Journal of Virological Methods 228 (2016) 48–54

Contents lists available at ScienceDirect

Journal of Virological Methods

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

# Expression, characterisation and antigenicity of a truncated Hendra virus attachment protein expressed in the protozoan host *Leishmania tarentolae*

Kerstin Fischer[a], Vinicius Pinho dos Reis[a], Stefan Finke[b], Lucie Sauerhering[c], Eileen Stroh[a], Axel Karger[b], Andrea Maisner[c], Martin H. Groschup[a], Sandra Diederich[a], Anne Balkema-Buschmann[a,*]

[a] *Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Novel and Emerging Infectious Diseases, Greifswald-Insel Riems, Germany*
[b] *Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Molecular Virology and Cell Biology, Greifswald-Insel Riems, Germany*
[c] *Institute of Virology, Philipps University of Marburg, Marburg, Germany*

*Article history:*
Received 17 August 2015
Received in revised form 5 November 2015
Accepted 10 November 2015
Available online 29 November 2015

*Keywords:*
Hendra virus
Attachment protein
*Leishmania tarentolae*
Protein expression
Binding assay

A B S T R A C T

Hendra virus (HeV) is an emerging zoonotic paramyxovirus within the genus *Henipavirus* that has caused severe morbidity and mortality in humans and horses in Australia since 1994. HeV infection of host cells is mediated by the membrane bound attachment (G) and fusion (F) glycoproteins, that are essential for receptor binding and fusion of viral and cellular membranes. The eukaryotic unicellular parasite *Leishmania tarentolae* has recently been established as a powerful tool to express recombinant proteins with mammalian-like glycosylation patterns, but only few viral proteins have been expressed in this system so far. Here, we describe the purification of a truncated, *Strep*-tag labelled and soluble version of the HeV attachment protein (sHeV G) expressed in stably transfected *L. tarentolae* cells. After *Strep*-tag purification the identity of sHeV G was confirmed by immunoblotting and mass spectrometry. The functional binding of sHeV G to the HeV cell entry receptor ephrin-B2 was confirmed in several binding assays. Generated polyclonal rabbit antiserum against sHeV G reacted with both HeV and Nipah virus (NiV) G proteins in immunofluorescence assay and efficiently neutralised NiV infection, thus further supporting the preserved antigenicity of the purified protein.



## 1. Introduction

Hendra virus (HeV) is an emerging virus of the *Henipavirus* genus in the Paramyxoviridae family that has first been isolated in Australia in 1994, causing severe respiratory and neurological diseases in horses and humans with high fatality rates (Barclay and Paton, 2000; Selvey et al., 1995). Infections with Nipah virus (NiV), belonging to the same genus, have caused high morbidity and mortality rates in pigs and also humans in Southeast Asia since 1998 (Chua et al., 2000). In experimental challenge studies, a large variety of mammalian species was shown to be susceptible to infection by HeV (Pallister et al., 2011; Weingartl et al., 2009). This wide host range may be due to its usage of the highly conserved ephrin-B2 (EFNB2) as a viral entry receptor (Bonaparte et al., 2005; Bossart et al., 2008). HeV binding to the host cell is mediated by the attachment protein G (HeV G), a type II transmembrane glycoprotein of 604 amino acids that shares structural similarities with the attachment glycoproteins of other paramyxoviruses (Bowden et al., 2008; Xu et al., 2008). HeV G elicits a potent neutralising antibody response in the infected hosts. Due to these facts, HeV G has become a main target in vaccination, diagnostic and therapeutic strategies to prevent and fight HeV infection (Gao et al., 2015; Middleton et al., 2014; Mire et al., 2014).

*Leishmania tarentolae* is a protozoan parasitic organism that naturally infects the lizard *Tarentola anularis* (Elwasila, 1988). In 2002, Breitling et al. established a novel eukaryotic protein expression system that was based on the stable transfection of *L. tarentolae* promastigotes with the respective DNA into the small ribosomal subunit rRNA gene of the parasite (Breitling et al., 2002). As a result of their parasitic lifestyle in mammalian hosts, oligosaccharide structures of the parasitic glycoproteins are often similar to those of mammalians (Basile and Peticca, 2009; Parodi, 1993).

* Corresponding author.
*E-mail address:* anne.buschmann@fli.bund.de (A. Balkema-Buschmann).

http://dx.doi.org/10.1016/j.jviromet.2015.11.006

Glycosylation has a crucial impact on the conformation and thus on the functionality of a protein (Phan et al., 2009; Soleimani et al., 2007). Therefore, besides the easy handling similar to bacterial protein expression and growth rates to high cell densities up to $1.4 \times 10^{\circ}$ cells/ml, the mammalian-like N-glycosylation pattern is a great advantage of this system. Several mammalian glycoproteins such as human laminin-332 and biologically active human erythropoietin (EPO) have been successfully expressed in this system with an exceptionally homogenous N-glycosylation (Phan et al., 2009; Breitling et al., 2002). So far, only a few viral proteins have been expressed in the *L. tarentolae* system, namely the hepatitis E capsid protein, the human papillomavirus16 E7 protein and the influenza virus hemagglutinin (Baechlein et al., 2013; Salehi et al., 2012; Pion et al., 2014). Thus, the advantages over conventional cell culture expression systems in combination with benefits in mammalian-like glycosylation patterns could substantially improve the development of glycoprotein-based diagnostic assays as well as scientific analysis of glycoprotein-dependent functions in receptor binding, entry and immune reactions of the host.

Here, we report the expression and purification of a truncated soluble HeV G protein (sHeV G) in stably transfected *L. tarentolae* cells. Functionality of sHeV G as a receptor-binding protein was confirmed in binding and co-precipitation assays. Furthermore, antiserum raised in rabbits against sHeV G reacted with G proteins from HeV and NiV, and neutralised NiV infection in cell culture experiments. In conclusion, the *L. tarentolae* system proofs to be a suitable host for the functional expression of viral glycoproteins.

## 2. Materials and methods

### 2.1. Sequence and plasmid construction

For efficient expression in *L. tarentolae*, the HeV G coding sequence (GenBank accession no. NC-001906) was codon optimised for *L. tarentolae* and a truncated G coding sequence (amino acids 71–604) lacking the transmembrane domain and cytoplasmic tail together with an N-terminally fused double *Strep*-tag coding sequence (iba GmbH, Germany; aa sequence: WSHPQFEKGGGSEGGSGGGSWSHPQFEK) was synthesised (GeneArt AG/Thermo Fisher Scientific Inc., Germany). This codon-optimised truncated HeV G sequence was submitted to GenBank under the accession no. KU050076. The product was cloned into the vector pLEXSY-sat2 (Jena Bioscience, Germany) using SalI and NotI restriction sites to yield pLEXSYHeVG. After ligation, *E. coli* DH5alpha were transformed with the respective construct and cultivated under ampicillin selection at 33 °C for up to 2 days. Resistant clones were picked and plasmid identities were confirmed by sequencing.

### 2.2. Cultivation, transfection and clonal selection of L. tarentolae

*L. tarentolae* strain P10 (Jena Bioscience) was cultivated in Brain–Heart Infusion (BHI) medium according to the manufacturer's instructions. Before transfection, 20 μg of the plasmid pLEXSYHeVG were digested with SwaI for linearisation and purified using the QIAquick Nucleotide Removal kit (Qiagen, Germany) following the manufacturer's instructions. At day 2 of cultivation, 4.5 ml of a culture of *L. tarentolae* in log growth phase (optical density ($OD_{600}$) 1.5) were centrifuged at $200 \times g$ for 7 min at 4 °C to recover the cells. After resuspension in 350 μl of BHI medium, cells were mixed with the linearised plasmid DNA and incubated for 10 min on ice. The mix was then transferred to a 4.0 mm cuvette (BioRad, Hercules, USA) and electroporated by two pulses (0.3 ms) at 1500 V. After another incubation period of 10 min on ice, cells were transferred to 10 ml of fresh BHI medium and further incubated at 26 °C.

Clonal selection was started at 48 h after transfection. Cells were centrifuged at $330 \times g$ for 7 min at 4 °C and resuspended in 10 ml fresh BHI medium containing the selection marker nourseothricin (Jena Bioscience) in a final concentration of 100 μg/ml. Then, 100 μl of this cell suspension were added per well of a 96-well plate and screened for cell vitality during the following days. Cells were grown in liquid culture by continuously increasing the culture volume. Successful transfection and genomic integration was confirmed by PCR with primers F3001 and A1715 (Jena Bioscience). Glycerol stocks were prepared and stored at −80 °C.

### 2.3. Expression and purification of Strep-tagged sHeV G

Cell lysates as well as cell culture supernatant of 5 ml densely grown cultures were investigated by immunoblot as described below for initial screening of protein expression. The total protein from the supernatant was precipitated by addition of 50% trichloracetic acid (TCA) to a final concentration of 10% followed by two washes with 80% acetone. For purification of *Strep*-tagged sHeV G protein from cell lysates, stably transfected *L. tarentolae* cells were cultivated in 1 L batches for 3–4 days until an $OD_{600}$ of 2.0–2.5 was reached. Then, cells were harvested by centrifugation for 15 min at $1200 \times g$ and 4 °C followed by resuspension in 10 ml of lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 0.5% TritionX-100, 1× protease inhibitor cocktail (Roche, USA) and incubation for 40 min on ice. Cell extracts were centrifuged for 35 min (20,817 × *g*, 4 °C). Afterwards, the supernatant was incubated overnight with 1 ml (0.5 g/ml) of *Strep*-Tactin® Sepharose® (iba GmbH) in lysis buffer at 4 °C. Four washing steps with 3 ml of washing buffer (50 mM Tris–HCl pH7.4, 150 mM NaCl, 2 mM $CaCl_2$) were performed by centrifugation and resuspension before the resin was transferred into a disposable column (Pierce, USA) followed by five additional washing steps. Bound proteins were sequentially eluted with six 0.5 ml aliquots of elution buffer (100 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM D-Desthiobiotin). Incubation of the third fraction of elution buffer on the column was extended to 60 min at 4 °C to increase protein yield.

### 2.4. Immunoblot analysis, silver staining and glycosidase treatment

Purified protein samples were separated on 7% SDS-polyacrylamide gels (PAGE) under reducing conditions and transferred to PVDF membranes for immunoblot analysis. After 1 h of blocking in 10% skim milk diluted in PBS/0.05% Tween-20 (PBST), the membrane was incubated for 1 h with a monoclonal mouse anti-*Strep*MAB-Classic (*Strep*MAB; iba GmbH) at a 1:3000 dilution. After three washing steps with PBST, the secondary antibody goat anti-mouse-horseradish peroxidase (HRP; polyclonal, 1:3000; Dianova, Germany) was added for 1 h followed by three washes. Immunoblot was developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA). Signals were visualised using the Quantity One analysis software (BioRad, Germany). Proteins in the elution fractions were separated by 10% SDS-PAGE (Laemmli, 1970) and visualised by silver staining (Chevallet et al., 2006). To analyse glycosylation of sHeV G, 2 μg of purified protein were incubated with 1 unit PNGase F (New England Biolabs, Germany) for 2 h at 37 °C and further analysed by immunoblot as described above.

### 2.5. Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-TOF/TOF MS)

Peptide mass fingerprint spectra were acquired using standard procedures for in-gel protein digestion (Shevchenko et al., 2006).