression plasmids (100 ng each), coding VP1, VP3, VP4, VP6, VP7 and NS2, followed by the second transfection with ten BTV T7 transcripts (50 ng each) using Lipofectamine 2000 Reagent (Life technologies) as described previously [16]. At 6 h post second transfection, the culture medium was replaced with fresh DMEM containing 5% FBS and the plates were incubated at 35 °C in 5% CO_2 for 3 days to allow cytopathic effects (CPE) to appear. Note that as the recovery efficiency should be low due to uncapped transcription in the second transfection, the plaque-purification procedure was not performed after transfection in this study.

2.6. BTV replication assay

To observe CPE, 10 μl of VP6-truncated BTV recovered from transfected BSR-VP6 cells was inoculated to normal BSR cells and the supernatant, containing viruses, was replaced with 1.5% low-melting agar in D-MEM supplemented with 5% FBS. At 2 days post-inoculation, cells were stained with 0.5% of crystal violet.

To determine the virus replications, each 100 μl of VP6-truncated BTV recovered from transfected BSR-VP6 cells were once amplified in BSR-VP6 cells (P0), followed by the second (P1) and the third (P2) amplification in normal BSR cells. The virus titer of each collected supernatant ($P0 \sim P2$) was determined by plaque assay using BSR-VP6 cells.

3. Results

3.1. Expression of VP6 in E. coli and structural characterization by NMR

To facilitate the NMR study, BTV-10 VP6 was overexpressed with an N-terminal his-tag in *E. coli*. The proteins were expressed well in soluble form (data not shown). The NMR analysis was first performed on the full-length VP6 (329 residues). Sequence specific assignment of NMR spectra of ^{15}N, ^{13}C-labelled VP6 could be completed for over half the protein sequence (Fig. 1A), whilst the remaining sequence formed two large dynamic loops. Conformational exchange on intermediate timescale within these regions broadened the majority of the NMR signals beyond detection. As shown in Fig. 1B, the chemical shift index (CSI) plot for backbone atoms of the full-length VP6 was obtained based on the sequential resonance assignment of the triple-labeled protein. There were two gaps in the assigned sequence (from aa 33 to aa 130 and aa 184 to aa 231), which likely indicate the presence of dynamic loops that exhibit conformation exchange, as the corresponding cross-peaks did not appear in the spectra.

3.2. Design of the truncated BTV VP6 variants

The presence of the two dynamic loops poses a significant challenge for the high-resolution structural study of VP6, especially as their signals are undetectable in NMR spectra and the