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Productive replication of peste des petits ruminants virus Nigeria 75/1 vaccine strain in vero cells correlates with inefficiency of maturation of the viral fusion protein

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ABSTRACT

Peste des petits ruminants virus (PPRV), a member of the genus *Morbillivirus*, in the family *Paramyxoviridae* expresses two membrane glycoproteins, the fusion (F) and haemagglutinin (H) glycoproteins which mediate virus-to-cell fusion and cell-to-cell fusion leading to the induction of syncytia in PPRV infected cells. In the context of the characterization of the virulent lineage IV strain PPRV Kurdistan 2011, isolated from wild goats from the Kurdistan region in Iraq, we observed that both PPRV Kurdistan 2011 and the PPRV Nigeria 75/1 vaccine strain led to induction of large syncytia in Vero-dogSLAM cells within 48 h whereas both failed to induce detectable cell-cell fusion events in two Vero cell lines of differing passage histories. We were unable to detect syncytium formation in transiently transfected cells expressing PPRV F or H alone whereas co-expression of F and H induced large syncytia – in Vero-dogSLAM cells only. In $Vero_{Montpellier}$ cells expressing PPRV F and H, fused cells were rarely detectable indicating that PPRV mediated cell fusion activity is impaired in this cell line. Surprisingly, on Vero-dogSLAM cells the vaccine strain grew to titers of $10^{5.25}$ $TCID_{50}$/ml, whereas infectious virus yield was about 200-fold higher on $Vero_{Montpellier}$ and Vero-76 cells. In contrast, the virulent Kurdistan 2011 strain grew to a maximum titer of $10^{7.0}$ $TCID_{50}$/ml on Vero-dogSLAM cells and only $10^{4.5}$ TCID50/ml on normal Vero cells. This was as expected since Vero cells lacking the SLAM receptor for PPRV are regarded as not so permissive for infection. To elucidate the divergent productive replication behaviour of PPRV Nigeria 75/1 vaccine strain on Vero vs Vero-dogSLAM cells, we examined whether intracellular transport and/or maturation of the viral envelope glycoproteins F and H might be implicated with this phenomenon. The results indicate that F in contrast to the H glycoprotein matures inefficiently during intracellular transport in $Vero_{Montpellier}$ cells, thus leading to an absence of detectable syncytia formation. However, in the case of the PPRV Nigeria 75/1 vaccine strain this did not impair efficient virus assembly and release.

1. Introduction

Peste des petits ruminants (PPR) also known as goat plague or pseudo-Rinderpest of small ruminants, is a highly contagious disease, notifiable to the World Organization for Animal Health (OIE, 2013). PPR initially emerged as a fatal disease leading to high mortalities in sheep and goats in the Ivory-coast (Cote D'Ivoire) in West-Africa (Gargadennec and Lalanne, 1942). The causative agent "peste des petits ruminants virus" (PPRV), has recently been re-classified as sole member of the *Small Ruminant Morbillivirus*, in the genus *Morbillivirus* in the family *Paramyxoviridae* together with other animal morbilliviruses such as rinderpest virus (Gibbs et al., 1979; Maes et al., 2019). The disease affects domestic and wild small ruminants and camels (Lefevre and Diallo, 1990; Kinne et al., 2010; Parida et al., 2015). PPR is a particularly fast spreading disease characterized by a morbidity reaching 90% and even higher, and a mortality ranging between 50% and 80% (reviewed in Baron et al., 2011; Albina et al., 2013). Devastating epidemics of PPR have occurred previously in Africa and Asia, the Middle East with outbreaks in Europe restricted only to Turkey and Bulgaria (Banyard et al., 2010; Kwiatek et al., 2011; Albina et al., 2013; Libeau et al., 2014; OIE-WAHIS, 2018). Emergence of PPR into new territories has been reported in Africa and Asia following the global eradication of

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the closely related rinderpest in 2011.

The genome of PPRV is comprised of a non-segmented, single stranded RNA of negative polarity and is 15.948 nucleotides (nts) in length. It contains six non overlapping transcription units encoding six structural proteins (Bailey et al., 2005; Chard et al., 2008). In addition, two nonstructural proteins (C and V) are found in infected cells and are derived from the P protein transcription unit by RNA editing (Mahapatra et al., 2003). While only one serotype of PPRV exists, different strains can be divided into four genetic lineages (I, II, III and IV) according to differences either in a partial sequence of F- (Forsyth and Barrett, 1995; Shaila et al., 1996; Dhar et al., 2002; Ozkul et al., 2002), N- (Couacy-Hymann et al., 2002; Kwiatek et al., 2007) or H- (Kaul, 2004; Balamurugan et al., 2010) genes.

The fusion protein (F) and haemagglutinin (H) glycoproteins are integrated in the viral envelope. The H glycoprotein promotes attachment of the virus to the host cells and, in addition, possesses haemagglutinating activity for red blood cells (Seth and Shaila, 2001). Therefore, HA and HI tests are performed as cheap, rapid and easy diagnostic methods for differentiation between PPRV and RPV (Wosu, 1991; Ezeibe et al., 2004; Osman et al., 2008). A 1830 nucleotide open reading frame (ORF) encodes the PPRV H glycoprotein of 609 amino acids (aa) with a predicted apparent molecular mass of 68 KDa (Bailey et al., 2005; Dhar et al., 2006). The PPRV F glycoprotein mediates fusion of the viral envelope with the host cell plasma membrane allowing penetration of the virus and release of the ribonucleocapsid into the cytoplasm, where virus replication takes place (Seth and Shaila, 2001). Expression of F glycoprotein from wild-type strains also mediates fusion between infected and adjacent non-infected cells resulting in syncytia formation and viral spread within the host without need for completely assembled viral particles (Lamb, 1993). In addition, purified F glycoprotein of PPRV can cause hemolysis (Devireddy et al., 1999). The ORF encoding the F glycoprotein is 1641 bases and the 546 aa primary sequence has a predicted apparent molecular mass of about 59.137 KDa (Bailey et al., 2005; Dhar et al., 2006). The F glycoprotein is synthesized as F_0, a fusion-inactive precursor which is cleaved following intracellular transport by host cell trypsin-like proteases into F_1 (438 amino acids) and F_2 (89 amino acids) subunits which are covalently linked by two disulphide bonds. This cleavage event is essential for the virus to become fusogenic and thus infectious. F_1 possesses a highly conserved N-terminal segment which mediates cell fusion whereas the conserved C-terminal region is proposed to mediate interactions between M and F proteins (Scheid and Choppin, 1977; Meyer and Diallo, 1995; Chard et al., 2008). The interaction of F and H glycoproteins with the host cell membrane mediates initiation of the viral infection. These glycoproteins are involved in the induction of a strong protective antibody and cell mediated immune responses. Of note, neutralizing antibodies are directed mainly against the H glycoprotein (Diallo et al., 2007; Chen et al., 2010).

Since cleavage of paramyxovirus F_0 is known to be essential for fusogenicity and thus for productive replication which results in induction of syncytia in infected cell cultures, it was surprising that experiments designed to analyze efficacy of shRNA against PPRV (Nizamani et al., 2011) showed that PPRV induced plaques in Vero$_{Montpellier}$ cells did not contain detectable syncytia. To elucidate the divergent productive replication behaviour of PPRV Nigeria 75/1 vaccine strain on the differing Vero cell lines, we examined whether the phenotype might be associated with intracellular transport and/or maturation of the viral envelope F and H glycoproteins. Here we present an analysis of the expression and intracellular maturation of PPRV F and H glycoproteins in Vero cells using newly generated monospecific antisera against both glycoproteins which proved to be suitable for application in immunoblotting and immunoprecipitation assays.

2. Materials and methods

2.1. Virus strains and cells

The vaccine strain PPRV Nigeria 75/1, lineage II (Diallo et al., 1989) was kindly provided by Geneviève Libeau and Emmanuel Albina (CIRAD, Montpellier, France). PPRV Kurdistan 2011, a virulent lineage IV strain, isolated recently from wild goats "*Capra aegagrus*" from the Kurdistan region of Iraq (Hoffmann et al., 2012; Wernike et al., 2014) was kindly provided by Michael Eschbaumer (FLI, Insel Riems, Germany).

Three clones of Vero cell lines were used for experiments throughout the study. Vero$_{Montpellier}$ (Vero-M) cells, refers to a clone of Vero cells obtained from Montpellier, France (kindly provided by Emmanuel Albina, CIRAD, Montpellier, France). Vero-76 cells, a clone of Vero cells obtained from FLI (kindly provided by the Collection of Cell Cultures for Veterinary Medicine, FLI, Insel Riems). Vero-dogSLAM (Vero-dS) cells, refers to Vero cell clone stably expressing the canine SLAM (signaling lymphocyte activation molecule) receptor for morbilliviruses that permits virus entry into cells (kindly provided by Michael Eschbaumer, FLI, Insel Riems). Rabbit kidney cell line 13 (RK13), originated from kidneys of a five-week-old rabbit (*Oryctolagus cuniculus*) and human embryonic kidney 293T (HEK293T) cell line (kindly provided by the Collection of Cell Lines in Veterinary Medicine, FLI, Insel Riems) were used for transient protein expression experiments. Cell lines were maintained in a 1:1 mixture of Minimum essential medium [MEM Eagle (Hank's salts) (Sigma) and MEM (Earle's salts)] supplemented with 10% fetal calf serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin and grown at 37°C in a humidified atmosphere in a cell culture incubator containing 5% CO_2.

2.2. Plasmids and antibodies

Plasmids F.PC and H.PC which contained the ORFs encoding the F and H glycoproteins of PPRV Nigeria 75/1, monoclonal antibodies directed against H (named α-H-2/3 MAb) and F (named α-F-2/4-9 MAb) and PPRV antibody positive and negative goat sera were kindly provided by Geneviève Libeau and Emmanuel Albina (CIRAD Montpellier, France).

Cloning of cDNA encompassing the ORFs for F and H of PPRV Nigeria 75/1 and Kurdistan 2011 into plasmid vector pSP73 is described elsewhere (Osman et al., 2018a). All cloning procedures for expression of fusion proteins in bacteria were done following standard procedures (Sambrook and Russell, 2001). Selected fragments from the PPRV Nigeria 75/1 strain F and H ORFs encoding amino acids 392–490 of F, amino acids 57–396 and 57–609 of H were amplified using primer pairs listed in Table 1. Amplicons were cleaved with either *Hin*dIII or *Hin*dIII and *Xba*I (see Table 1) and integrated in frame to the maltose binding protein (MBP) ORF contained in plasmid pMal-p2X (New England Biolabs, Dreieich, Germany) which had also been digested with the respective enzymes. Correct sequences of the resulting plasmids pMal-PPRV-F392–490 from F ORF, pMal-PPRV-H57-396 and pMal-

Table 1
Primers used for PCR amplification of partial PPRV-F and -H ORFs and sequencing.

Primer Name	Sequence 5' to 3'
PPRV-F392+ (for) (*Xba*I)	taa*tctaga*ggagcaggagcaaagtgttacacaacggagacagttatc
PPRV-F490- (rev) (*Hin*dIII)	tag*aagctt*aagctcctgctccgccactgaaaggtacccctttaacag
PPRV-H57+ (for) (*Xba*I)	taa*tctaga*ggagcaggagcagggatcaggcttcaccgagccac
PPRV-H396- (rev) (*Hin*dIII)	tag*aagctt*aagctcctgctccgccattgcaaaatgaaggaggtc
PPRV-H609- (rev) (*Hin*dIII)	tag*aagctt*aagctcctgctccgactggattacatgttacctctatac
pMal+	ccgccagcggtcgtcagactg
PPRV-H620+ (for)	agagcttacgctgaccctgatg

for: 5′→3′ primer orientation in ORF; rev: 3′→5′ primer orientation in ORF.

PPRV-H57–609 from H ORF were determined using primers pMal+ and PPRV-H620+ (Table 1). Expression and purifications of the MBP fusion proteins were performed as recommended by New England Biolabs (pMAL™ Protein Fusion & Purification System, www.neb.com) (Fig. S1).

For transient expression experiments, the ORFs encoding F and H of PPRV Nigeria 75/1 and Kurdistan 2011 strains were excised from the respective pSP73 based plasmids with *Bgl*II and *Hin*dIII, respectively, blunt ended using Klenow DNA polymerase and integrated into expression vector pCAGGS (Niwa et al., 1991), which had also been blunt ended after cleavage with *Eco*RI. The resulting plasmids were named pCAGGS-PPRV-$F_{N(Nigeria\ 75/1)}$ and -$F_{K(Kurdistan\ 2011)}$; and pCAGGS-PPRV-H_N and -H_K, respectively.

2.3. Preparation of PPRV-F and PPRV-H antisera in rabbits

Purified fusion proteins Mal-PPRV-F392–490, pooled Mal-PPRV-H57–396 and Mal-PPRV-H57–609 were used for the immunization of 4 rabbits, each 2 rabbits with 1.0 mg of Mal-PPRV-F or 0.8 mg of Mal-PPRV-H pool purified fusion proteins, respectively, dissolved in 1000 µl PBS- and mixed with an equal volume of Freund's complete adjuvants for the first injection. Rabbits were boostered 4 times in 4 weeks intervals with the same amount of antigen, mixed with Freund's incomplete adjuvants. The animals were exsanguinated and serum was collected after centrifugation of the blood at 3000 rpm for 5 min, inactivated at 56°C for 30 min and stored in aliquots at −20°C.

2.4. Indirect immunofluorescence (IIF) assay

For indirect immunofluorescence (IIF), cells were fixed with 3.7% formaldehyde in PBS- for 10 min and permeabilized using 0.2% Triton X100 for 10 min then washed twice with PBS- containing 10% FCS. Fixed cells were incubated with 1:1000 dilutions of monospecific anti-PPRV-H or anti-PPRV-F rabbit sera, 1:100 dilution of α-H-2/3 MAb or 1:250 dilution of α-F-2/4-9 MAb for 1 h at room temperature followed by washing thrice with PBS- for 5 min. Bound antibodies were visualized with 1:1000 diluted Alexa Fluor® 488 (green fluorescence) or Alexa Fluor® 568 (red fluorescence) conjugated anti-species IgG after incubation for 1 h at room temperature and washing thrice with PBS-. Nuclei were stained with Höchst 33258 diluted 1:10,000 in PBS-, incubated for 5 min and washed twice for 5 min each with PBS-. Fluorescence was recorded with Nikon T100 fluorescence microscope with CCD camera and NIS software or with a Leica SP5 confocal laser scan microscope, all images were acquired with an oil immersion HCX PL APO 63x/1.40-0.60 objective and a R 9624 photomultiplier detector. Brightness and contrast were slightly modified using ImageJ software.

2.5. Transient expression of PPRV-H and PPRV-F in mammalian cells

The expression plasmids for PPRV Nigeria 75/1 [F (pCAGGS-PPRV-F_N) and H (pCAGGS-PPRV-H_N)] and PPRV Kurdistan 2011 [F (pCAGGS-PPRV-F_K) and H (pCAGGS-PPRV-H_K)] were used for transient protein expression. Vero-dogSLAM, $Vero_{Montpellier}$, RK13 and HEK293T cells were seeded into 24- or 6-well cell culture plates (Corning, USA) and transfected with 0.625 or 2.5 µg of pCAGGS-PPRV-$F_{N/K}$ or pCAGGS-PPRV-$H_{N/K}$ expression plasmid per well, respectively, using polyethyleneimine (PEI) transfection as described recently (Osman et al., 2018b). Cells were transfected with expression plasmids encoding for PPRV F and H alone or in combination (F and H).

2.6. Single step growth kinetics

Growth kinetics experiments were performed for PPRV Nigeria 75/1 vaccine and Kurdistan 2011 strains on different Vero cell lines. Confluent monolayer of Vero-dogSLAM, $Vero_{Montpellier}$ and Vero-76 cells in 24-well cell culture plates (Corning, USA) were infected with the respective PPRV strains at a MOI of 0.1 then incubated at 37°C for 2 h. Cells were washed once with medium and extracellular virions were inactivated by incubation with 0.5 ml of Citrate buffer, pH 3.0 (40 mM citric acid, 10 mM KCl, 135 mM NaCl) for 2 min at room temperature, then the buffer was removed and cells were washed twice with 0.5 ml medium and finally incubated with 250 µl MEM medium at 37°C. Supernatant were collected and infected cells were trypsinized at the indicated time point and stored at −70°C. Scheduled times for harvest of PPRV from infected cells were as follows: 24, 48, 72, 96, 120 and 144 h post infection (p.i.).

Virus titrations were performed on Vero-dogSLAM cells grown in 96-well cell culture plates (Corning, USA). Serial 10-fold dilutions of the virus in medium were prepared, 100 µl of each dilution were added to 2 wells of the plate. After incubation at 37°C and 5% CO_2 for 1–2 h, the inoculum was replaced by cell culture medium. Plates were incubated for 6 days, wells which showed CPE were considered infected. Titrations were repeated 4 times and virus titres were estimated as $TCID_{50}$/ml.

In parallel, wells of PPRV infected cells were fixed at the indicated time points using 3.7% formaldehyde in PBS- for 10 min and permeabilized using 0.2% Triton X100 for 10 min, subsequently indirect immunofluorescence (IIF) was performed for demonstration of syncytia induced by PPRV strains.

2.7. Pulse-chase metabolic labeling and radioimmunoprecipitation

$Vero_{Montpellier}$ cells in 6-well cell culture plates were infected with PPRV Nigeria 75/1 at a MOI of 1 for 36 h. RK13 or HEK293T cells in 24-well cell culture plates were transfected with 0.625 µg/well of pCAGGS-PPRV-$H_{N/K}$ or pCAGGS-PPRV-$F_{N/K}$ for 18–20 h. Cells were pulse labelled for 60 min with 92 µci/ml [^{35}S]methionine in methionine-free medium and then chased in normal cell culture medium for the times indicated. Cells were then lysed at different times post infection/transfection in 500 µl of 1X Rita lysis buffer (10 mM Tris, 150 mM NaCl, 1% Na-desoxycholate, 1% SDS, 2 mM Methionin, 1 mg/ml Ov.alb. Grade V, 0.02% NaN_3, 1 mM PMSF and 1% NP40). Cell lysates were sonicated at 40 W for 20 sec, incubated for 30 min on ice and sonicated again. Cell debris was pelleted by centrifugation at 40,000 rpm for 30 min at 4°C using a Beckman-Coulter TLA 55 Rotor. Supernatants were collected in new tubes. 200 µl cell lysate was mixed with 10 µl of α-PPRV-H or α-PPRV-F sera and incubated at 25°C for 1 h and then on ice for 60 min. After centrifugation at 14,000 rpm in an Eppendorf R5430 centrifuge for 30 min at 4°C. Supernatants were mixed with 25 µl *Staph. Aureus* cells (Pansorbin, Calbiochem) and incubated for 30 min on ice. Samples were washed 4 times by centrifugation at 14,000 rpm for 20 sec. Pellets, resuspended in 500 µl 1X RITA, were centrifuged through 500 µl of 30% Sucrose at 14,000 rpm for 2 min. Pellets were resuspended in 50 µl SDS-PAGE protein buffer, heated at 56°C for 5 min then centrifuged at 14,000 rpm for 2 min. 25 µl of the supernatants were mixed with 0.75 µl mercaptoethanol and proteins were separated by SDS-10% PAGE along with 7 µl of BenchMark™ pre-stained protein Marker (Invitrogen). Gels were fixed, dried at 80°C for 1 h and exposed to image plate (Fuji BAS Cassette 2325 Imaging Plates, Raytest, Germany). Labeled proteins were visualized using a Fuji FLA-3000 Scanner.

2.8. SDS-PAGE and Western blotting

Bacterial and eukaryotic cell pellets were resuspended in protein sample buffer and sonicated 3 times for 20 sec at 40 W in a Branson ultrasonic water bath. ß-mercaptoethanol was added to 4% end concentration. After incubation at 85°C for 10 min, proteins were separated by sodium dodecyl sulfate-10%polyacrylamide gel electrophoresis (SDS-10%PAGE) and transferred to nitrocellulose membranes using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) at 20 V for 45 min.

Membranes were incubated in PBS-/6% skimmed milk powder for 1 h at room temperature and probed with 1:5000 dilutions of anti-PPRV-H or anti-PPRV-F in PBS- containing 0.1% Tween 20 and 0.6% skimmed milk powder for 1–2 h at RT or overnight at 4°C. Bound antibodies were visualized by incubation with peroxidase-conjugated affinipure F(ab')2 fragment goat anti-rabbit IgG (H + L) (Jackson ImmunoResearch Laboratories, USA) diluted 1:20,000 in PBS- containing 0.1% Tween 20 for 1 h followed by 3 washing steps with PBS- containing 3% and 1% Tween 20 and incubation with Super Signal West Pico Chemiluminescent Substrate and Super Signal West Pico Stable Peroxidase Solution (Thermo scientific, USA). Images were recorded and analyzed using VersaDoc™ Imaging System 4000 M P (Bio-Rad).

3. Results

3.1. Generation of rabbit monospecific sera directed against PPRV-F and PPRV-H

Investigations into PPRV glycoproteins have been hampered by a paucity of antibodies that are reactive in Western blots and/or immunoprecipitation assays. We therefore cloned partial open reading frames (ORFs) of F and H glycoproteins of PPRV Nigeria 75/1 vaccine strain in-frame with the maltose binding protein (MBP) ORF in the bacterial expression vector pMal-p2×. Selection of the ORF segments was based on in-silico analyses of hydrophilicity, surface probability and antigenic index of the respective deduced amino acid sequences using GCG (Genetics Computer Group (GCG), Wisconsin Package, Version 10.3) "peptidestructure" program. Testing of multiple constructs expressing partial F and H protein sequences showed that only pMal-PPRV-F392–490 (V_{131} to D_{164}); pMal-PPRV-H57–396 (K_{19} to D_{132}) and pMal-PPRV-H57–609 (K_{19} to L_{203}) yielded detectable amounts of the respective MBP-fusion proteins upon induction in E.coli (Fig. S1). Of note, proteins encoded by Mal-PPRV-H57–396 and Mal-PPRV-H57–609 were found to be unstable, resulting in only low amounts of purified full length fusion proteins (Fig. S1). Purifications of both proteins were therefore pooled and used for the immunization of two rabbits as was done with the purified Mal-PPRV-F392–490 fusion protein. The rabbit sera prepared against PPRV F "anti-PPRV-F #431 and anti-PPRV-F #444" were obtained from the 2 rabbits immunized with the purified protein Mal-PPRV-F392–490 whereas rabbit sera prepared against PPRV H "anti-PPRV-H #436 and anti-PPRV-H #438" were obtained from the other 2 rabbits immunized with the pooled Mal-PPRV-H57–396 and Mal-PPRV-H57–609 purified proteins.

3.2. Testing the reactivity of the rabbit sera against their target antigens in PPRV infected $Vero_{Montpellier}$ cells

To test the specificity of the resulting antisera, $Vero_{Montpellier}$ cells were infected with PPRV Nigeria 75/1 or vaccinia virus WR as a control (Fig. 1A). Infected cultures were fixed after the development of plaques, permeabilized and incubated with the rabbit sera (α-PPRV-F #444/α-PPRV-F #431 or α-PPRV-H#438/α-PPRV-H #436) spiked with mouse monoclonal antibodies against PPRV-F (α-F-2/4-9 MAb) or PPRV-H (α-H-2/3 MAb). This showed (Fig. 1A) that the rabbit sera did not bind to vaccinia virus infected $Vero_{Montpellier}$ cells but reacted specifically with PPRV infected cells and indicated that sera α-PPRV-F #444 and α-PPRV-H #438 (green fluorescence) yielded slightly better fluorescence intensities than their respective pairs [α-PPRV-F #431 and α-PPRV-H #436] and thus were used in further experiments (Fig. 1A). The rabbit sera directed against MBP-PPRV-F and MBP-PPRV-H fusion proteins detect their target antigens in PPRV infected $Vero_{Montpellier}$ cells.

To elucidate whether antibodies in the monospecific sera bind to their target proteins on the surface of infected cells, cultures with PPRV induced plaques were fixed and non-permeabilized prior to immunostaining. Fig. 1B shows that the epitopes for α-PPRV-F #444 and α-PPRV-H #438 (green fluorescence) are exposed on the surface of PPRV infected cells and suggests that both sera recognize correctly transported mature F and H, respectively, as do monoclonal antibodies α-H-2/3 and α-F-2/4-9 (green fluorescence).

3.3. Assessment of the capacity of PPRV Nigeria 75/1 and PPRV Kurdistan 2011 strains to induce syncytium formation in Vero cell lines

To assess the capacity of PPRV Nigeria 75/1 to induce syncytium formation, Vero-dogSLAM cells were infected with PPRV strains Kurdistan 2011 and Nigeria 75/1 at a MOI of 0.1 and incubated for 48 h p.i. (Fig. 2A). We observed that both PPRV Kurdistan 2011 and the PPRV Nigeria 75/1 vaccine strain led to induction of large syncytia in Vero-dogSLAM cells within 48 h (Fig. 2A).

Cell fusion activity induced by PPRV was further investigated during different time points in PPRV Kurdistan 2011 or Nigeria 75/1 infected Vero-dogSLAM (Fig. 2B) or $Vero_{Montpellier}$ (Fig. 2C) cultures. Vero-dogSLAM and $Vero_{Montpellier}$ cells were infected with PPRV strains Kurdistan 2011 and Nigeria 75/1 at a MOI of 0.1 and incubated for 24 h p.i. (Fig. 2B) and 6 d p.i. (Fig. 2C), respectively. Cells were fixed at 24 h p.i. and 6 d p.i, respectively, and PPRV F and H were visualized by indirect immunofluorescence using a mixture of H- (α-PPRV-H #438) and F- (α-PPRV-F #444) monospecific antibodies followed by Alexa Fluor® 488-labeled secondary antibody (Fig. 2B, 2C). Shortly after infection, in Vero-dogSLAM cells infected with the PPRV Kurdistan 2011 strain, small syncytia appeared which progressively developed into large syncytia at 24 h p.i. (Fig. 2B). In contrast, in Vero-dogSLAM cells infected with PPRV Nigeria 75/1 vaccine strain, small syncytia could only be observed at 24 h p.i. (Fig. 2B). Induction of syncytia by PPRV Nigeria 75/1 in Vero cells has been described previously by Seth and Shaila (2001) and Mahapatra et al. (2006) but was not observed in infected $Vero_{Montpellier}$ cell cultures (Fig. 2C). However, PPRV Nigeria 75/1 was able to infect Vero-dogSLAM cell cultures (von Messling et al., 2003) with large syncytia observed at 24 h p.i. (Fig. 2B) and 48 h p.i. (Fig. 2A) which were comparable in size to the syncytia induced by PPRV Kurdistan 2011. Monitoring of cell-cell fusion by indirect immunofluorescence revealed that both strains induced syncytium formation in Vero-dogSLAM cells but not in normal $Vero_{Montpellier}$ cells suggesting that PPRV mediated cell-to-cell fusion activity is impaired in infected $Vero_{Montpellier}$ cells (Fig. 2C).

3.4. Assessment of the cell fusion activity of PPRV strains Nigeria 75/1 and Kurdistan 2011 in transfected Vero cell lines

To find an explanation for the impairment of the cell fusion activity by PPRV in $Vero_{Montpellier}$ cells, Vero-dogSLAM or $Vero_{Montpellier}$ cell cultures were transfected with expression plasmids for PPRV Nigeria 75/1 [F (pCAGGS-PPRV-F_N) and H (pCAGGS-PPRV-H_N)] and PPRV Kurdistan 2011 [F (pCAGGS-PPRV-F_K) and H (pCAGGS-PPRV-H_K)]. Cells were transfected with expression plasmids encoding for PPRV F and H alone or in combination as indicated (Fig. 3). In contrast to Seth and Shaila (2001) who reported PPRV F mediated cell-cell fusion in CV-1 cells, we were unable to detect syncytium formation in transfected cells transiently expressing F alone (Fig. 3A, 3B). Expression of PPRV H also did not result in cell-to-cell fusion, whereas co-expression of PPRV F and H induced large syncytia in Vero-dogSLAM cells (Fig. 3A). In $Vero_{Montpellier}$ cells expressing PPRV F and H, fused cells were rarely detectable and when present contained only a few nuclei (Fig. 3B), indicating that PPRV mediated cell fusion activity is impaired in this cell line.

3.5. Growth kinetics of PPRV strains Nigeria 75/1 and Kurdistan 2011 in differing Vero cell lines

The growth kinetics of PPRV Nigeria 75/1 and Kurdistan 2011 were examined in Vero-dogSLAM, $Vero_{Montpellier}$ and Vero-76 cultures (Fig. 4). Surprisingly, the PPRV Nigeria 75/1 vaccine strain grew to

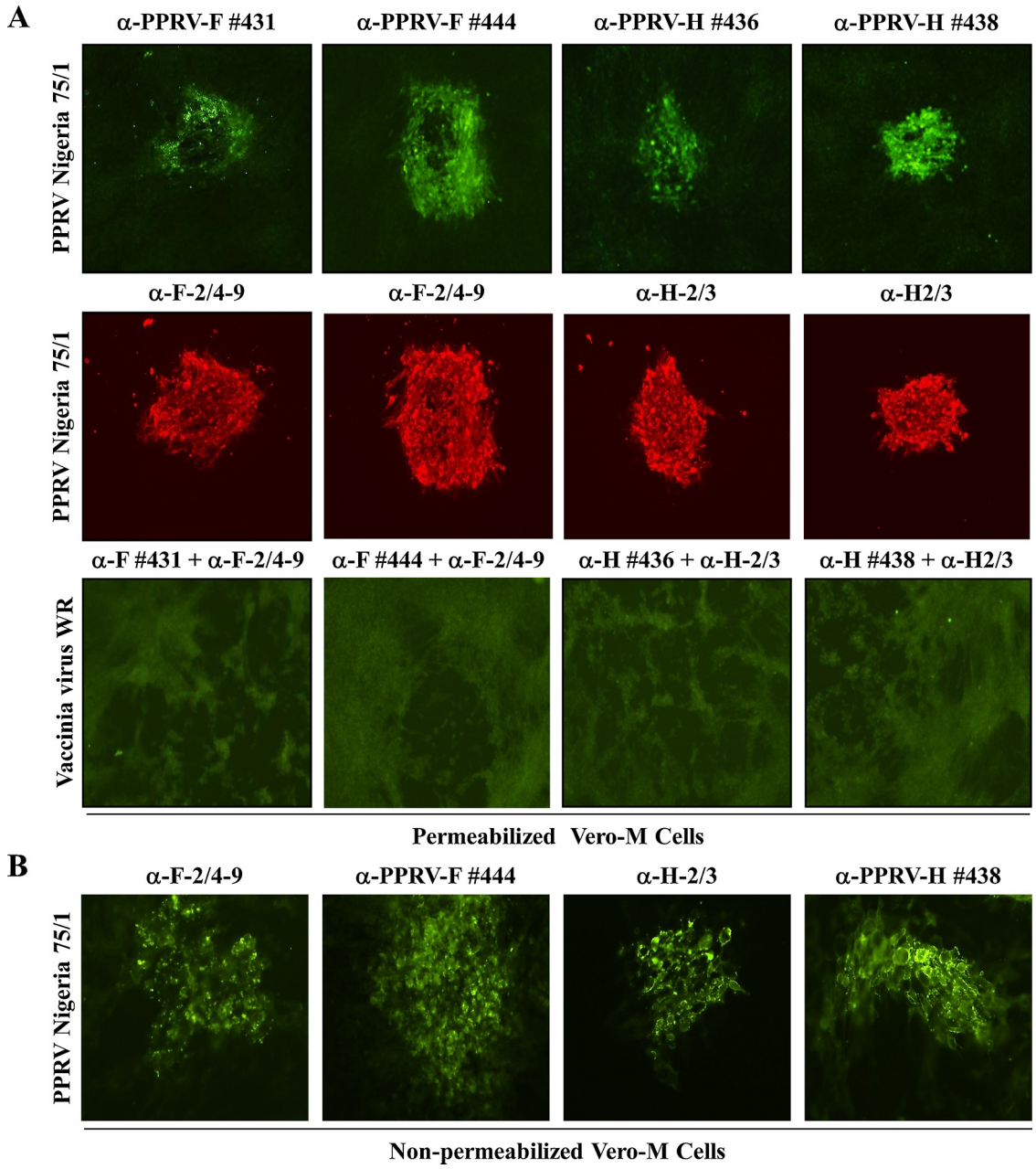

Fig. 1. The rabbit sera directed against MBP-PPRV-F and MBP-PPRV-H fusion proteins detect their target antigens in PPRV infected Vero$_{Montpellier}$ cells. Vero$_{Montpellier}$ cells in 24-well cell culture plates were infected with a MOI of 0.1 of PPRV Nigeria 75/1 vaccine strain or vaccinia virus strain WR as noted. After development of plaques, cells were fixed with 3.7% formaldehyde in PBS- and permeabilized with 0.2% Triton X100 (A) or fixed with 3.7% formaldehyde only (B). (A) Cultures were incubated with the indicated 1:1000 diluted rabbit sera α-PPRV-F #431 or #444 spiked with 1:250 diluted monoclonal antibodies α-F-2/4-9 or 1:1000 diluted rabbit sera α-PPRV-H #436 or #438 spiked with 1:100 diluted monoclonal antibodies α-H-2/3. Bound antibodies were labeled with Alexa Fluor® 488 goat α-rabbit IgG (green fluorescence) and Alexa Fluor® 568 goat α-mouse IgG (red fluorescence). (B) Cultures were incubated with 1:1000 diluted rabbit sera or monoclonal antibodies α-F-2/4-9 and α-H-2/3 as indicated, bound antibodies were labeled with Alexa Fluor® 488 goat α-rabbit IgG or Alexa Fluor® 488 goat α-mouse IgG (green fluorescence). Fluorescing cells were photographed using a Nikon T100 fluorescence microscope with CCD camera and NIS software, respectively.

titers of $10^{5.25}$ $TCID_{50}$/ml on Vero-dogSLAM cells whereas infectious virus yield was about 200-fold higher on Vero$_{Montpellier}$ and Vero-76 cells.

In contrast, the virulent strain Kurdistan 2011 grew to a maximum titer of $10^{7.0}$ $TCID_{50}$/ml on Vero-dogSLAM cells and only $10^{4.5}$ $TCID_{50}$/ml on normal Vero cells (Fig. 4). The latter result was not unexpected since Vero cells lack a receptor for wild-type PPRV.

3.6. Monitoring the intracellular maturation and transport of PPRV -F and -H glycoproteins in Vero$_{Montpellier}$ cells infected by PPRV Nigeria 75/1 strain

The absence of detectable syncytium formation in Vero$_{Montpellier}$ cells was investigated further by monitoring the intracellular maturation and transport of F and H glycoproteins in pulse/chase experiments (Fig. 5A, B). This showed that the F precursor molecule (pF) exhibits an apparent molecular mass of 55 kDa (Fig. 5A, lanes 2–7). The F precursor was converted into a 59 kDa form during the 150 min chase period, indicating that pF reached the Golgi apparatus where N-glycans are converted from the mannose-rich to the complex form and/or O-

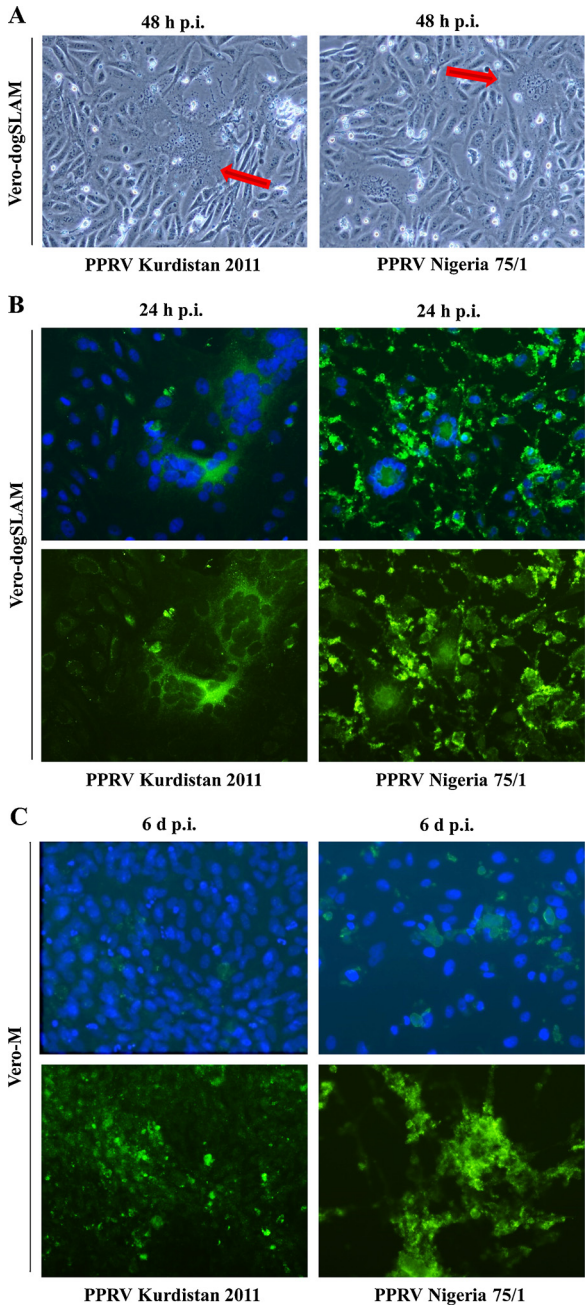

Fig. 2. Assessment of the capacity of PPRV Nigeria 75/1 and PPRV Kurdistan 2011 strains to induce syncytium formation in Vero cell lines. (A) Vero-dogSLAM cells were infected with PPRV Nigeria 75/1 vaccine strain (right) and PPRV Kurdistan 2011 virulent strain (left) at a MOI of 0.1. Cultures were photographed at 48 h p.i. using a Nikon T100 microscope with CCD camera and NIS software. Red arrows point to representative syncytia. (B) Vero-dogSLAM or (C) $Vero_{Montpellier}$ cultures were infected with PPRV strains Nigeria 75/1 (right) or Kurdistan 2011 (left) as indicated. Cells were fixed at 24 h p.i. (B) and 6 d p.i. (C), respectively, and PPRV F and H were visualized by indirect immunofluorescence using a mixture of H- and F-monospecific antibodies and Alexa Fluor® 488-labeled secondary antibodies. Nuclei were stained with Höchst 33258. Cultures were photographed using laser scan confocal microscope (Leica SP5) and LAS AF software for Confocal Imaging.

glycans are added resulting in uncleaved F_0. However, cleavage into the F_1 and F_2 subunits in the *trans*-Golgi network (TGN) did not occur efficiently because conversion of F_0 into faster migrating proteins was not apparent (Fig. 5A). This conclusion is supported by the absence of subunits after a longer time period which resulted in a more diffusely migrating fusion protein form (Fig. 5A, lane 8).

In contrast, conversion of the 63 kDa precursor of H (pH) (Fig. 5B, lanes 2–7) to the mature 68 kDa form, which was still incomplete after a 150 min chase (Fig. 5B, lane 7), appeared to be completely converted into the mature 68 kDa H glycoprotein in the sample metabolically labeled from 36 to 120 h p.i. (Fig. 5B, lane 8).

3.7. Monitoring the kinetics of PPRV-F and -H glycoprotein expression after low MOI infection of $Vero_{Montpellier}$ cells

The conclusion that F_0 remains only partly or entirely uncleaved during PPRV Nigeria 75/1 infection of $Vero_{Montpellier}$ cells was supported by monitoring the kinetics of F and H glycoprotein expression from 18 h p.i. until 120 h p.i. (5 days after infection) using α-PPRV-F #444 or α-PPRV-H #438. Antibodies in both sera reacted efficiently in Western blots and facilitated detection of F and H glycoproteins at 48 h p.i. (Fig. 6). The respective signals slowly increased in abundance until 120 h p.i., suggesting a relatively slow productive replication with no evidence for cleavage of F_0 even at 5 d p.i. (Fig. 6A). Expression of the H glycoprotein was found to follow comparable kinetics (Fig. 6B). In addition, slower migrating proteins with about twice the size of each of F or H became detectable by α-PPRV-F #444 and α-PPRV-H #438, respectively, at about 96 h p.i.

3.8. Monitoring the transient protein expression of PPRV-F and -H in transfected RK13 cells

These slower migrating forms, which can also be observed in Fig. 5 are not detectable in Western blots analyzing transient expression of PPRV F and H in RK13 cells transfected with plasmids encoding F and H genes from PPRV Nigeria 75/1 and Kurdistan 2011 strains (Fig. 7). Thus, the formation of the antisera-reactive high molecular weight proteins may be time dependent or may need expression of additional viral functions. In lane 4 in Fig. 7 showing α-PPRV-F#444-reactive proteins from PPRV infected $Vero_{Montpellier}$ cells a minor protein band appears below the predominant F_0 signal. The apparent molecular mass of the corresponding protein is around 48 kDa and thus could represent the F_1 subunit (Herbert et al., 2014; Rahman et al., 2003). This protein may however be a degradation product of F_0.

3.9. Monitoring the cleavage activity of PPRV-F after high MOI infection of Vero cells

The cleavage activity of PPRV Nigeria 75/1 F was examined in Vero-dogSLAM (Nig75/1-dS) or $Vero_{Montpellier}$ (Nig75/1-M) cells in which a MOI of 10 was used. Cells were harvested at 48 h p.i., lysed and PPRV F and H expression was analyzed by Western blotting using F- (α-PPRV-F #444) and H- (α-PPRV-H #438) monospecific antibodies. It appears that the cleavage of F_0 is completed only in Vero-dogSLAM cells following high MOI infection (Fig. 8). These results indicate that F in contrast to the H glycoprotein matures inefficiently during intracellular transport in $Vero_{Montpellier}$ cells, leading to an absence of detectable syncytia formation. However, in the case of the PPRV Nigeria 75/1 vaccine strain this did not impair efficient virus assembly and release.

4. Discussion

PPRV F and H are integral membrane proteins and are pivotal for initiation of infection for which H performs attachment to the target cell and F mediates fusion between the viral and cellular membranes (Seth and Shaila, 2001; reviewed in Kumar et al., 2014). The N-glycosylated F precursor F_0 is cleaved by furin at the consensus cleavage site R-R-T-R-R where R stands for arginine, and T for threonine. Cleavage of F_0 is regarded as essential for virus infectivity and membrane fusion (reviewed in Kumar et al., 2014) which in Vero cells infected with PPRV Nigeria

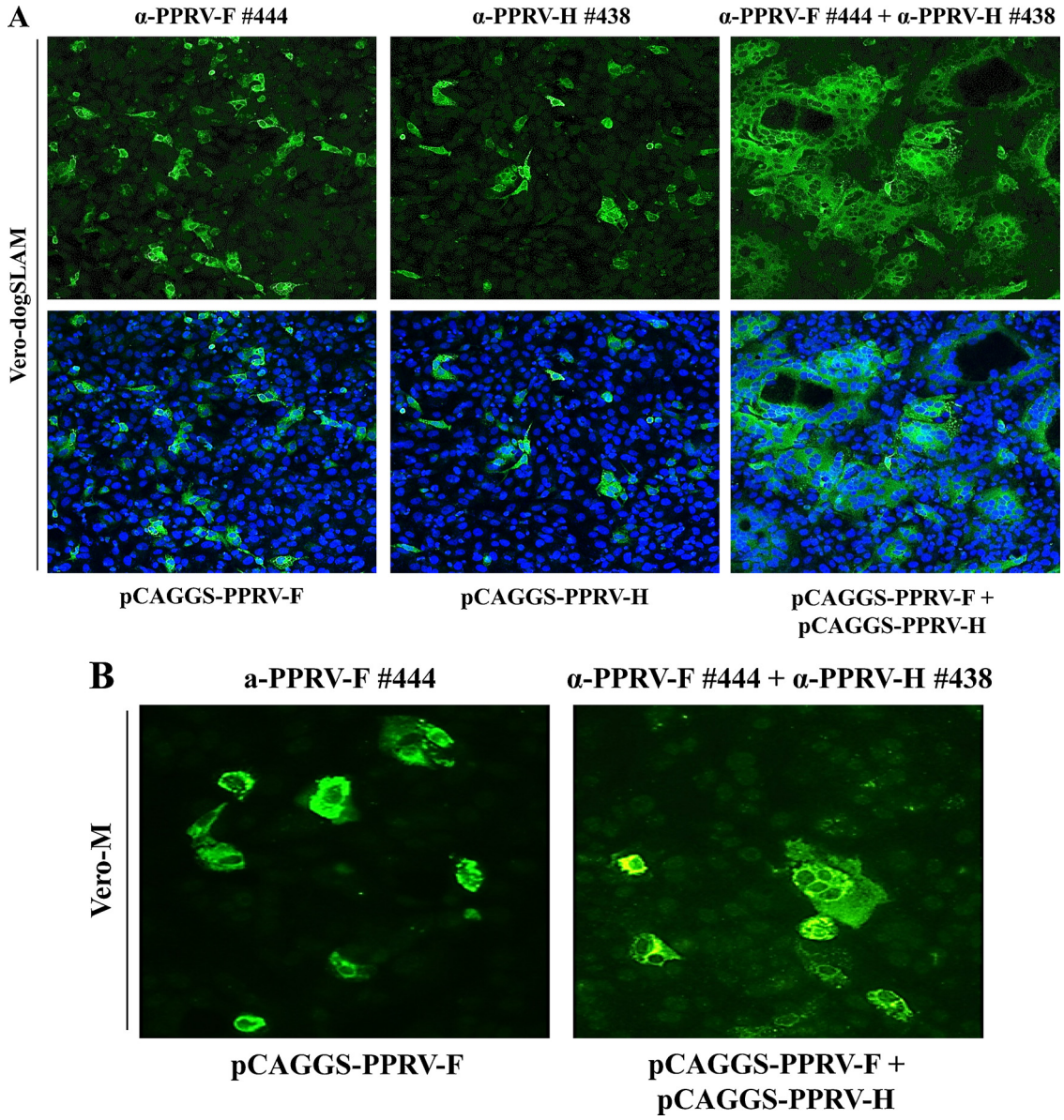

Fig. 3. Assessment of the cell fusion activity of PPRV strains Nigeria 75/1 and Kurdistan 2011 in transfected Vero cell lines. Vero-dogSLAM cells (A) and $Vero_{Montpellier}$ cells (B) were transfected with expression plasmids encoding for pCAGGS-PPRV-$F_{N/K}$ and pCAGGS-PPRV-$H_{N/K}$ alone or in combination (pCAGGS-PPRV-$F_{N/K}$ + pCAGGS-PPRV-$H_{N/K}$) as indicated. Cultures were fixed at 28 h p.tr. and PPRV F and H were visualized by indirect immunofluorescence using α-PPRV-F #444, α-PPRV-H #438 or a mixture of both monospecific antibodies and bound antibodies were labeled with Alexa Fluor® 488 goat α-rabbit IgG secondary antibodies (green fluorescence). Nuclei were stained with Höchst 33258. Fluorescing cells were photographed using laser scan confocal microscope (Leica SP5).

75/1 or in CV-1 cells transfected with F-encoding expression plasmids results in formation of syncytia (Mahapatra et al., 2006; Seth and Shaila, 2001).

Interestingly, syncytium formation in $Vero_{Montpellier}$ cell cultures was not observed after infection with PPRV Nigeria 75/1 although the virus did grow to titers up to $10^{7.5}$ $TCID_{50}$/ml. We therefore addressed the question of whether processing of F and/or H might be involved in this apparently specific feature of $Vero_{Montpellier}$ cells. H was included in the analyses because for some paramyxoviruses the presence of the attachment glycoprotein is beneficial or even necessary for induction of membrane fusion. Since available monoclonal antibodies α-F-2/4-9 and α-H-2/3 were suitable for specific detection of F and H by indirect immunofluorescence within and on the surface of PPRV-infected cells but failed to recognize the respective glycoproteins in Western blots and immunoprecipitation assays, monospecific sera were raised in rabbits which proved capable of binding to their target proteins in the applications mentioned above. Indirect immunofluorescence experiments demonstrated that the epitopes recognized by the sera are, as expected from their respective positions in the proteins, exposed on the outside of the cell membrane. Specificity of the monospecific rabbit sera was demonstrated by their reactivity with transiently expressed F and H from PPRV Nigeria 75/1 vaccine strain and the wild-type Kurdistan 2011 isolate. Again, neither F- or H- expression of either strain alone revealed evidence for syncytium formation. In contrast, co-expression of F and H induced large syncytia in Vero-dogSLAM cells only. These results indicate that F cell-cell fusion activity needs at least the concomitant expression of H.

Since intracellular transport of glycoproteins can be followed by pulse/chase experiments using [^{14}C]-labeled (Schlesinger and Schlesinger, 1972) or [^{35}S]-labeled (König et al., 2004) amino acids, maturation of F and H glycoproteins in PPRV infected $Vero_{Montpellier}$ cells was monitored after a 60 min pulse with [^{35}S]-methionine. As expected for a type II integral membrane protein, the H precursor molecules with an apparent molecular mass of 63 kDa was modified

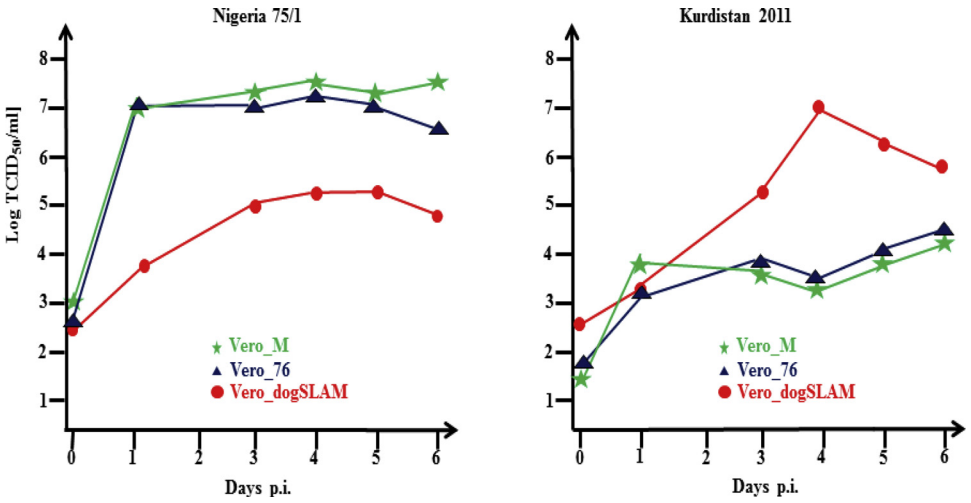

Fig. 4. Growth Kinetics experiments for PPRV strains Nigeria 75/1 and Kurdistan 2011. Vero-dogSLAM, $Vero_{Montpellier}$ and Vero-76 cultures in 24-well cell culture plates were infected with PPRV strains Nigeria 75/1 (left) or Kurdistan 2011 (right) at a MOI of 0.1 as indicated. After 2 h incubations, cells were washed with medium and extracellular virions were inactivated by incubation with 0.5 ml of Citrate buffer (pH 3.0) for 2 min then cells were washed twice with 0.5 ml medium and finally incubated with 250 µl MEM medium. Cells and supernatants were harvested at 24, 48, 72, 96, 120 and 144 h p.i., stored at −70°C and titrated on Vero-dogSLAM cells after thawing. Titrations were repeated 4 times and virus titers were estimated as $TCID_{50}$/ml.

during intracellular transport to the probably 68 kDa apparent molecular mass mature protein. Surprisingly, the putative 55 kda F precursor molecules was modified to the 59 kDa F_0 which, however, was apparently not cleaved into the expected 48 kDa F_1 (Herbert et al., 2014; Rahman et al., 2003) during the chase period or after long time labeling (Fig. 5) which suggest that F maturation is impaired with regard to cleavage in PPRV Nigeria 75/1 infected $Vero_{Montpellier}$. This interpretation is supported by the analyses of the apparent molecular masses of F expressed in the time course after low MOI infection of $Vero_{Montpellier}$ cells or after transient expression in RK13 cells which did not reveal unequivocal presence of the F_0 cleavage products (Figs. 5–7). These results suggest that other viral proteins could be required for an effective F_0 cleavage.

Since cleavage of paramyxoviral F_0 protein is regarded to be essential for membrane fusion activity, F_0 cleavage deficiency in $Vero_{Montpellier}$ cells could lead to the observed absence of syncytia after infection with PPRV Nigeria 75/1 or after transient expression mediated by transfected plasmids. Monitoring the steady state levels of F and H after infection and transient transfection and transport/maturation in pulse chase experiments following infection demonstrates that F, in contrast to H, matures inefficiently in $Vero_{Montpellier}$ cells which may be due to hampered intracellular transport since the pulse/chase experiments gave no indication for a timely processing of the putative F_0 protein. In addition, steady state levels in infected or transfected cells revealed mainly uncleaved F_0. In contrast, steady state levels of F_0 and the large subunit F_1 appeared as to be expected in infected or transiently transfected Vero-dogSLAM cells.

In summary, we showed that F_0 of PPRV Nigeria 75/1 is not cleaved efficiently in $Vero_{Montpellier}$ cells which coincides with the absence of detectable syncytium formation despite development of plaques after low MOI inoculation and a complete CPE around 6 days after infection with a MOI of 0.2 to 0.5. These results provide evidence that the inefficient processing of the PPRV fusion protein is beneficial for the productive replication of the PPRV Nigeria 75/1 vaccine strain in normal Vero cells. The molecular mechanism leading to processing hindrance in cells which apparently process type I glycoproteins of other viruses normally needs to be elucidated. Ovine Nectin-4 (PVRL4) cell receptor is responsible for PPRV entry into epithelial cells and when overexpressed permits efficient viral replication (Birch et al., 2013). Whether the observed phenotype - productive virus infection in absence of syncytia formation - is correlated to Nectin-4 expression mediated entry of PPRV Nigeria 75/1 grown on $Vero_{Montpellier}$ cells as has been described by Birch et al. (2013) for infection of sheep kidney epithelial cells with PPRV Ivory Coast 1989 strain needs to be elucidated.

Author's contribution

Osman N.A.: performed most of the laboratory work including cell culture work, RT-PCR, molecular cloning, DNA sequencing, induction of fusion proteins in E.coli, protein purification, preparation of the

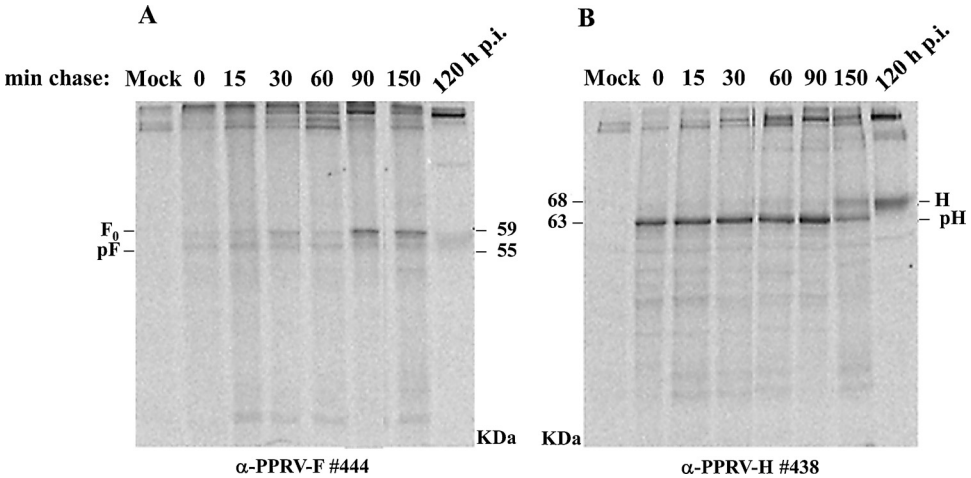

Fig. 5. Intracellular maturation and transport of PPRV-F and -H glycoproteins in $Vero_{Montpellier}$ cells infected by PPRV Nigeria 75/1 strain. $Vero_{Montpellier}$ cells in 24-well plates were infected with PPRV Nigeria 75/1 vaccine strain at a MOI of 0.1. At 36 h p.i. cell culture medium was replaced by 125 µl methionine-free culture medium supplemented with 1 µCi $^{[35]}$S-methionine per ml. Label was removed 1 h later and cells were washed twice and further incubated with normal cell culture medium for 0 min (lane 2), 15 min (lane 3), 30 min (lane 4), 60 min (lane 5), 90 min (lane 6) 150 min (lane 7). As controls, noninfected cells (Mock) were labeled for 1 h and chased for 150 min before lysis (lane 1) or infected cells labeled from 36 h p.i. until 120 h p.i. with 125 µCi $^{[35]}$S-methionine per ml (lane 8). Cell lysates were incubated with α-PPRV-F #444 (A) or α-PPRV-H #438 (B) and immunoprecipitated proteins were separated by SDS-10% PAGE. The positions of the putative F precursor (pF), the uncleaved fusion protein (F_0) and the precursor and mature form of the haemagglutinin (pH and H, respectively) are shown as well as the respective apparent molecular masses in kilo Dalton (kDa).

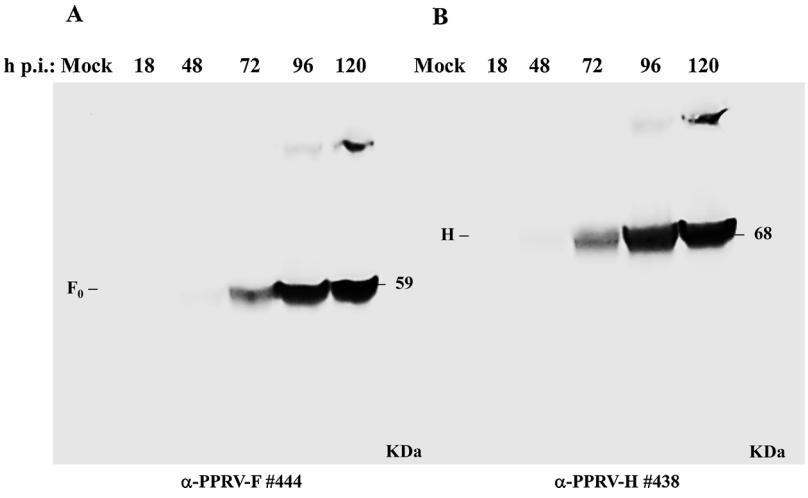

Fig. 6. Intracellular transport and cleavage of PPRV F_0 is inefficient after low MOI infection of $Vero_{Montpellier}$ cells. $Vero_{Montpellier}$ cells in 24-well plates were infected with PPRV Nigeria 75/1 vaccine strain at a MOI of 0.1 or left uninfected (lane 1: Mock). Cells were harvested at 18 h p.i. (lane 2), 48 h p.i. (lane 3), 72 h p.i. (lane 4), 96 h p.i. (lane 5), and 120 h p.i. (lane 6). Lysed-cell proteins were separated by SDS-10%PAGE and transferred to nitrocellulose membranes. Filters were probed with 1:5000 dilutions of the α-PPRV-F #444 (panel A) or the α-PPRV-H #438 (panel B) serum. The positions of F_0 of the fusion protein and the haemagglutinin (H) are shown on the left of the corresponding panel.

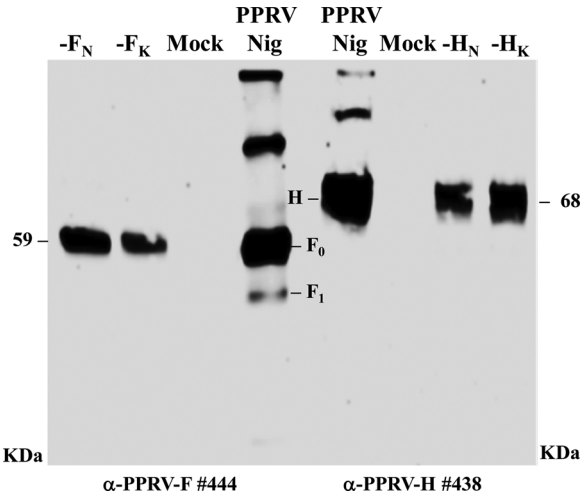

Fig. 7. Intracellular transport and cleavage of PPRV F_0 is inefficient after transfection of RK13 cells. $Vero_{Montpellier}$ cells in 24-well plates were infected with PPRV Nigeria 75/1 vaccine strain at a MOI of 0.1 for 5 days (lanes 4 and 5) or left uninfected (lanes 3 and 6). RK13 cells were transfected with pCAGGS-PPRV-F_N (lane 1), pCAGGS-PPRV-F_K (lane 2), pCAGGS-PPRV-H_N (lane 7), or pCAGGS-PPRV-H_K (lane 8) and harvested at 48 h after transfection. Lysed cell proteins were separated by SDS-10%PAGE and transferred to nitrocellulose membranes. Filters were probed with 1:5000 dilutions of α-PPRV-F #444 (panel A) or α-PPRV-H #438 (panel B) serum. The positions of F_0, F_1 and H are indicated.

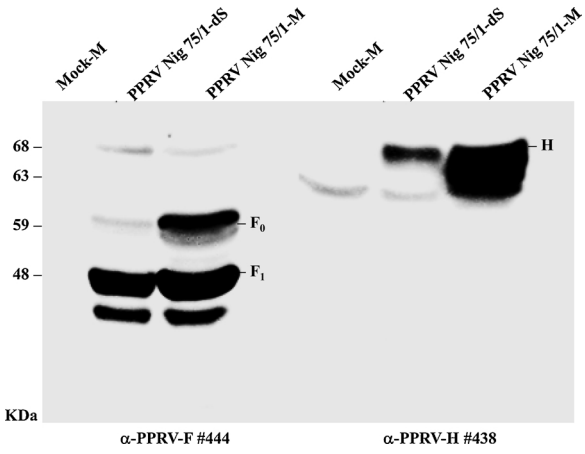

Fig. 8. Cleavage of F_0 is complete only in Vero-dogSLAM cells after high MOI infection. PPRV strain Nigeria 75/1 was used to infect Vero-dogSLAM (Nig75/1-dS) or $Vero_{Montpellier}$ (Nig75/1-M) cultures at a MOI of 10. Cells were harvested at 48 h p.i., lysed and PPRV F and H expression was analyzed by Western blotting using H- (α-PPRV-H #438) and F- (α-PPRV-F #444) monospecific antibodies.

rabbit antisera, DNA transfection, transient protein expression, indirect immunofluorescence, Pulse-chase metabolic labeling and radioimmunoprecipitation, SDS-PAGE and Western blotting, data analysis, results interpretation, prepared the drafted and the final manuscript; Portugal R.: contributed by capturing and preparing the confocal images; Giesow K.: helped with molecular cloning and fusion protein purification; Keil G.M.: was responsible for the design and supervision of this research project, performed viral infection of cells, virus titration and growth kinetics experiments, results interpretation, revised and finalized the manuscript.

Potential conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.05.012.

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