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Title

Expression and Immunogenicity of Secreted Forms of Bovine Ephemeral Fever Virus Glycoproteins Applied to Subunit Vaccine Development

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Running headline

Expression and immunogenicity of BEFV glycoproteins

Abstract

Aims: Vaccines for bovine ephemeral fever virus (BEFV) are available but are difficult to produce, expensive, or suffer from genetic instability. Therefore, we designed constructs encoding C-terminally truncated forms (transmembrane anchoring region deleted) of glycoproteins G and G_{NS} such that they were secreted from the cell into the media to achieve high-level antigen expression, correct glycosylation pattern, and enable further simple purification with the V5 epitope tag.

Methods and Results: In this study, synthetic biology was employed to create membrane-bound and secreted forms of G and G_{NS} glycoprotein. Mammalian cell culture was employed as an antigen expression platform, and the secreted forms of G and G_{NS} protein were easily purified from media by using a highly effective, single-step method. The V5 epitope tag was genetically

fused to the C-termini of the proteins, enabling detection of the antigen through immunoblotting and immunomicroscopy. Our data demonstrated that the C-terminally truncated form of the G glycoprotein was efficiently secreted from cells into the cell media. Moreover, the immunogenicity was confirmed in mice test.

Conclusions: The immuno-dot blots showed that the truncated G glycoprotein was present in the total cell extract, and was clearly secreted into the media, consistent with the western blotting data and live-cell images. Our strategy presented the expression of secreted, epitope-tagged, forms of the BEFV glycoproteins such that appropriately glycosylated forms of BEFV G protein was secreted from the BHK-21 cells. This indicates that high-level expression of secreted G glycoprotein is a feasible strategy for large-scale production of vaccines and improving vaccine efficacy.

Significance and Impact of the Study: The antigen expression strategy designed in this study can produce high-quality recombinant protein and reduce the amount of antigen used in the vaccine.

Keywords

Bovine ephemeral fever virus, G glycoprotein, Vaccin, Infectious diseases.

Introduction

Bovine ephemeral fever virus (BEFV) is an arthropod-borne negative-stranded RNA virus (genus *Ephemerovirus*, family Rhabdoviridae) (Murphy *et al*. 1972). Currently, the virus prevalent throughout Africa, the Middle East, the Indian Sub-continent, South and South-East Asia, the Russian Federation, Japan and Australia - but not, as yet, in America or Europe (except western parts of Turkey) (Walker and Klement 2015). Whilst unapparent infections have been reported in Cape buffalo, hartebeest, waterbuck, wildebeest, deer and goats, clinical disease is only observed in cattle and water buffalo (Liao *et al*. 1998; Li *et al*. 2015; Walker and Klement 2015). Symptoms include a bi-phasic fever, salivation, ocular / nasal discharge, anorexia, muscle stiffness,

recumbency and lameness. Typically, both the onset and recovery of disease is rapid (1-3 days), although sequelae of paralysis and ataxia have been reported (St George 2004). The economic impacts of BEFV mainly in cessation of lactation in dairy cattle, loss of condition in beef cattle and water buffalo (Walker 2005; Aziz-Boaron *et al*. 2014). BEFV is non-contagious, with some evidence of transmission by many species of biting midges and mosquitoes (Walker and Klement 2015).

Recent epizootics found in the Henan Province of China in 2004, 2005 and 2011 had case-fatality rates of ~18%, the causative virus strain being closely related to BEFV isolated from China, Taiwan and Japan (Zheng and Qiu 2012; Trinidad *et al*. 2014). In Taiwan, however, since 1967 there has been a clear trend towards more frequent epizootics with falling morbidity rates - but increasing case-fatality rates. Mortalities due to disease and culling exceeded 10% in most epizootics since 1989–1990, but reached 50% in 2002 (Wang *et al*. 2001; Hsieh *et al*. 2005; Hsieh *et al*. 2006; Ting *et al*. 2014).

Natural BEFV infection has been reported to result in durable immunity: a strong neutralising antibody response follows natural or experimental BEFV infection, developing by the third day of clinical disease with antibody titres increasing during recovery (Mackerras *et al*. 1940; St George 1985). Colostral antibody has also been shown to provide passive protection in cattle against BEFV infection (St George *et al*. 1986). Five types of vaccine against BEFV have been produced: (i) live-attenuated vaccines (developed by passage in suckling mice), (ii) inactivated vaccines (bulk growth in BHK-21 cells, β-propiolactone inactivation), (iii) subunit G protein-based vaccines (G protein detergent-released from particles) and (iv) recombinant vaccines (Neethling strain of lumpy skin disease virus expressing BEFV G protein) (v) plasmid DNA vaccine (encoding G1 epitope of BEFV) (Uren *et al*. 1994; Walker and Klement 2015; Pasandideh *et al*. 2018). These vaccines, however, suffer from either genetic instability, low efficacy, the need

for multiple vaccinations, or, are simply too expensive for farmers in low-middle income countries.

In the downstream of the virus glycoprotein (G), the genomes of *Ephemeroviruses* encode a non-structural glycoprotein gene (G_{NS}) followed by a series of 'accessory' proteins: in the case of BEFV comprising a viroporin (α1) and small proteins of unknown function (designated α2, α3, β and γ; Fig. 1A) (Walker *et al*. 1992; Joubert *et al*. 2014). BEFV G and G_{NS} glycoproteins play important roles in autophagy induction and cell entry (Cheng *et al*. 2015; Cheng *et al*. 2019). In this study, we cloned and expressed either full-length G / G_{NS} proteins (Fig. 1B), or, versions of these glycoproteins (Fig. 1C) with a C-terminal truncation (transmembrane anchoring / cytoplasmic tail domains deleted) so that these variants would be secreted from transfected cells into the tissue culture media. The V5 epitope tag, genetically fused to the C-termini of these proteins (Fig. 1), allowed detection of the antigen by immuno-blotting and immuno-microscopy. This tag will also enable purification of the expressed truncated G antigen from the cell media. Furthermore, we created a genetic fusion of each of the virus glycoproteins with green fluorescent protein (GFP) linked *via* a 2A 'ribosome skipping' sequence to create a 'self-processing' polyprotein (de Felipe and Ryan 2004; de Felipe *et al*. 2006) encoding a GFP-2A-BEFV glycoprotein (Fig. 2).

Our strategy was to use mammalian cell culture as antigen expression platform, since post-translational modifications in these cells produces the correct glycosylation pattern. We designed constructs encoding C-terminally truncated forms (transmembrane anchoring region deleted) of these BEFV glycoproteins such that they would be secreted from the cell into the media to achieve high-level antigen expression and improving vaccine efficacy.

Materials and Methods

Polymerase chain reaction (PCR)

The transmembrane domains of the BEFV G and G_{NS} glycoproteins were predicted using the TMHMM 2.0 algorithm (http://www.cbs.dtu.dk/services/TMHMM/). The full-length BEFV G gene, and the C-terminally truncated form of the gene, was PCR-amplified from a full-length cDNA copy of the BEFV genome (pHCW1; manuscript in preparation) based upon the sequence of the isolated Bovine/China/Henan1/2012 (Zheng and Qiu 2012). The nucleotide sequences of the primers (IDT Technologies, Leuven, Belgium), together with predicted sizes of the PCR products, are shown in Table 1. The longer primers were designed to be complementary to the C-terminal regions of the BEFV glycoproteins, but also needed to encode the TEV cleavage site and the V5 epitope tag, as appropriate (see below). Such primers were used directly as supplied by the manufacturer - in all cases using a 'standard' PCR protocol (described below). The binding sites of all primers are shown in Fig. 1B and primer sequences in Table 1. The full-length form of BEFV G was amplified from pHCW1 using oligonucleotide primers LisaG-For and pLisa1-V5_rev. The C-terminally truncated form was amplified from pHCW1 using primers LisaG-For and LisaG-Rev2. The full-length form of BEFV G_{NS} was PCR-amplified from pHCW1 using primers LisaG_{NS}-For and pLisa2-V5_rev. The C-terminally truncated form of the gene was amplified from pHCW1 using primers LisaG_{NS}-For and LisaG_{NS}-Rev2.

Reverse primers used to construct both the full-length and C-terminally truncated forms of the BEFV G and G_{NS} proteins encode a C-terminal extension of (i) a Tobacco Etch Virus proteinase cleavage site (-ENLYFQG-), (ii) a short linker sequence (-GSDQTENSG-) and (iii) the V5 epitope tag (-GKPIPNPLLGLDST-$_{COOH}$). PCR reactions were performed in a final volume of 50 µl: GoTaq® 10× Reaction Buffer (contains 15 mmol l^{-1} $MgCl_2$ for a final concentration of 1.5 mmol l^{-1} in a 1× reaction), 0.2 mmol l^{-1} each dNTP, 0.2 µmol l^{-1} of forward and reverse primers, plasmid pHCW1 template DNA (50 ng), 1.25 units of GoTaq® DNA Polymerase (Promega, Southampton, UK) and nuclease-free water to 50 µl. The three-step PCR-cycle: denaturation

(94°C for 30 s), annealing (55°C for 30 s), and elongation (71°C for 1 min) was repeated 25 times.

Construction of plasmids

PCR products corresponding to the full-length and the truncated forms of the BEFV G and G_{NS} genes were purified and initially cloned into the vector pGEM-T Easy (Promega, Southampton, UK) as per the manufacturer's instructions. Restriction enzyme fragments and PCR products were resolved by 1% agarose gel electrophoresis and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Southampton, UK) as per the manufacturer's instructions. All clones were verified by automated Sanger sequencing (GATC Biotech, Cambridge, UK).

The plasmids containing the encoding sequence full-length and truncated forms of BEFV-G and BEFV-G_{NS} were extracted and purified using the E.Z.N.A. ® Plasmid Mini Kit II (Omega Bio-tek, GA, USA) according to the manufacturer's instructions. The pJC3 expression vector (Luke and Ryan 2013; Minskaia *et al*. 2013) was pcDNA3.1-based with *Thosea asigna* virus 2A (T2A) linking GFP and monomeric cherry fluorescent protein (mCherryFP) genes (Fig. 2). Sequences encoding mCherryFP were excised by restriction with *Apa*I and *Pst*I (New England Biolabs, Hitchin, UK). Sequences encoding full-length and truncated forms of BEFV-G and BEFV-G_{NS} were similarly excised from pGEM-T Easy and ligated into the pJC3 restriction fragment (Fig. 2). This created clones encoding either the full-length BEFV glycoproteins (pLisa1V5 - encodes [GFP-2A-BEFV-G]; pLisa2V5 - encodes [GFP-2A-BEFV-G_{NS}], or, the truncated forms of the glycoproteins (pLisa3V5 - encodes [GFP-2A-BEFV-Gtrunc]; pLisa4V5 - encodes [GFP-2A-BEFV-G_{NS}trunc]). All clones were verified by automated Sanger sequencing (GATC Biotech, Cambridge, UK).

Cells and virus

Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection (ATCC, Teddington, UK) and propagated in Dulbecco's Modified Eagle's Medium (DMEM;

Sigma-Aldrich Ltd, Dorset, UK) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco™, Loughborough, UK), incubated at 37ºC in 5% CO_2. To aid in the differentiation between transfected and non-transfected cells, BHK-21 cells stably expressing a nuclear-restricted red fluorescent protein (mKate2; RFP), were generated. 6-well plates were seeded and left to adhere for 24 h. Cell monolayers were infected with IncuCyte NucLight Lentivirus Reagent (Essen BioScience, Welwyn Garden City, UK) at a MOI of 3 and left to incubate for 24 h. Following incubation, media was removed and replaced with fresh growth media before incubating for an additional 24 h. RFP expression was monitored sporadically during this incubation step using the EVOS® FL Imaging System (Thermo Fisher Scientific, Basingstoke, UK). To select for cells expressing RFP, media was removed and replaced with fresh media containing 0.4 μg ml^{-1} Zeocin (Invitrogen™, Loughborough, UK). Cells were incubated for a total of 72 h with the media replaced every 48 h. Successfully transformed cells (designated BHK-21_RFPNuc) were maintained in selection media to preserve a stable population.

For BEFV propagation, virus was cultured in BHK-21 cells (Cheng *et al*. 2019). BEF virus titers were determined by the Reed and Muench method (1938) and expressed as $TCID_{50}$ ml^{-1}. Briefly, viruses were serially ten-fold diluted and applied to wells containing 2×10^4 cells $well^{-1}$ in 96-well plates. The virus stocks were titrated as a positive control while DMEM served as a negative control. The plates were incubated at 37°C, 5% CO_2 for at least three days and observed daily for cytopathic effect.

IncuCyte microscopy

For live-cell imaging experiments, plasmid DNA (0.75 μg) was transfected into BHK-21_RFPNuc cell monolayers (1×10^5 cells $well^{-1}$ of a 12-well plate: 70–80% confluent) using Lipofectamine™ 2000 (Invitrogen™, Loughborough, UK), as per the manufacturer's instructions. The images of transfected cells were captured at 4 h intervals between 0–64 h post-transfection using an IncuCyte

ZOOM kinetic live-cell imaging system (Essen BioScience, Welwyn Garden City, UK) housed within an incubator maintained at 37°C and 5% CO_2. Images were captured from 9 regions per well in a 12-well plate using the 10× objective. The average GFP intensity per well was measured using the IncuCyte ZOOM image processing software (Tulloch *et al*. 2014). The values were pooled from all nine regions and averaged across three replicates.

Detection and purification of secreted protein

The media and cell extract were collected at time points 24, 36, 48 and 60 h post-transfection of BHK-21 cells. Cell culture media was removed from each well then clarified by centrifugation (2000 *g*, at 4°C for 15 min) and the supernatant decanted to a new tube before storing at –20°C. Supernatants were thawed on ice before spotting onto nitrocellulose blotting membranes (pore size 0.2 μm; Amersham™ Hybond™ and Amersham Protran™, GE Healthcare). Membranes were then air-dried at room temperature for 15 min prior to image analysis using the Odyssey® CLx Imaging System (CI-COR® Biosciences, Cambridge, UK). Analyzed by dot blot using a monoclonal antibody against the very widely used V5 epitope tag (1:1000; primary antibody) (kind gift from Professor Richard E. Randall; also commercially available from a number of companies) and IRDye® 680RD goat anti-mouse secondary antibody (1:5000; LI-COR Bioscience Ltd, Cambridge, UK).

To purify the truncated form of BEFV G, the media were collected at time point 48 h post-transfection of BHK-21 cells. The clarified supernatant was applied to a V5-tagged Protein Purification Kit Ver.2 (MBL, Japan) according to the manufacturer's protocol. The eluted protein fractions containing truncated form of BEFV G were pooled and concentrated using an Amicon® ultra centrifugal filter with a pore size of 30 kDa (Merck Millipore, Burlington, MA). The protein concentration was determined by a Pierce™ 660 nm protein assay (Thermo Fisher Scientific, Basingstoke, UK).

Cell-associated protein: cell extract preparation

At 24, 36, 48 and 60 h post-transfection of BHK-21 cells, the cell culture media was removed and each well washed once with ice-cold phosphate-buffered saline (PBS). Cells were harvested using a cell scraper in 1 ml PBS per well. Samples were centrifuged at 2000 *g*, 4°C for 5 min to pellet cells. The supernatant was removed and the pellet re-suspended in 100 µl RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Basingstoke, UK) supplemented with a mixture of protease inhibitors (cOmplete™, EDTA-free Protease Inhibitor Cocktail Tablets; Roche Diagnostics Ltd, Burgess Hill, UK), as per the manufacturer's instructions. Samples were left on ice for 25–30 min and briefly vortexed every 5 min before centrifugation (13000 *g*, 4°C for 15 min). The supernatant was transferred to a new microfuge tube before storing at –20°C.

Near-infrared fluorescent western blotting

Proteins were resolved using precast gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 4–20%; Expedeon Ltd, Cambridge, UK) and run at a constant voltage (120 V). Proteins resolved by SDS-PAGE were transferred to nitrocellulose membranes at 20 V for 7 min using an iBlot® Gel Transfer Device (Invitrogen™, Loughborough, UK) as described by the manufacturer. Membranes were washed in PBS for 2 min and blocked for 1 h at room temperature in Odyssey® PBS blocking buffer (CI-COR®, Cambridge, UK) diluted 1:1 in PBS. Membranes were probed with mouse anti-V5 and rabbit anti-GAPDH (2B Scientific, Oxfordshire, UK) antibodies, with each antibody diluted 1:1000 in Odyssey PBS blocking buffer. Membranes were incubated overnight at 4°C with gentle shaking. The following day, membranes were washed 3× 5 min in PBS-T (PBS, 0.1% [v/v] Tween-20), and the primary antibodies detected with IRDye® 800CW Goat anti-Rabbit IgG and IRDye® 680RD Goat anti-Mouse IgG (1:5000 dilution in Odyssey PBS Blocking Buffer). Membranes were incubated in the secondary antibody for 1 h at room temperature with gentle shaking in the dark. Membranes (still kept in

darkness) were then washed 3× 5 min in PBS-T. Finally, membranes were rinsed with PBS to remove residual Tween-20 and imaged with an Odyssey® CLx Imager (auto-scan settings, 700 and 800 nm channels).

Cell localisation using DeltaVision microscopy

BHK-21 cells were fixed using the protocol described in Minskaia *et al*. 2013. Briefly, the media was aspirated, coverslips were washed twice with 1 ml of PBS, fixed with 4% paraformaldehyde for 10 min and subsequently washed twice with deionized water, before mounting using VECTASHIELD® mounting medium with DAPI (Vector Laboratories Ltd, Peterborough, UK). Images were obtained using a DeltaVision microscope (Applied Precision, Marlborough, UK) fitted with a 60× oil-emersion objective Olympus lens and a Photometric CH300 CCD camera. Fluorescence was detected with an excitation wavelength of 488 nm for GFP (FITC filter), 587nm for RFP (TRITC filter) and 350 nm for DAPI (DAPI filter). Images were acquired under oil immersion using the 60× lens and analysed using the softWoRx® Resolve 3D software package (Applied Precision, Marlborough, UK). Experiments were done in triplicates.

Immunization and sample collection

Vaccine preparation: purified truncated form of BEFV G was formulated with the oil adjuvant ISA 206 (SEPPIC, Paris, France) at the suggested ratios, and final concentration of truncated form of BEFV G in each dose was 4 or 0.4 μg.

Fifteen 4-week-old BALB/c mice (LASCO, Taiwan) were randomly assigned to three groups (five mice each), and 200 μl of each vaccine was injected via the intramuscularly route. The mice were immunized twice (on day 0 and 14) and boosted with the same vaccine, the mice injected with PBS-ISA 206 served as the control group. Serum samples were collected on day 28 after the primary immunization to determine the immunogenicity of recombinant protein through immune-dot blot analysis. The animal trial protocols were approved by the Animal Care and Uses

Committee of the National Pingtung University of Science and Technology (NPUST). The experiments were conducted according to the Ethical Rules and Laws of NPUST.

Antibody analysis

To determine the immunogenicity of recombinant protein, supernatant of BEFV-infected BHK-21 cells was two-fold serially diluted with PBS and blotted onto nitrocellulose membrane by suction through the dot-blotting equipment GFE9600 (Bio-East Technology, Taiwan). For negative control, supernatant of BHK-21 cells was used. After washing with PBS-T the membranes were blocked using Hyblock Blocking Buffer (GOALBIO, Taiwan) for 1 min at room temperature. Thereafter, 1:1000 diluted mouse serum samples or rabbit anti-BEFV antiserum (served as a positive control; kind gift from Dr. Hsian-Yu Wang) (Cheng *et al*. 2019) was added, and the membranes were incubated for 1 h at 37°C. After washing, HRP-conjugated goat anti-mouse IgG (1:5000) or HRP-conjugated goat anti-rabbit IgG (1: 10,000) (KPL, Gaithersburg, MD) was added to membranes, and the membranes were incubated for 1 h at 37°C. The membrane was then washed again before fluorescence signal development using the Immobilon® Crescendo Western HRP Substrate (MILLPORE, MA, USA).

Results

Molecular cloning of full-length and truncated forms of BEFV G and BEFV G_{NS} glycoproteins

PCR amplification of the BEFV glycoproteins produced products of the predicted sizes (Table 1). The PCR products were initially cloned into the pGEM-T Easy vector system and verified by automated Sanger sequencing before being excised (using *Apa*I and *Pst*I) from this vector and being transferred to the pJC3-based expression system - similarly digested to remove sequences encoding mCherryFP (Fig. 2). The single open reading frame (ORF) was preserved such that each clone encoded a 'polyprotein' comprising [GFP-T2A-BEFV Glycoprotein].

Evaluation of the expression of full-length and truncated forms of BEFV G and BEFV G_{NS} glycoproteins using live-cell imaging

Live-cell expression studies were conducted in BHK-21 cells stably expressing RFP targeted to the nucleus (BHK-21_RFPNuc), such that cells transfected with the various pLisa constructs (fluorescing green) could be differentiated from non-transfected cells (fluorescing red alone) to monitor and compare cell transfection efficiencies for each of the constructs (Fig. 3). BHK-21_RFPNuc cells were transfected with plasmids pLisa1 (full-length BEFV G), pLisa2 (full-length BEFV G_{NS}), pLisa3 (truncated BEFV G) and pLisa4 (truncated BEFV G_{NS}) and GFP expression monitored at 4 h intervals between 0–64 h post-transfection. The red object count showed that the mock-transfected, pLisa1 and pLisa3 transfected cells showed a gradual decline throughout the course of the experiment (Fig. 3, Panel A1). Calculation of the total integrated GFP fluorescence intensity showed the typical pattern of transient expression. Maximal expression (GFP fluorescence) of the full-length G protein (pLisa1) occurred at ~28 h, whereas maximal expression (GFP fluorescence) of the truncated G protein (pLisa3) occurred at ~40 h. The truncated form was expressed at ~2 fold higher than the full-length form (Fig. 3, Panel A2). The data from pLisa2 and pLisa4-transfected BHK-21_RFPNuc cells showed similar kinetics of that of pLisa1 and pLisa3 transfected cells (Fig. 3, Panel B1). In the case of the full-length G_{NS} protein (pLisa2), maximal expression (GFP fluorescence) occurred at ~50 h, whereas maximal expression (GFP fluorescence) of the truncated G_{NS} protein (pLisa4) occurred at ~60 h. Again, the truncated form was expressed at ~1.8 fold higher than the full-length form (Fig. 3, Panel B2).

Evaluation of the expression of full length and truncated forms of BEFV G and BEFV G_{NS} glycoproteins using immuno-dot blots

To determine if the C-terminally truncated forms of the BEFV G and G_{NS} proteins (lacking the transmembrane anchoring domain) were secreted into the cell culture media, immuno-dot blot

assays were performed on the media and cell extracts using a monoclonal antibody raised against the V5 epitope tag, present at the C-terminus of each of the BEFV protein constructs. The immuno-dot blots showed that the truncated G glycoprotein (encoded by pLisa3) was present in the total cell extract, but was clearly secreted into the media. This secreted form of the G protein appeared to be stable in the cell media for the duration of the experiment (60 h), consistent with the western blotting data described below. In contrast, the truncated form of the G_{NS} protein (encoded by pLisa4), was also present in the cell extracts, but was not secreted into the media: the faint signal observed we attribute to low-level cell lysis releasing G_{NS} from the cell into the media (Fig. 4). Immuno-dot blot testing of the full-length BEFV G and G_{NS} proteins did not show any secretion to the media (data not shown).

Evaluation of the expression of truncated forms of BEFV G and BEFV G_{NS} glycoproteins using western blot analysis

Western blotting analyses (24, 36, 48 and 60 h post-transfection) of transfected BHK-21 cell extracts showed the maximal expression of the truncated G protein at ~48 h (Fig. 5), consistent with our GFP fluorescence analyses (Fig. 3, Panel A2). The protein band was diffuse, associated with different stages of protein glycosylation. Analysis of the truncated G_{NS} transfected cells showed maximal expression between 48 and 54 h in agreement with our live-cell imaging.

Sub-cellular localisation of full-length and truncated G / G_{NS} proteins

The DeltaVision permits the use of multiple filters and the observation of micro-anatomical details. The truncated G and G_{NS} proteins were located within the exocytic pathway (Fig. 6B and 6D) with full-length G also located within the plasma membrane (Fig. 6A). Similarly, cells expressing full-length forms of both G and G_{NS} proteins were also located within the exocytic pathway (Fig. 6A and 6C). In cells transfected with the control plasmid, pJC3, mCherry and GFP were diffuse throughout, in contrast to cells transfected with the plasmids generated in this study

(Fig. 6E). Additionally, cells transfected with full-length G (at the latter time-points post-transfection, ≥ 48 h) displayed a multi-nuclear phenotype which suggested cell fusion, a feature common with G proteins from other RNA viruses (Fig. 7) (Roche and Gaudin 2002; Roche *et al*. 2007; Roche *et al*. 2008).

Evaluation of the immunogenicity of truncated form of BEFV G using immune-dot blots

In order to test the immunogenicity of truncated form of BEFV G, mice were immunized with 4 or 0.4 μg truncated form of BEFV G and the antiserum produced was evaluated by immune-dot blot. Results showed that the antiserum which immunized with 4 μg truncated form of BEFV G can recognize BEFV particle, clear signals can be observed. Moreover, background signals from BHK-21 cell culture were minimal (Fig. 8).

Discussion

In this study we have designed a strategy of antigen expression that can be readily adapted for new outbreak strains of BEFV, and for closely related viruses (e.g. Kimberley virus, Berrimah virus, Kotonkan virus, Adelaide River virus and Obodhiang virus) (Walker and Klement 2015). To aid our expression studies, we created a genetic fusion of each of the viral glycoproteins with GFP linked *via* a 2A 'ribosome skipping' sequence to create a 'self-processing' polyprotein encoded by a single ORF (encoding a [GFP-2A-BEFV glycoprotein]), such that upon translation within transfected cells the individual translation products were predicted to be [GFP-2A] and the specific form of the BEFV glycoprotein in question. It has been previously demonstrated that this strategy does not impair the function of signal peptide sequences downstream of 2A (de Felipe and Ryan 2004; de Felipe *et al*. 2006). This strategy enables (i) identification of transfected cells by GFP fluorescence, (ii) real-time, live-cell, quantification of expression by measuring GFP fluorescence signal intensities and (iii) subsequent fluorescence activated cell sorting of stably-transfected cells.

Our strategy was to use mammalian cell culture as our antigen expression platform, since post-translational modifications in these cells produces the correct glycosylation pattern: (i) carbohydrate and glycopeptidic epitopes are vital for the correct antigenic structure of the protein and (ii) exposure of peptidic epitopes of glycoproteins is also frequently modulated by glycosylation (Zhu 2012). Our data showed that the full-length V5 epitope tagged G and G_{NS} glycoproteins (bearing transmembrane anchor sequences) were detected in the exocytic pathway and the plasma membrane (Fig. 6A and 6C), consistent with our observation that transfected cells could fuse with adjacent cells (Fig. 7), as previously reported for the glycoprotein of rabies and vesicular stomatitis virus (Roche and Gaudin 2002; Roche *et al*. 2007; Roche *et al*. 2008). This type of direct cell-to-cell transmission, without the release of particles, enables BEFV to transmit from cell-to-cell without being exposed to circulating antibodies (Wikel 1999). In contrast, this cell fusion was not observed in the case of cells expressing the BEFV G_{NS} glycoprotein (data not shown).

However, expression of the C-terminally truncated forms produced contrasting data. The truncated BEFV G glycoprotein was detected throughout the exocytic pathway, but our immuno-blotting data showed this truncated form of the G glycoprotein was also secreted into the tissue-culture media (Fig. 4). The western blot analyses showed a 'diffuse' banding pattern (rather than a discrete band), characteristic of glycosylation (Fig. 5A). Our immuno-blotting data showed the full length form of the G_{NS} glycoprotein remained cell-associated. Unexpectedly, however, the immuno-blotting data showed that the truncated form of the BEFV G_{NS} protein was not secreted into the media, but also remained cell associated (Fig. 4). High resolution microscopy showing that truncated BEFV G_{NS} localised to the exocytic pathway, primarily the endoplasmic reticulum (Fig. 6D). Possibly this was due to simple mis-folding of the G_{NS} protein, preventing it's transit through the Golgi apparatus to the plasma membrane, or, in the case of the truncated form of the

G_{NS} protein, it's secretion from the cell. Our Western blot analyses of transfected cell extracts (using the anti-V5 monoclonal antibody) showed a discrete protein of the predicted molecular mass (66.8 kDa) was observed for the G_{NS} protein with no lower molecular mass breakdown products, suggesting the G_{NS} protein was not degraded. It is interesting to speculate that BEFV G_{NS} and the other 'accessory' proteins (α1, α2, α3, β and γ) may be required for, and function in, the replication and/or transmission by the arthropod vector.

The BEFV antigens we have expressed are virus glycoproteins. The effects of glycolsylation are broad: glycosylation may 'inactivate' peptidic epitopes, or, may be required for their reactivities with antibodies. Evidence is increasing that similar effects of glycosylation pertain to T-cell dependent cellular immune responses: glycosylated peptides can be bound and presented by MHC class I or II molecules and elicit glycopeptide-specific T-cell clones. Expression within mammalian cells produces compositionally more complex *N*-glycans containing terminal sialic acids, whilst other eukaryotic expression systems (e.g. insect cells) mostly produce simpler *N*-glycans with terminal mannose residues. The correct glycosylation of such antigens is, therefore, essential to elicit a protective immune response. The immunogenicity was confirmed in mice test which immunized with 4 μg truncated form of BEFV G glycoprotein can recognize BEFV particle (Fig. 8). Our strategy designed in this study can produce high-quality recombinant protein and reduce the amount of antigen used in the vaccine.

The experimental strategy presented in this paper describes the expression of secreted, epitope-tagged, forms of the BEFV glycoproteins such that appropriately glycosylated forms of BEFV G protein was secreted from the BHK-21 cells and may be purified by epitope tags by a simple procedure. At this stage in our program of research we wished to simply demonstrate the 'proof-of-principal' that the C-terminally truncated forms of BEFV G and G_{NS} glycoproteins (lacking the transmembrane anchoring domains) would be secreted from the cell into the medium.

Our immuno-dot blots showed that this certainly was the case - the full-length forms, bearing the anchoring domains, remained cell-associated. The next stage will modify these constructs to include selectable markers (e.g. dihydrofolate reductase; DHFR) that would allow for enhancement of expression, *via* forcing gene duplication using selection (increasing methotrexate) within Chinese hamster ovary (CHO) cells – where the yield is a critical factor. CHO produce correct glycosylation and are the most common mammalian cell line used for mass production of therapeutic / antigenic proteins.

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Author contribution

M.D.R. and C.Y.C. obtained financial support and designed the study. Y.T.L., F.T., H.C.W. and G.A.L. performed the experiments. Y.T.L. and F.T. analysed data. M.D.R. and H.C.W. wrote the paper. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1 The genome structure of BEFV and PCR amplification of regions encoding the G and G_{NS} glycoproteins. Coding regions are indicated as arrows (Panel A). The cloning strategy and oligonucleotide primers for genes encoding the full-length G and G_{NS} proteins (boxed areas) is shown, together with the signal peptide (grey shaded areas) and the transmembrane anchoring domains (blue shaded areas). The cloning strategy and oligonucleotide primers for genes encoding the C-terminally truncated (transmembrane anchoring domains deleted) forms of the G and G_{NS} proteins (boxed areas) are shown, together with the signal peptide (grey shaded areas) and the C-terminal V5 epitope tag (white boxes) (Panel B). The NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) was used to find the N-glycosylated proteins identified from full-length G and G_{NS} proteins. Sequences having N-glycosylation potential > 0.5 were considered as cut-off value (Panel C).

Figure 2 Cloning of BEFV proteins into expression vectors. Plasmid pJC3 encodes a [GFP-T2A-mCherryFP] polyprotein encoded by a single open reading frame. Transcription is driven by the human cytomegalovirus enhancer/promoter and the mRNA polyadenylated by the bovine growth hormone polyadenylation signal. Sequences encoding mCherryFP were excised by

restriction with *Apa*I and *Pst*I, then replaced by the various forms of BEFV virus proteins (excised with *Apa*I and *Pst*I from the corresponding pGEM-T Easy sub-clones of the individual PCR products) to create plasmids pLisa1 (encodes a [GFP-T2A-BEFVG] polyprotein), pLisa2 (encodes a [GFP-T2A-BEFVG_{NS}] polyprotein), pLisa3 (encodes a [GFP-T2A-BEFVG_{trunc}] polyprotein), and pLisa4 (encodes a [GFP-T2A-BEFV$G_{NS\text{-}trunc}$] polyprotein). In this manner we were able to quantify cell transfection, and monitor the expression of the BEFV glycoproteins - within live cells *via* GFP fluorescence.

Figure 3 Quantitation of GFP expression. BHK-21_RFPNuc cells stably expressing RFP in the nucleus were transfected and the kinetics of BEFV G and G_{NS} expression monitored by GFP fluorescence using an IncuCyte ZOOM fluorescent microscope. Fluorescence experiments performed after transfecting BHK-21_RFPNu cells with plasmid pLisa1 encoding BEFV full-length G protein and plasmid pLisa3 encoding BEFV truncated G protein, respectively (Panel A). Fluorescence experiments were also performed with plasmid pLisa2 encoding BEFV full-length G_{NS} protein and plasmid pLisa4 encoding BEFV truncated G_{NS} protein, respectively (Panel B). Cells were mock transfected using the identical transfection protocol (Opti-MEM / Lipofectamine™ 2000, as per the manufacturer's instructions) but omitting plasmid DNA. At the time point indicated, images were captured and the GFP fluorescence quantified for each plasmid construct over a 64 h period: data shown as the red object count/mm^2 (Panels A1 and B1) or as the total integrated GFP fluorescence intensity (Panels A2 and B2). The total Green Integrated Intensity (GCU x µm2/Image: Y-axis) represents the total sum of the objects' fluorescent intensity in the image and is plotted against time post-transfection (X-axis). Data points/error bars shown are derived from two independent transfections, with four replicates for each transfection. ( ) pLisa1; ( ) pLisa2; ( ) pLisa3; ( ) pLisa4; ( ) Mock.

Figure 4 Immuno-dot blot analyses of cell media and cell extracts. Tissue-culture media (M)

and cell extracts (C) from BHK-21 cells transfected with truncated G (pLisa3) and G_{NS} (pLisa4) proteins at the time points indicated; analyzed by dot blot using a monoclonal antibody against the V5 epitope tag (1:1000; primary antibody) and IRDye 680RD goat anti-mouse secondary antibody (1:5000) to check whether truncated G and G_{NS} proteins were secreted. Mock was untransfected BHK-21 cells.

Figure 5 Western blot analyses of transfected cell extracts. Samples of cellular extracts were prepared and analysed (4-20% SDS-PAGE) prior to the transfer as described above. Transfer membranes were probed using the mouse anti-V5 monclonal antibody (1:1000) to detect cellular expression products: truncated G (Panel A) and truncated G_{NS} (Panel B) – both red, together with anti-GAPDH antibodies (1:1000) to provide a loading control (green). Lane M: prestained protein marker, lane mock: untransfected BHK-21 cells.

Figure 6 Sub-cellular protein localisation: high-resolution microscopy. BHK-21 cell monolayers were formed on glass coverslips and transfected with 750 ng of plasmid DNA within 24-well plates. At 24 and 48 h (data not shown) post-transfection cells were fixed, blocked and probed using a mouse primary anti-V5 antibody and subsequently a goat anti-mouse Texas Red labelled secondary antibody, as described above. Coverslips were imaged under oil immersion using the 60× lens. Both proteins were predominantly located within the exocytic pathway with full-length G also located within the plasma membrane (denoted by white arrow, Panel A). Plasmid pJC3 was used as a control demonstrating diffuse expression of mCherry and GFP throughout the cell (Panel E). Images represent one well from triplicate transfections. Scale bar represent 0.5 μm.

Figure 7 High resolution convoluted microscopy of BHK-21 cells transfected with pLisa1 expressing full-length G protein. Cell monolayers within 24-well plates were transfected with of pLisa1 plasmid DNA (750 ng) and harvested at 48 h post-transfection. Coverslips were fixed,

blocked and probed with the mouse primary anti-V5 antibody and the goat anti-mouse Texas Red labelled secondary antibody. G protein was located within the exocytic pathway as shown in the main body of the text. Here we observed the appearance of multi-nucleated cells, quite possibly due to cell-cell fusions of adjacent transfected cells - mediated by the cell surface localization of the BEFV G protein. Another observation was multinuclear cell localisation, likely due to cell fusion performed by the G protein. Data represents one well from triplicate transfections. Scale bar represents 0.5 μm.

Figure 8 Antibody analysis using Dot blot assays. Dot blot assays were set up with raised mouse anti-truncated form BEFV G or rabbit anti-BEFV antiserum as primary antibodies. Two-fold serial dilutions of BEFV samples, or BHK-21 cell supernatant, were applied for antibody detection.

Table 1 Oligonucleotide primers used to amplify genes encoding the G and G_{NS} proteins

Oligonucleotide primer	Nucleotide sequence (5' to 3')[a]	Position	Predicted PCR product size (bp)
LisaG-For	GCGCGC**GGATCCGGGCCC**ATGTTCAGGGTCCTAATAATTACCTTATTAGTCAATGG	1-38	
pLisa1-V5_rev	CTGGATAT**CTGCAG**TCAGGTGCTATCCAGGCCCAGCAGCGGGTTCGGAATCGGTTTGCCgccgctg ttctcggtctggtcgctgccGCCCTGAAAATACAGGTTTTCATGATCAAAGAACCTATCATCACCGATTGGT TTACTCCC	1831-1869	1994[b]
LisaG-Rev2	GCGCGCGC**CTGCAG**TCAGGTGCTATCCAGGCCCAGCAGCGGGTTCGGAATCGGTTTGCCgccgct gttctcggtctggtcgctgccGCCCTGAAAATACAGGTTTTCTAAACCAACCTACAACAGCAGATAAAACC TTTTTCCCTCC	1552-1590	1715[c]
$LisaG_{NS}$-For	GCGCGC**GGGCCC**ATGTTCCTGCAACTCTTTAACCTCATATTAGTG	1-33	
pLisa2-V5_rev	CTGGATAT**CTGCAG**TCAGGTGCTATCCAGGCCCAGCAGCGGGTTCGGAATCGGTTTGCCgccgctg ttctcggtctggtcgctgccGCCCTGAAAATACAGGTTTTCATAATCTAACTTAAAGAATCTTTGTACTCTG TG	1726-1758	1877[d]

LisaG$_{NS}$-Rev2	GCGCGCGC**CTGCAG**TCAGGTGCTATCCAGGCCCAGCAGCGGGTTCGGAATCGGTTTGCCgccgct gttctcggtctggtcgctgccGCCCTGAAAATACAGGTTTTCCCAATATTCCTTATTCTGTATGGTTTGTTTC TC	1588-1620	1739[e]

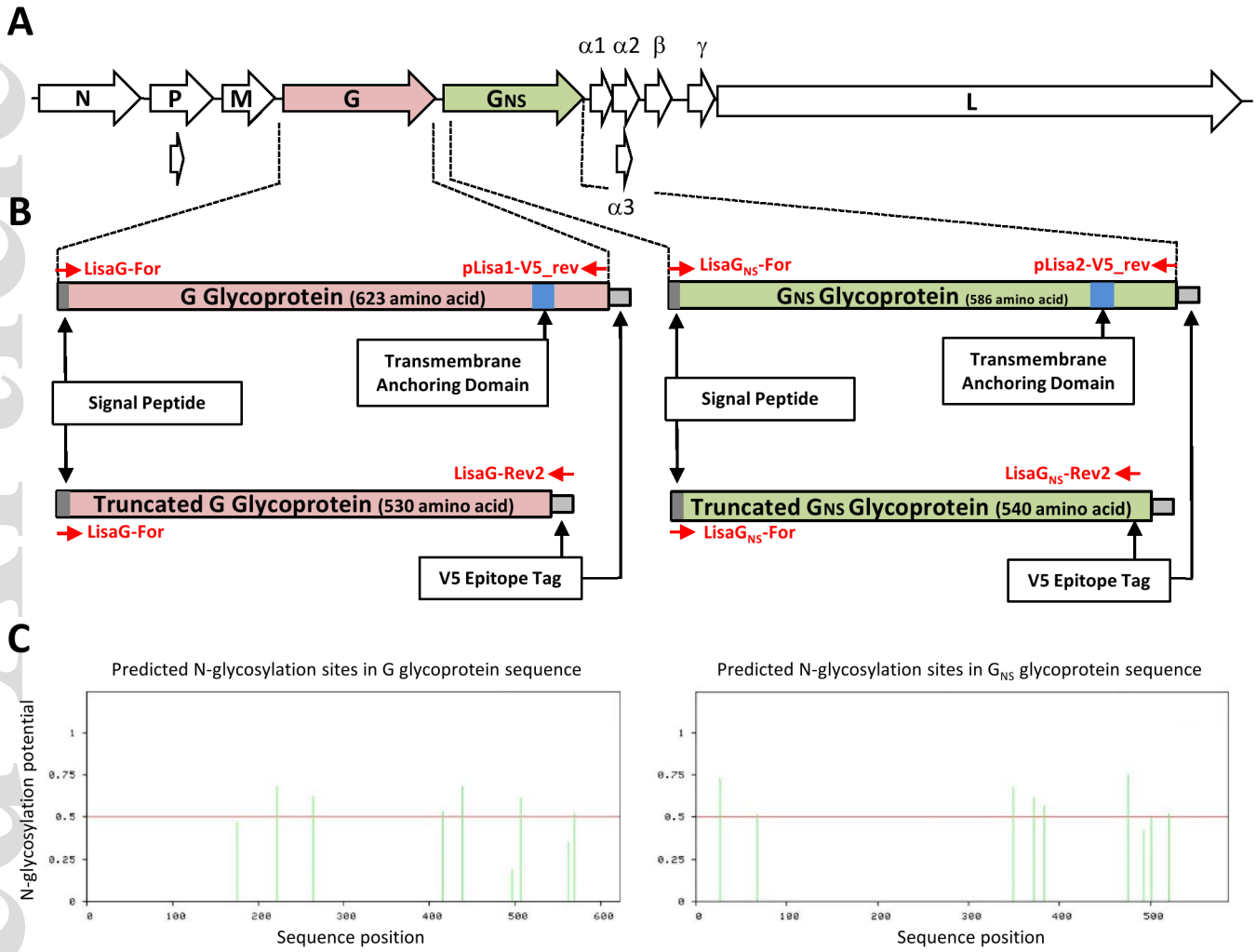
[a] Sequences which indicate a V5 epitope tag, a linker or a TEV cleavage site are underlined, lower case or boxed, respectively. Sequences represent various restriction sites are in bold; GGATCC (*Bam*H Ⅰ), GGGCCC (*Apa* Ⅰ), and CTGCAG (*Pst* Ⅰ).

[b] The primers LisaG-For and pLisa1-V5_rev for amplification of full-length BEFV G.

[c] The primers LisaG-For and LisaG-Rev2 for amplification of truncated BEFV G.

[d] The primers LisaG$_{NS}$-For and pLisa2-V5_rev for amplification of full-length BEFV G$_{NS}$.

[e] The primers LisaG$_{NS}$-For and LisaG$_{NS}$-Rev2 for amplification of truncated BEFV G$_{NS}$.



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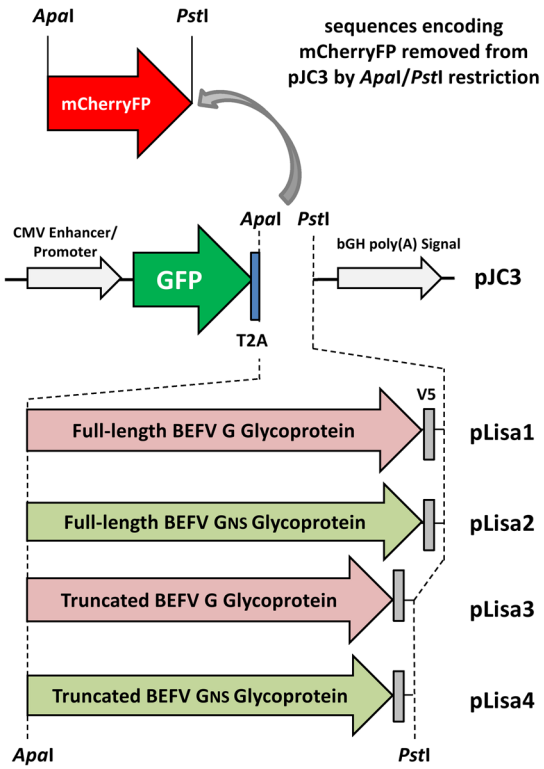

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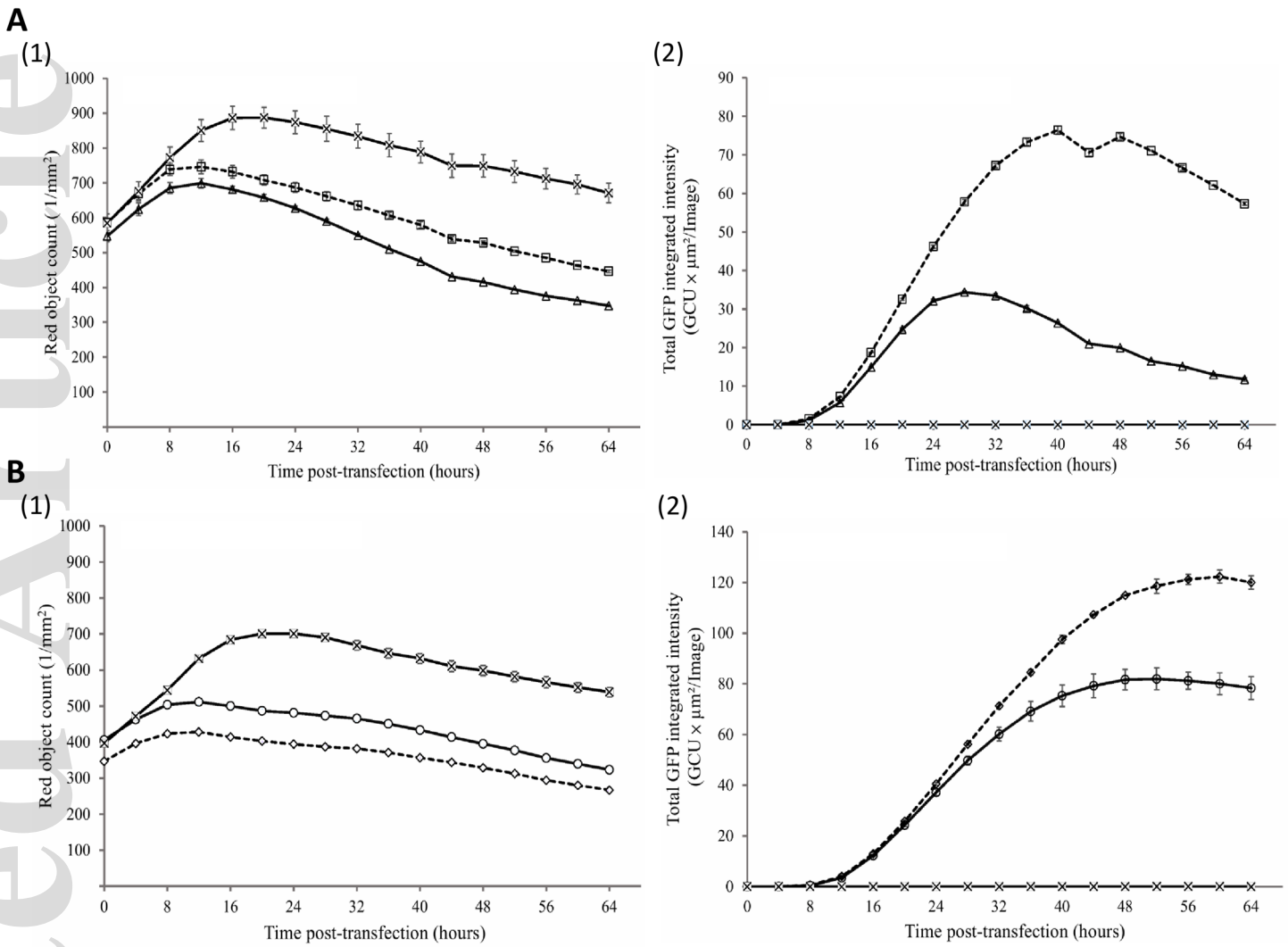

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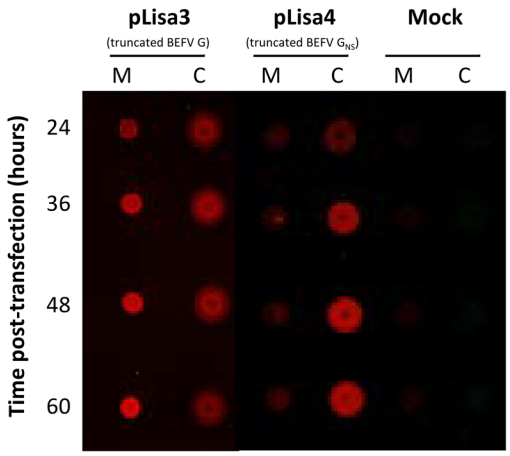

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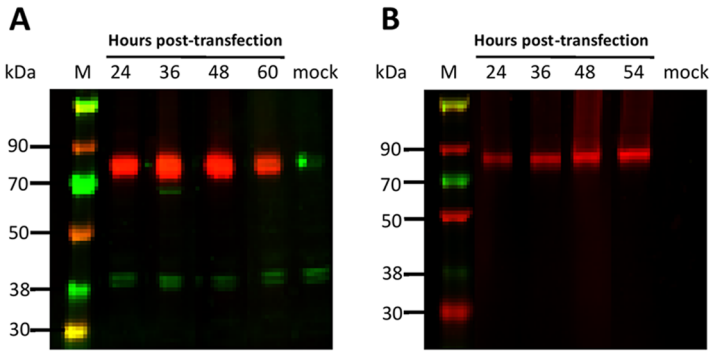

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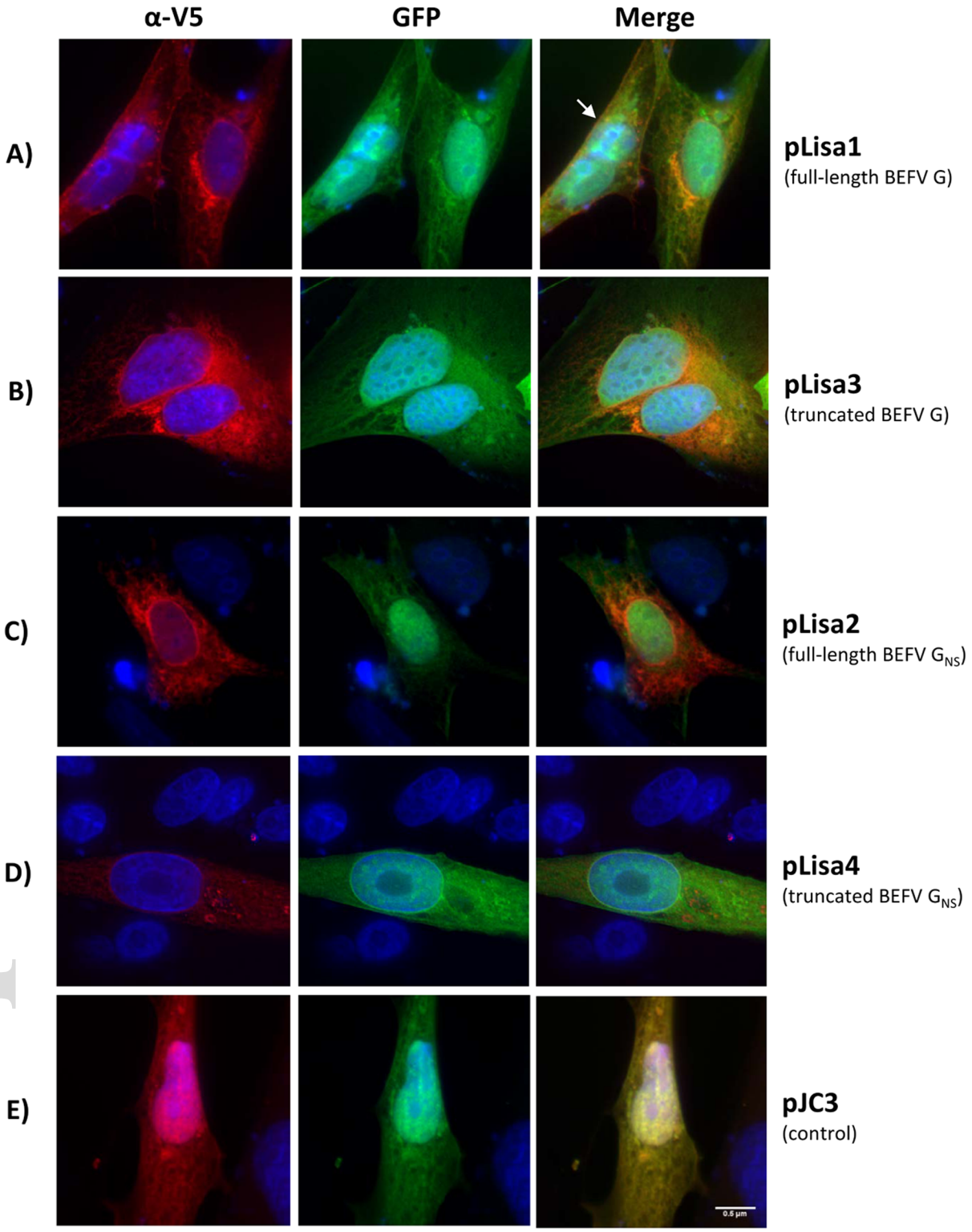

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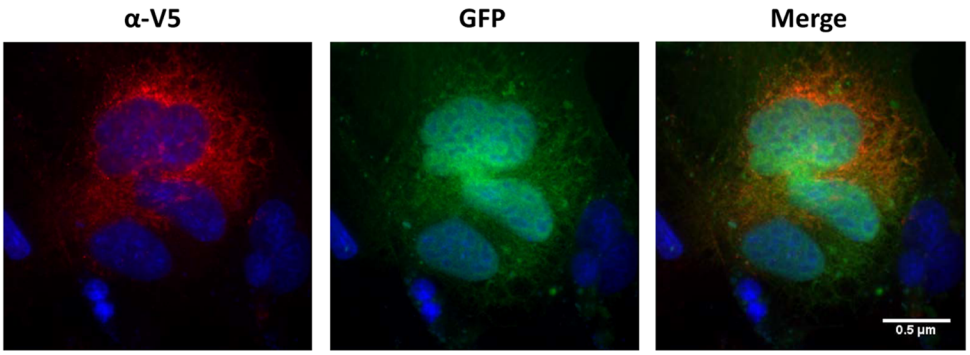

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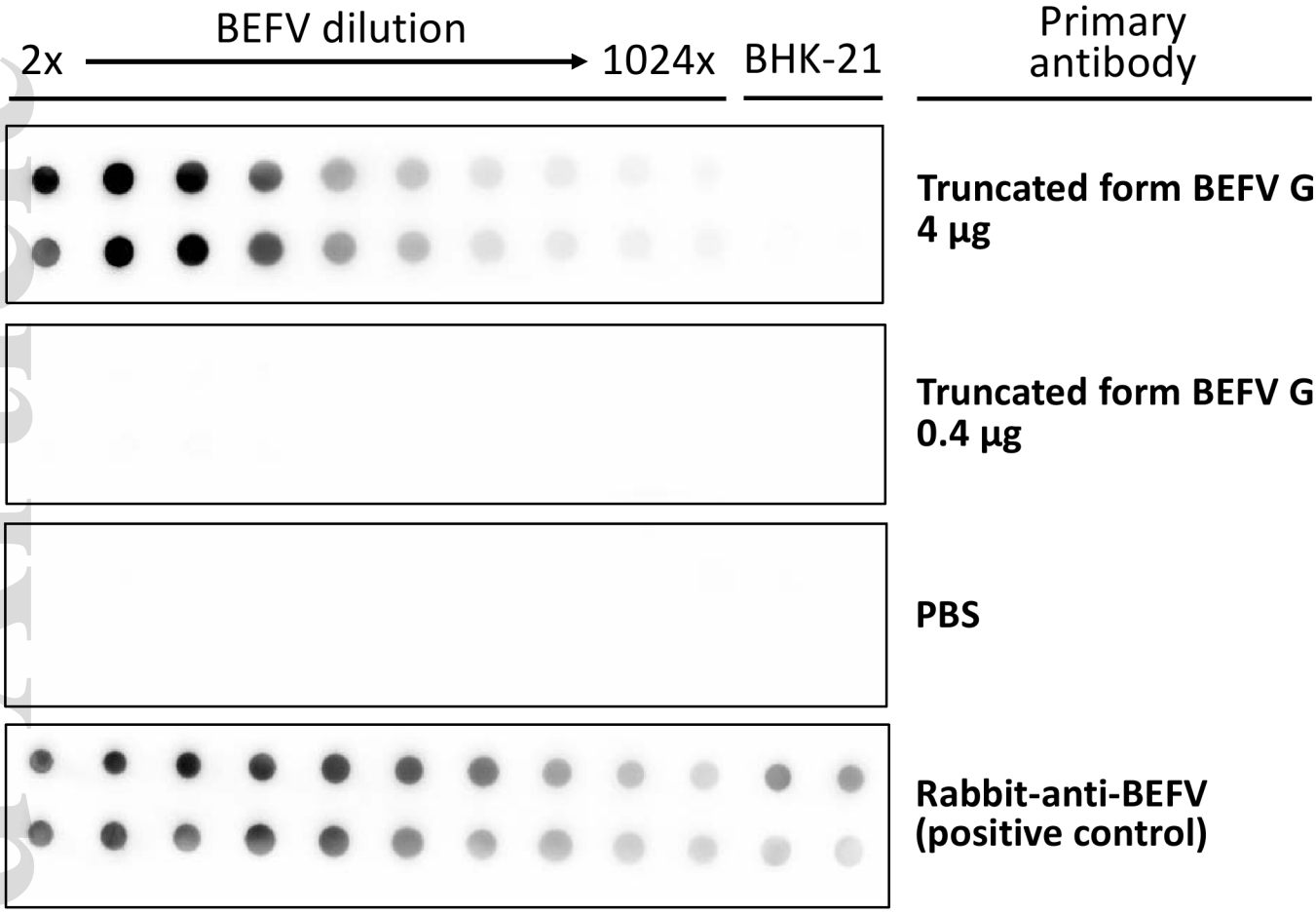

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