Journal of Virological Methods 187 (2013) 159–165

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

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

Multiple phosphorylable sites in the Zaire Ebolavirus nucleoprotein evidenced by high resolution tandem mass spectrometry

Jérémy Peyrol[a], Céline Thizon[a,1], Jean-Charles Gaillard[b], Charles Marchetti[c], Jean Armengaud[b], Françoise Rollin-Genetet[a,*]

[a] CEA, iBEB, SBTN, LDCAE, F-30207 Bagnols-sur-Cèze, France
[b] CEA, iBEB, SBTN, LBSP, F-30207 Bagnols-sur-Cèze, France
[c] CEA, iBEB, SBTN, LICB, F-30207 Bagnols-sur-Cèze, France

Article history:
Received 20 April 2012
Received in revised form 4 September 2012
Accepted 3 October 2012
Available online 13 October 2012

Keywords:
Nucleoprotein
Zaire Ebolavirus
Stable cell line
Phosphorylation
Mass spectrometry

A B S T R A C T

The 739-amino-acid nucleoprotein (NP) of Zaire Ebolavirus (ZEBOV) plays a key role in Ebola virion formation and replication. A stable HEK-293 cell line capable of producing an N-ter 6His-tagged recombinant form of NP – ZEBOV was created. Production of this protein was triggered in batch culture using microcarriers. Because NP Ebola phosphorylation has been shown to occur but localization of the modified residues remained to be established, the phosphorylation status of recombinant NP – ZEBOV was investigated through extensive characterization by high-resolution tandem mass spectrometry. The NP – ZEBOV sequence may well be covered by the use of multiple proteases. NP was found to be phosphorylated in two different amino acid stretches: [561–594] and [636–653]. Furthermore, residues Thr_{563}, Ser_{581}, Ser_{587} and Ser_{647} were accurately identified as phosphorylated sites. These data highlight how high resolution tandem mass spectrometry is a method of choice for characterizing post-translational modifications of viral proteins. Because these four phosphorylable sites are conserved among Ebolavirus and Marburgvirus NPs, their modification may play a modulatory role in viral RNA synthesis.

1. Introduction

Ebola and Marburg viruses belong to the Filoviradae family in the order Mononegavirales, which comprises viruses with a non-segmented, single-strand RNA of negative polarity. These viruses are responsible for severe hemorrhagic fever with an extremely high mortality rate in human and nonhuman primates (Ascenzi et al., 2008; Feldmann and Geisbert, 2011). In the case of Ebolavirus, at least seven structural proteins are encoded by the RNA. Four of these proteins, namely nucleoprotein (NP), VP35, VP30 and RNA-dependent RNA polymerase (L), form the ribonucleoprotein complex (RNP) which is required for transcription and replication of the viral genome (Mahanty and Bray, 2004). Three other proteins, namely glycoprotein (GP), VP40 and VP24, are membrane-associated proteins (Noda et al., 2005, 2010). NP is the main RNP component and interacts with the other nucleocapsid proteins (Johnson et al., 2006). Expression of NP in eukaryotic cells in the absence of viral RNA and any other viral proteins leads to the formation of recombinant NP–RNA complexes (Watanabe et al., 2006). These complexes form helices that are very similar to authentic nucleocapsids, but differ in having an approximate diameter of 20 nm instead of 50 nm (Noda et al., 2010). The structural and architectural description of Ebolavirus has been provided using cryo-electron tomography (Bharat et al., 2012). In terms of protein sequences, LVmv and ZEBOV NPs show significant differences. Both sequences share only 38% of identical amino acids (Ascenzi et al., 2008). Functional mapping of Ebolavirus nucleoprotein has been completed (Watanabe et al., 2006). Ebolavirus NP is a protein with 739 amino acids. Its N-terminal part (first 450 amino acid residues) is required for NP–NP interaction in NP–RNA structures. Its C-terminal part (amino acid residues 451–739) is hydrophilic (Ascenzi et al., 2008). It influences the conformation of recombinant NP helices and is crucial for incorporating nucleocapsids in virions (Shi et al., 2008). Unusual behavior has been observed for NP upon electrophoresis, showing an increase in its apparent molecular weight which was found to be higher than expected (Shi et al., 2008). This behavior is due to a highly acidic stretch of amino acids located within its C-terminal half and predicted to be a disordered region. Specifically, two motifs (amino acids 439–492 and 589–739) have been found to be responsible for these unusual characteristics.

Both Ebola and Marburg viral NPs were found to be post-translationally modified. *O*-glycosylated and sialylated modifications have been shown in Ebolavirus NP (Huang et al., 2002;

* Corresponding author. Tel.: +33 466 791 913; fax: +33 466 791 905.
E-mail address: francoise.rollin-genetet@cea.fr (F. Rollin-Genetet).
[1] Present address: BiotechDeva, ENSTBB-IPB, 33000 Bordeaux, France.

0166-0934/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jviromet.2012.10.003

Watanabe et al., 2006). Glycosylations have been shown to be required for nucleocapsid assembly. Marburg virus NP has been found to be phosphorylated and studied extensively. While its N-terminus is not modified, its C-terminus comprises several phosphorylated serine and threonine residues (Lotfering et al., 1999). In Marburg virions, only the phosphorylated form of NP has been detected (Becker et al., 1994).

With respect to Ebolavirus NP, phosphorylations have been detected (Elliott et al., 1985), but identification of the phosphorylated residues has not yet been reported. Some authors have reported that C terminus Ebolavirus NP is not modified after extensive analysis of the C-terminus, including evaluation of all the peptides by MALDI-TOF mass spectrometry (Shi et al., 2008). However, the location of NP – ZEBOV phosphorylation is still unknown and deserves to be established.

To characterize the exact position of NP – ZEBOV phosphorylation sites, extensive characterization by high-resolution tandem mass spectrometry was performed. A comprehensive biochemical and structural characterization of viral proteins requires pure material in large quantities. Current technology for producing Ebola NP uses transient expression in mammalian cell lines, by plasmids (Noda et al., 2010; Sanchez et al., 1989; Watanabe et al., 2006) or baculovirus expression systems (Becker et al., 1994; Saijo et al., 2001). Recently, stable mammalian cell lines expressing EBOV protein transmembrane glycoprotein (GP), the soluble glycoprotein (sGP) and matrix protein (VP40), by producing EBOV VLPs, were created and shown to be an efficient way of obtaining material for further characterization (Melito et al., 2008). Protein production by transient transfection of mammalian cells is time consuming and expensive. Because this technology requires multiple batch preparations, it could lead to variability of the purified protein. To continue with the biochemical characterization of Ebolavirus NP, a full-length protein in a stable cell clone was produced and its post-translational modifications investigated using state-of-the-art mass spectrometry. This NP was characterized extensively by high-resolution tandem mass spectrometry and several phosphorylation sites were located.

2. Materials and methods

2.1. Cells and cloning strategy

Human embryonic kidney cells Flp-In™ T-Rex™-293 are HEK-293 derivatives from Invitrogen. They were used to produce the NP – ZEBOV as a stable expressing clone. A pFRT/lacZeo plasmid is already transfected in these cells, providing a unique FRT recombination site. The pcDNA5/FTR plasmid encodes the resistances to hygromycin (HG) and blasticidin (BL). It was designed to express a gene of interest from an inducible promoter with a strong tetracycline repressor. The whole open reading frame encoding NP – ZEBOV, complemented with a 6His tag and a TEV cleavage site at its N-terminus, was cloned into this vector. Evidence of insertion of the gene of interest was indicated by the loss of Zeocin resistance. The resulting plasmid was cotransfected with the pOG44 vector, which mediates pcDNA5/FTR recombination with the integrated pFRT/lacZEO. The recombinant NP – ZEBOV sequence was verified by sequencing.

2.2. Production of stable cell lines

HEK-293 cells were grown in 24-well plates to 90% confluence in DMEM-Glutamax base culture medium containing 10% fetal calf serum (FCS) (GIBCO, Saint Aubin, France), at 37 °C at 9% CO_2. The insert and pOG44 plasmids were mixed with pcDNA5/FTR and opti-MEM 1 (GIBCO, Saint Aubin, France) (9:1, vol/vol). 1 μg DNA in 50 μl opti-MEM1 was added per well. A mixture combining 2 μl lipofectamin and 50 μl Opti-MEM1 was added and incubated for 30 min. The medium was removed from the wells and replaced with 400 μl DMEM-Glutamax containing 10% FCS and 1% Pen-Strep (PS) (GIBCO, Saint Aubin, France). 100 μl of transfection mix was added to each well and the cells were incubated at 37 °C and 9% CO_2 for 24 h. Finally, the transfected cells were transferred to a new, 6-well plate, to which 2 ml DMEM-Glutamax containing 10% FCS and 1% PS were added. They were incubated for 24 h at 37 °C and 9% CO_2 before selection of the candidate clone. The culture medium was removed and replaced by the selection medium consisting of DMEM-Glutamax containing 10% FCS, 1% PS, 100 μg/ml HG and 15 μg/ml BL (Invitrogen, Cergy Pontoise, France). The medium was renewed until the hygromycin-resistant transfected cells were identified and non growing cells removed. The polyclonal cell bank was frozen in nitrogen, in 90% FCS containing 10% DMSO, for further use.

2.3. Monoclonal bank production

The cell count and viability of the polyclonal bank were evaluated using the well-established Trypan Blue exclusion method with the automated cell counting system, Cedex (Roche Innovatis, Mannheim, Germany). The cells were mixed and diluted with DMEM-Glutamax containing 10% FCS, 1% PS, 100 μg/ml HG and 15 μg/ml BL in a volume allowing for 1 cell per 100 μl media. Volumes of 100 μl of diluted culture mix were dispensed into wells from a 96-well plate, at 37 °C and 9% CO_2. When the selected cells had reached 90% confluence, all the cells in the same well were amplified in an upscaling process in a flask. As the Flp-In™ T-Rex™-293 cells carry a single FRT site linked to the Zeocin-resistance gene, evidence of insertion of the gene of interest is indicated by the loss of Zeocin resistance. Candidates from the monoclonal bank production step were grown in 6-well plates in DMEM-Glutamax containing, initially, 10% FCS, PS, HG, and BL, and then in DMEM-Glutamax containing 10% FCS, 1% PS, 100 μg/ml Zeocin and 15 μg/ml BL. Cells which showed both resistance to HG and sensitivity to Zeocin, indicating correct insertion of the gene of interest, were identified and saved as production resources. The cells, considered to be monoclonal at this stage, were resuspended in FCS comprising 10% DMSO, and frozen in liquid nitrogen for further use. These cells were then grown in 6-well plates at 37 °C and 9% CO_2 in DMEM-Glutamax containing 10% FCS, 1% PS, 100 μg/ml HG and 15 μg/ml BL. When the cells reached 90–95% confluence, the medium was removed and the cells were harvested using nonenzymatic cell-dissociation solution (Sigma, Saint Quentin, France). The cells were centrifuged for 10 min at 1200 rpm and 4 °C. They were then resuspended at a concentration of 14×10^6 cells per ml in hypotonic buffer consisting of 50 mM sodium phosphate, pH 7.5, containing 10 mM imidazole, 1 mM $MgCl_2$, 10 U/l benzonase-nuclease (Novagen), 1:3000 protease inhibitor cocktail set III, EDTA-free (Calbiochem, Villeneuve d'Asq, France), 100 μM sodium orthovanadate (Sigma, Saint Quentin, France) and 0.2 mg/ml RNase A (Qiagen, Hilden, Germany). The cells were disrupted in this buffer using a Basic Z cell disruptor (Constant System, Northants, UK) operated at a pressure of 1 kbar. After disruption, samples were centrifuged for 30 min at 4 °C and 16,000 × *g* and NP expression was verified by anti-6His Western blotting.

2.4. NP production and capture

Selected HEK-293 cells were cultured at 37 °C and 9% CO_2 in DMEM-Glutamax supplemented with 10% FCS, 1% PS, 100 μg/ml HG and 15 μg/ml BL. The cells were cultured in flasks to a density sufficient to inoculate the cytocultor at 1.6×10^5 cells per ml, then mixed with cultisphere-S microcarrier (3 g/l microcarrier, Sigma,