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

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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).

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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).

More detailed information referring to MS analysis has been deposited in the Supplementary data.

2.6. Binding of recombinant human EFNB2 to sHeV G

A 96-well plate (Medisorp, Thermo Fisher Scientific Inc., Germany) was coated with 100 μl of a dilution of 2 μg/ml of sHeV G in PBS overnight at 4 °C. Plates were blocked with 2% BSA in PBST for 1 h at 37 °C, followed by three washes with PBST. All reagents were diluted in 0.5% BSA/PBST. Increasing amounts of recombinant soluble human ephrin-B2/Fc-Tag (EFNB2/Fc) (Sino Biological Inc., USA) from 0.625 ng to 20 ng were added per well. One porcine serum and a serum from a HeV infected pig (kindly provided by Hana Weingartl, National Centre for Foreign Animal Disease, Canada) were used in a 1:100 dilution as negative and positive controls. To estimate influences of unspecific binding between EFNB2/Fc and Leishmania-derived proteins, *Strep*-tag purified cell extract of untransfected Leishmania cells was coated to control wells. Plates were incubated for 1 h at 37 °C, followed by three washes with PBST. Protein G-HRP conjugate (Calbiochem, USA) (1:5000) was added and after another 1 h of incubation and three washes, the reaction was developed with an 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate (Roche, USA), stopped with 1% SDS and absorbance was measured at 405 nm in a Tecan infinite 200Pro ELISA Reader (Tecan Deutschland GmbH, Germany).

For co-precipitation of sHeV G by EFNB2/Fc, 50 μl (the equivalent of 1.5 mg) of Dynabeads Protein G (novex by life technologies, Norway) were incubated under rotation with 4 μg of EFNB2/Fc for 30 min at room temperature (RT). After washing with 200 μl PBS/0.02% Tween-20 (0.02% PBST), 20 μg sHeV G diluted in 200 μl 0.02% PBST were added, followed by another 30 min of rotating incubation at RT. Then, the complex was washed two times using 200 μl of 0.02% PBST, resuspended in 100 μl of 0.02% PBST and transferred to a clean tube. Elution was carried out using 30 μl of 4× Laemmli buffer (Laemmli, 1970) complemented with 10% β-mercaptoethanol and a final heating step at 70 °C for 10 min. Samples were run on a 7% SDS-PAGE and transferred to PVDF membrane for Western blot analysis of sHeV G protein as described above. In control reactions, a polyclonal rabbit αEFNB2 antibody (Sigma–Aldrich, Germany) was used in a dilution of 1:500. As secondary antibodies, goat α-mouse-HRP and goat α-rabbit-HRP antibodies (Dianova) were used at a 1:3000 dilution.

2.7. Cells and transfection

Rabbit Kidney 13 (RK-13), Chinese Hamster Ovary K1 (CHO-K1) and Human Embryonic Kidney 293T (HEK293T) cells (Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut, Insel Riems, Germany) were cultivated in Minimal Essential Medium (MEM, Earl's and Hank's salts 1:1) supplemented with non-essential amino acids and 10% foetal calf serum. Vero 76 cells (ATCC #CRL1587) were cultivated in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (FCS), 100 U of penicillin/ml, 0.1 mg of streptomycin/ml, and 4 mM glutamine (Life Technologies).

Transfection of HEK293T cells was performed using polyethylenimine (PEI; Sigma–Aldrich) as described previously (Bauer et al., 2014).

2.8. Cell adhesion assay

The cell adhesion assay was performed using RK-13 cells, endogenously expressing EFNB2 receptors, as well as CHO-K1 cells, which lack these receptors. Initially, 96-well plates were coated with dilutions of sHeV G ranging from 0.1 to 20 μg/ml in PBS at 4 °C overnight and then blocked with 2% BSA in PBS for 1 h at 37 °C. Wells without sHeV G were only coated with 2% BSA and served as blocking control. As positive adhesion control, wells were coated with poly-L-lysine solution only (Sigma–Aldrich). Cells were detached with PBS containing 10 mM EDTA, subsequently suspended in serum-free MEM and seeded in a density of 1×10^5 cells/ml to each well. After incubation for 1 h at 37 °C, unbound cells were removed by stringent washing and attached cells were fixed with 3% paraformaldehyde (PFA; in PBS) for 45 min. Following two washes, cells were stained with 0.5% crystal violet for 30 min. After extensive washing with distilled water, colour was extracted in 100 μl of 0.1 M sodium citrate in 50% ethanol. The absorbance was measured at 550 nm.

2.9. Generation of polyclonal rabbit antiserum against sHeV G

Antiserum against sHeV G was produced in two rabbits by three consecutive immunisations. For each rabbit, the first inoculation contained 100 μg of sHeV G in elution buffer mixed in a 1:1 ratio with complete Freund's adjuvant. The total volume of 1 ml was injected subcutaneously at four different sites. The second and third inoculations were performed after 3 and 6 weeks, by injection of 50 μg of sHeV G in incomplete Freund's adjuvant. Two weeks after the third inoculation, serum was collected and tested for reactivity. It is referred to as RAb4048.

2.10. Indirect immunofluorescence assay (IFA)

For indirect IFA, transfection of expression plasmids pCAGGS HeV G (unpublished) and pczCFG5 NiV G (Moll et al., 2004) into HEK293T cells was performed as described above. After 20 h, monolayer cultures were fixed with 3% PFA and permeabilised with 0.5% Triton-X100 (Sigma–Aldrich) in PBS. Immunodetection of HeV G and NiV G was performed using the polyclonal RAb4048 at a dilution of 1:2000 and 1:1000 respectively and Cy3-conjugated secondary antibodies (1:1000 in PBS; Dianova). All images were acquired with a Leica SP5 confocal laser scanning microscope. Images were processed with the ImageJ software version 1.45 s (Schneider et al., 2012).

2.11. Virus neutralisation

All NiV infection experiments were performed in the biosafety-level 4 (BSL-4) containment at the Institute of Virology, Philipps University of Marburg, Germany.

NiV was propagated and titrated in Vero 76 cells as described earlier (Weise et al., 2010). To test if the G-specific antiserum can neutralise replicative henipaviruses, 180 μl of heat-inactivated RAb4048 serum dilutions (diluted 1:2, 1:20 or 1:2000 in DMEM) were mixed with 20 μl of a NiV suspension containing 2×10^4 infectious units ($TCID_{50}$). After incubation for 45 min at 37 °C, the serum–virus mixture was added to confluent Vero 76 cells grown on coverslips (2×10^5 cells). Following virus adsorption for 45 min at 37 °C, cells were extensively washed and then supplemented with DMEM containing 2% FCS. After 22 h, cells were fixed and inactivated with 4% PFA for 48 h. After permeabilisation with methanol–acetone (1:1), virus-positive syncytia were stained with a NiV-specific guinea pig antiserum as described previously (Lamp et al., 2013).

3. Results

3.1. Cloning and genomic integration of pLEXSYHeVG

It was our aim to express a soluble and *Strep*-tagged variant of the HeV G-protein for future application in serological assays. For this purpose, codon usage of the desired viral sequence was

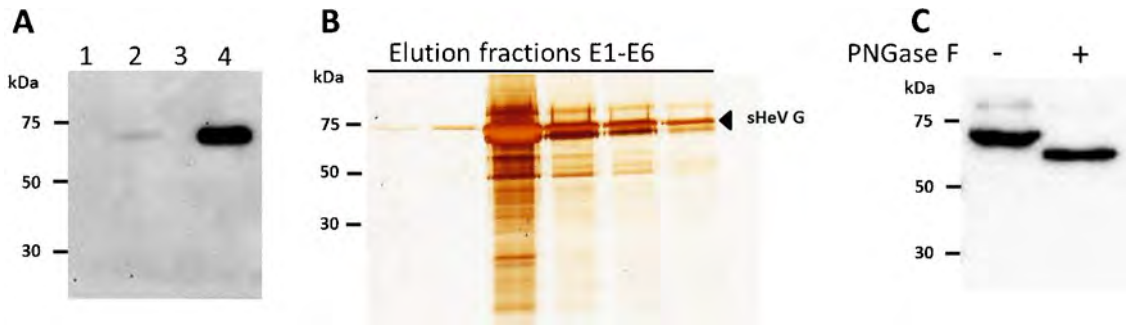

Fig. 1. Expression and purification of *Strep*-tagged sHeV G. (A) TCA-precipitated cell culture supernatant (lane 1), *L. tarentolae* cell lysate (lane 2), *Strep*-tag-purified cell culture supernatant (lane 3) and *Strep*-tag-purified cell lysate (lane 4) were separated by 7% SDS-PAGE and analysed in immunoblot using an α*Strep*MAB antibody. (B) Elution fractions E1–E6 of *Strep*-tag purified sHeV G were separated by 10% SDS-PAGE under reducing conditions and visualised by silver staining. (C) Immunoblot analysis of elution fraction E3 with (+) and without (−) PNGase F treatment using α*Strep*MAB.

optimised for *L. tarentolae*, a *Strep*-tag was added N-terminally and the sequence was fused in-frame to a secretory signal peptide (SP) of *Leishmania mexicana* secreted acid phosphatase (LMSAP), leading to the construct pLEXSYHeVG (Suppl. Fig. 1). Sequencing of the obtained clones revealed 100% sequence identity with the expected construct. Sequence-verified plasmids were subsequently electroporated into the expression host *L. tarentolae*. The correct genomic integration of the expression cassette into the small subunit rRNA gene (ssu locus) of *L. tarentolae* was confirmed by analytical PCR. Forty recombinant clones were identified, from which five were randomly selected for expression analysis.

3.2. Protein expression, purification and immunoblot analysis

Expression and secretion of sHeV G was analysed by immunoblot analysis of TCA-precipitated cell culture supernatant and cell lysates (Fig. 1, A, lanes 1 and 2). Although *Strep*-tagged sHeV G was readily expressed in *L. tarentolae*, it was not efficiently secreted into the growth medium. Purification of the recombinant protein by *Strep*-tag affinity chromatography from the cell supernatant and from cell lysates confirmed this finding (Fig. 1A, lanes 3 and 4). Due to the intracellular accumulation of the protein, cell lysates were subsequently used for further purification of the recombinant protein. After purification of sHeVG, the electrophoretic analysis of six elution fractions demonstrated the enrichment of the approximately 70 kDa comprising sHeV G protein (Fig. 1B). Up to 500 μg of recombinant sHeV G were purified from 1 L of densely grown static Leishmania culture after four days of cultivation. The glycosylation of the recombinant protein was tested by incubation with PNGase F, resulting in a mass shift of approximately 7–8 kDa of the sHeV G in comparison to the untreated control in the immunoblot analysis (Fig. 1C). Furthermore, the purified protein was analysed by MS. For this purpose, it was digested with trypsin, the resulting peptides were subjected to a peptide mass fingerprint analysis and 40 masses of the fingerprint spectrum were subjected to tandem MS analysis. The peptide spectra covered 71% of the sequence and 44.4% of the sequence could be confirmed by tandem MS (Suppl. Fig. 2A). Notably, the N- and C-termini were as well identified as the *Strep*-tag (AA 1–28). Moreover, the N-terminal tryptic fragment (WSHPQFEK) could be unambiguously identified by tandem MS of mass 1058.505 Da (Suppl. Fig. 2B) indicating that the N-terminus had been processed as expected. Tryptic peptides representing the SP of LMSAP were not detected.

3.3. Binding of sHeV G to soluble and cellular EFNB2

To assess whether the conformation of the purified sHeV G indeed allows binding to the HeV entry receptor, the efficiency

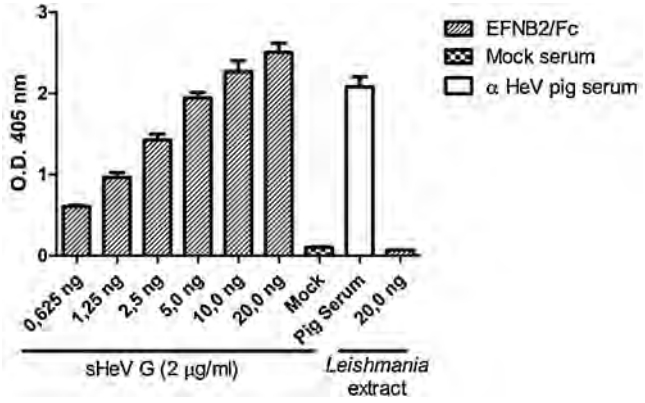

Fig. 2. Binding of recombinant soluble EFNB2/Fc-tag to sHeV G. Binding assay was performed as described in Section 2. Briefly, recombinant sHeV G was coated to the 96-well plate. Soluble EFNB2/Fc in the indicated amounts was applied. Anti-HeV pig antiserum (αHeV pig serum) and anti-HeV IgG negative pig serum (mock serum) served as controls. As a control for unspecific binding, 20 ng of EFNB2/Fc was applied to wells coated with *Strep*-tag-purified cell lysate of non-expressing *L. tarentolae* cells. OD was measured at 405 nm.

of sHeV G binding to EFNB2 was tested. After coating of an ELISA plate with sHeV G at a concentration of 2 μg sHeVG/ml, recombinant EFNB2/Fc was added in increasing amounts and binding of the receptor was visualised by final OD measurement. sHeV G indeed captured the EFNB2/Fc protein (Fig. 2), showing a dose-dependent interaction of EFNB2/Fc with the recombinant protein sHeV G expressed in *L. tarentolae*. The strong binding of the recombinant receptor to the *L. tarentolae* expressed sHeV G further indicates a conformation of the purified protein that is not in conflict with receptor binding.

To identify specific interaction between sHeV G and EFNB2, co-precipitation experiments were performed. As shown in Fig. 3, sHeV G was efficiently precipitated by recombinant EFNB2/Fc. Incubation of EFNB2/Fc with a *L. tarentolae* extract of cells lacking the HeV G gene did not result in precipitated proteins. Since sHeV G did not bind to the protein G dynabeads, we conclude that the Leishmania-expressed sHeV G interacts specifically with the HeV cell entry receptor EFNB2.

Finally, to assess if sHeV G is able to bind to the cellular HeV receptor EFNB2 in its native environment, a receptor-binding study was performed with EFNB2 positive and negative cell lines. RK-13 cells have been reported to be a very sensitive cell line to HeV infection (Halpin et al., 2000) and therefore were chosen as receptor-positive cells. In contrast, CHO-K1 cells, which are refractory to HeV infection, as they do not express EFNB2 (Negrete et al., 2005), were selected as negative control. As shown in Fig. 4, recombinant sHeV G bound to RK-13 cells in a dose-dependent and

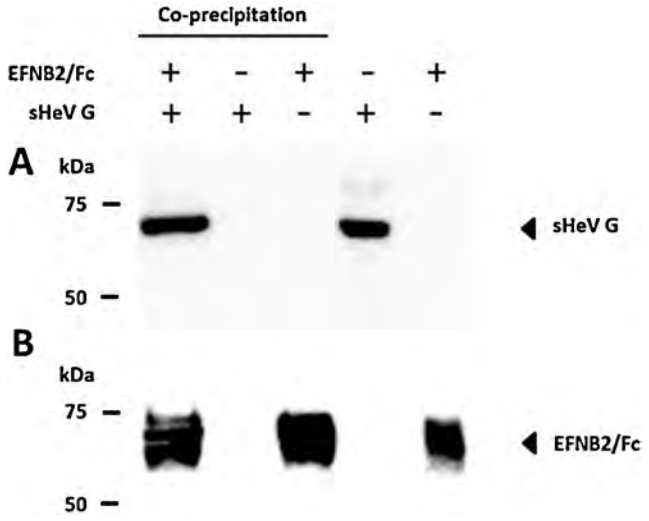

Fig. 3. Co-precipitation of sHeV G by EFNB2/Fc. To confirm the interaction of EFNB2 with sHeV G, a co-precipitation was conducted. Western Blot was performed detecting either (A) sHeV G via *Strep*MAB-Classic antibodies or (B) EFNB2/Fc using αEFNB2 antibody. EFNB2/Fc was bound to protein G dynabeads and incubated with sHeV G (lane 1). To test for unspecific binding of sHeV G to the dynabeads, sHeV G was incubated with dynabeads only (lane 2). *L. tarentolae* cell lysate of untransfected Leishmania cells was incubated with EFNB2/fc bound to dynabeads and served as negative control (lane 3). Controls for recombinant sHeV G and EFNB2/Fc are shown in the last two lanes.

saturable manner, thus confirming a specific interaction between the cellular EFNB2 receptor and the recombinant protein. In contrast, CHO-K1 cells did not bind to the recombinant protein. Altogether, these results argue for a correct folding of the recombinant protein expressed in *L. tarentolae* cells.

3.4. Generation of polyclonal rabbit antiserum against sHeV G

To investigate the immunogenic potential of purified sHeV G it was used to immunise rabbits. After three immunisations with 100 and 50 μg of sHeVG, the serum RAb4048 was obtained and tested in HeV G or NiV G expressing HEK293T cells. Serum dilutions of 1:2000 (HeV G) and 1:1000 (NiV G) resulted in convincing visualisation of both proteins, whereas no signals were obtained in cells transfected with empty vector as a negative control (Fig. 5). These data show that antibodies induced by sHeV G also bind to native HeV G proteins. Moreover, a strong cross-reactivity for both, HeV G and NiV G, was observed.

In agreement with the observed cross-reactivity, a dilution of 1:20 showed almost complete neutralisation of NiV-induced virus foci (Fig. 6).

4. Discussion

We here describe the expression, purification and characterisation of a recombinant truncated HeV G protein (sHeV G) expressed in *L. tarentolae*. With the yield of up to 0.5 mg sHeVG per litre

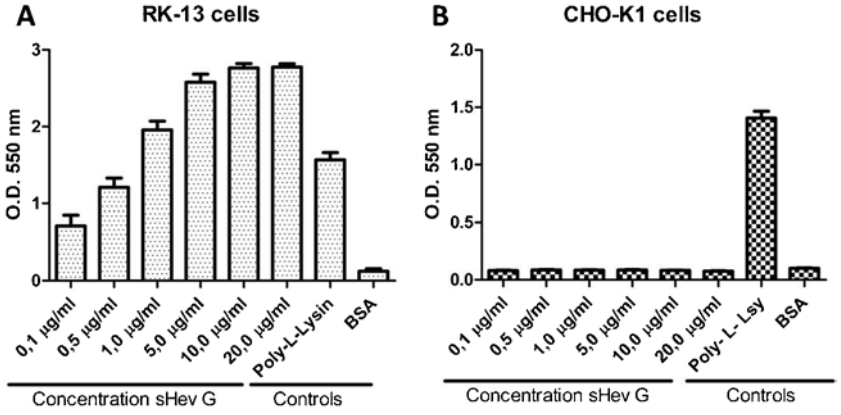

Fig. 4. Cell adhesion assay activity of sHeV G. 96-well plates were coated with increasing concentrations of purified sHeV G. Subsequently, 1×10^5 cells/well of (A) RK-13 cells and (B) of CHO cells were added and incubated for 1 h at 37 °C. After washing and fixation of attached cells, relative numbers of attached cells were assessed by intensity of crystal violet staining. Data are expressed as the means of triplicate results.

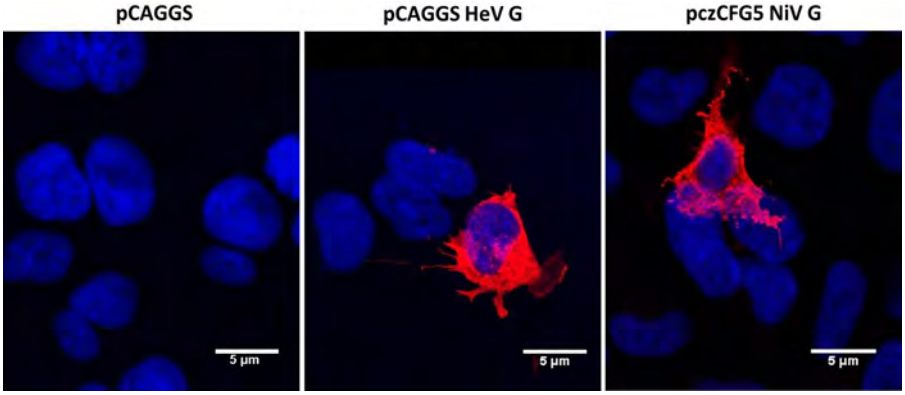

Fig. 5. Immunofluorescence assay (IFA) in transfected HEK293T cells. Mock transfected or HeV G- or NiV G-expressing HEK293T cells were fixed and stained with an anti-sHeV G rabbit antiserum (RAb4048) and a goat anti-rabbit Cy3-conjugate. Nuclei were counterstained with Hoechst 33342. Images were recorded with a Leica SP5 confocal laser scanning microscope. Magnification, 630×.

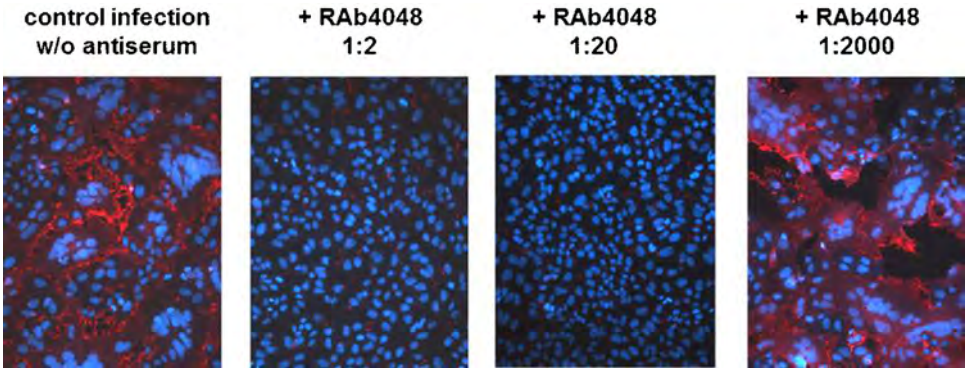

Fig. 6. Virus neutralisation. Vero 76 cells were inoculated with NiV at a multiplicity of infection (m.o.i.) of 0.1. Except for the control infection (w/o antiserum), virus inoculum was pre-incubated for 45 min at 37 °C with the indicated dilutions of the RAb4048 antiserum. Virus–serum mixtures were added to the cells and proceeded as described in Section 2. To visualise NiV-induced foci, the samples were stained with NiV-specific polyclonal guinea pig antiserum and AlexaFluor-568 labelled secondary antibodies. Nuclei were counterstained with DAPI. Images were taken with a Zeiss Axiophot microscope. Magnification, 200×.

Leishmania cell culture, the recombinant sHeV G protein was efficiently purified from cell extracts, indicating that the applied system is indeed suitable for the production of sHeV G protein, even though the initially intended secretion of the protein was not detectable. Most importantly, we here show that (i) the Leishmania-expressed sHeV G protein is able to bind to ephrinB2 as a cellular receptor molecule which is utilised by Hendra virus, (ii) it induces antibody production against Hendra virus G protein that are also cross-reactive against the related NiV G protein and (iii) NiV is neutralised by sHeV G derived rabbit sera.

The sHeV G protein was expressed as a cytoplasmic tail and transmembrane domain deleted HeV G protein in which the authentic HeV signal peptide was replaced by a secretory signal peptide (SP) of *L. mexicana* secreted acid phosphatase (LMSAP) for efficient targeting to the ER. Therefore, it was surprising that no secretion of the soluble protein into the cell culture supernatant was detectable. As mass spectrometry confirmed cleavage of the SP by the detection of the expected N-terminal peptide, it is most likely that the sHeV G protein was correctly synthesised and processed in the ER and that the observed retention is correlated to downstream secretory pathway steps. It has been shown that modifications in the LMSAP signal peptide sequence may increase the secretion of recombinant antibody fragments (Klatt and Konthur, 2012). However, since we assume that the majority of sHeV G protein is correctly cleaved by a signal peptidase, it is rather unlikely that in case of sHeV G protein modifications in the LMSAP signal peptide sequence could increase the secretion efficiency. Moreover, production of influenza virus hemagglutinin (HA) protein was impaired by fusion to the LMSAP signal peptide sequence, while using the authentic influenza virus SP sequence consistently lead to efficient HA production (Pion et al., 2014), indicating that the LMSAP signal peptide can also be inhibitory to glycoprotein secretion. Whether insertion of the authentic virus SP into the sHeV G expression constructs increases the sHeV G secretion in a similar way, or whether other factors are involved in G protein retention remains to be investigated. As earlier studies have shown that proteins can be expressed and purified in the *L. tarentolae* expression system in a range of 0.1–5 mg/L cell culture (Baechlein et al., 2013; Basile and Peticca, 2009; Klatt and Konthur, 2012), our yield of 0.5 mg of sHeV G protein/litre cell culture proved that the purification of intracellular sHeV G from cell lysates was an appropriate alternative to the purification of secreted protein.

The use of *L. tarentolae* expression system has considerable advantages as compared to conventional protein expression systems. Firstly, due to a doubling time of 4–5 h and cultivation in a complex medium without supplementation of CO_2, the handling of *L. tarentolae* is rather simple (Fritsche et al., 2007). Secondly, the N-glycosylation pattern in *L. tarentolae* is exceptionally homogenous with a high eukaryote-like bi-antennary oligosaccharide and $Man_3GlcNAc_2$ core structure lacking higher branched N-glycans (Breitling et al., 2002). It was shown that the complexity of glycosylation of recombinant HeV G proteins expressed in two different systems affected the affinity between the host cell receptor EFNB2 and soluble forms of recombinant HeV G (Colgrave et al., 2012). In comparison to baculovirus expressed HeV G from insect cells, HeV G produced in mammalian cells showed a more complex glycosylation pattern and bound more efficiently to the HeV receptor EFNB2. In contrast to this, it was recently shown that mutations in HeV G leading to the lack of glycosylation sites may even trigger a stronger binding of the resulting protein to the EFNB2 receptor (Bradel-Tretheway et al., 2015). Our data show that *L. tarentolae*-expressed sHeV G efficiently bound to recombinant EFNB2 and EFNB2 expressing cells indicating that the glycosylation pattern of the Leishmania-expressed protein is not detrimental to receptor binding.

Most importantly, functional integrity of sHeV G in terms of receptor binding strongly indicated a preserved HeV G-like protein conformation, which is crucial for the use for serological assays and the induction of neutralising antibodies against the virus. Similar to truncated G protein expressed in mammalian cells (Bossart et al., 2005), the polyclonal antiserum raised against Leishmania-expressed G was not only reactive against both the HeV and NiV G proteins in immunostaining but also blocked NiV infection. Similar cross-reactivity was reported for sera from experimentally HeV infected pigs that neutralised NiV in a plaque reduction neutralisation test (Li et al., 2010).

We therefore conclude that the here described procedure of HeV G expression and purification is a promising approach for the production of antigen for the establishment of diagnostic assays and antiviral reagents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2015.11.006.

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