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ORIGINAL ARTICLE

Highly efficient production of rabies virus glycoprotein G ectodomain in Sf9 insect cells

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Abstract

In the present study, we developed a complete process to produce in insect cells a high amount of the ectodomain of rabies virus glycoprotein G (G_E) as suitable antigen for detecting anti-rabies antibodies. Using the baculovirus expression vector system in Sf9 insect cells combined with a novel chimeric promoter (*polh-pSeL*), the expression level reached a yield of 4.1 ± 0.3 mg/L culture, which was significantly higher than that achieved with the standard *polh* promoter alone. The protein was recovered from the cell lysates and easily purified in only one step by metal ion affinity chromatography, with a yield of 95% and a purity of 87%. Finally, G_E was successfully used in an assay to detect specific antibodies in serum samples derived from rabies-vaccinated animals. The efficient strategy developed in this work is an interesting method to produce high amounts of this glycoprotein.

Keywords Baculovirus · Rabies virus glycoprotein · *polh-pSeL* promoter · Sf9 cells

Introduction

Rabies virus (RABV) is the etiologic agent of rabies, a globally distributed infectious disease that causes fatal encephalitis in mammals. RABV infection of livestock animals such as camelids, sheep, goats, and cows causes significant economic losses in the productive system (Jibat et al. 2016). In addition, this disease also affects humans, causing approximately 59,000 deaths per year (Hampson et al. 2015).

RABV is a negative-stranded RNA virus that belongs to the genus *Lyssavirus* and family *Rhabdoviridae*. Among its structural proteins, the rabies virus glycoprotein G (RABV-G) is the most antigenic and immunogenic surface protein involved in viral attachment (Wunner et al. 1988). The mature RABV-G protein (MW 65 kDa) is formed by an external domain (ectodomain, 1-439 amino acid) exposed on the surface of the virus, a transmembrane domain (440-461 amino acid), and a cytoplasmic domain (462-505 amino acid) (Huang et al. 2017). In particular, the ectodomain is responsible for the induction of anti-rabies antibodies in infected and vaccinated individuals (Perrin et al. 1985).

RABV infection is prevented through vaccination with the inactivated whole virus. Thus, there is a high demand for efficient and easy-to-handle methods to evaluate the

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immunity state of immunized animals, based on the quantification of anti-RABV-G antibodies (Wasniewski and Cliquet 2012). Several Enzyme-Linked ImmunoSorbent Assays (ELISAs) have been developed using the chemically inactivated whole virus or pure glycoprotein G as the target antigen (Cliquet et al. 2004; Feyssaguet et al. 2007). However, the use of the whole virus as the target antigen may produce non-specific reactions with other antibodies present in the serum sample. In addition, the purification process of a subunit of RABV is complex, requiring specialized containment laboratories and a high amount of rabies virus (Zhang et al. 2009). Thus, the use of recombinant RABV-G as a coating antigen to detect anti-rabies antibodies is an attractive technology that does not require of specialized facilities for virus manipulation (Qian et al. 2013). The full-length RABV-G has been expressed in diverse systems. In *Escherichia coli*, the RABV-G has been highly expressed as inclusion bodies but found to lack its immunogenicity (Yelverton et al. 1983), whereas in *Saccharomyces cerevisiae*, the same antigen proved to be hyperglycosylated and incorrectly folded (Sakamoto et al. 1999). In the baculovirus expression vector system (BEVS), the full-length RABV-G has been expressed anchored to the plasma membrane of insect cells with correct folding and biological properties similar to those of the native protein (Tuchiya et al. 1992; Ramya et al. 2011). However, the expression of transmembrane proteins in BEVS is difficult and produces low yields (van Oers 2011). In addition, the solubilization and extraction of transmembrane proteins constitute a bottleneck in all expression systems, because protein structure is usually lost during purification. This loss can be avoided using certain extraction agents, which in turn might impair the purification process itself (Carpenter et al. 2008). For these reasons, the purification processes of this kind of proteins are tedious, time-consuming, and difficult to scale-up (Qoronfleh et al. 2003). Alternatively, it has been possible to produce a soluble form through the expression of the RABV-G ectodomain (G_E). The G_E has been expressed in several expression systems: mammalian cells (Gupta et al. 2005), insect Sf21 cells (Sissoëff et al. 2005), and yeast cells (Qian et al. 2013; Picotto et al. 2017). In all cases, the soluble protein expressed was antigenic and immunogenic, and could be used to detect the immunological status of vaccinated hosts. Among the different expression systems available, insect cells present some advantages that support the choice of the BEVS platform for G_E production. For instance, the insect cells are easier to culture and more tolerant of osmolarity and by-product concentration than mammalian cells for large-scale protein (Ikonomou et al. 2003). In addition, BEVS offer a more suitable eukaryotic environment than yeast for glycoprotein production (Rendić et al. 2008). So far, the only work reporting the G_E expression in BEVS focused on G_E antigenicity characteristics, but provided little information about the production and purification process (Sissoëff et al. 2005). The traditional promoters used to express the foreign protein in this system are *polyhedrin* (*polh*) and *p10* (Martínez-Solís et al. 2019). However, alternative promoters have recently been developed to improve expression levels. Martinez Solís et al. (2016) designed a new strong chimeric promoter (*polh-pSeL*) by combining the *polh* promoter and 120 nt belonging to the promoter from the *orf46* of *Spodoptera exigua multiple nucleopolyhedrovirus* (*pSeL*). However, this promoter has not been tested for the expression of different proteins with therapeutic or industrial value.

In this work, we report for the first time a complete process to produce, using standard as well as the novel promoter, high amounts of purified G_E in Sf9 cells to obtain a suitable antigen for detecting anti-RABV Ig.

Materials and methods

Glycoprotein G cDNA sequence and cloning into a baculovirus vector

The detailed procedure for constructing the baculovirus vector is shown in Supplementary Fig. S1. The full-length glycoprotein G cDNA sequence from RABV CVS strain (GenBank Accession No. AJ506997.1) cloned into the pGEMT easy plasmid (Promega, Madison, USA) was kindly provided by Oscar Pérez (Servicio de Vacuna anti-rábica, Administración Nacional de Laboratorios e Institutos de Salud "Dr. Carlos Malbrán", Buenos Aires, Argentina). Using this vector as a template, the truncated glycoprotein G—composed only of the ectodomain (G_E) without its endogenous signal peptide from 58 bp to 1377 bp corresponding to amino acid residues 1-439 of the mature RABV-G—was amplified by PCR using two specific primers. These primers added *Bam*HI and *Eco*RI restriction sites and six histidine residues (His-tag): 5′-CGGATCCAAATT CCCTATTTACAC GATACC-3′ (Primer Sense; *Bam*HI site underlined) and 5′-CGA ATTCTCAGTGA**TGATGATGATGATGATAC** TTCCCCCAGTTCGGG-3′ (Primer antisense, EcoR1 site underlined). The reverse primer included a sequence encoding a His-tag (in bold). The PCR conditions (50 µL final volume) were as follows: 200 nM each primer, 1 × PFU buffer, 0.3 mM each dNTP, and 2.5 U PFU polymerase (Promega). The PCR program was 95 °C for 6 min, 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min/kb × 30 cycles. An additional extension step of 72 °C for 5 min was then applied. After the reaction, the PCR product was purified using the PCR WizardTM SV gel and PCR Clean-up System (Promega). The G_E sequence fused to the His-tag sequence was then cloned using the *Bam*HI and *Eco*RI sites of the pAcGP67-B vector (BD Biosciences, San Diego, USA) to construct pAcGP67-G_E, which contained the nucleotide sequence that encode

the baculoviral glycoprotein 64 leader peptide (GP64; syn.: GP67). Using pAcGP67-G_E as a template, the G_E fused with the GP64 signal peptide (gpG_E) was amplified following the protocol described above by PCR using two specific primers, which added the *Eco*RI and *Xba*I restriction sites: 5′-CGAA TTCATGCTACTAGTAA ATCAGTCAC-3′ (primer sense, *Eco*RI site underlined) and 5′- GTCTAGATCAGTG ATG ATGATGATGATG-3′ (primers antisense, *Xba*I site underlined). The gpG_E cassette and the enhanced green fluorescent protein (EGFP) cDNA were cloned into the pFastBacDual vector (Thermo Fisher Scientific, Waltham, USA) under the *polh* and *p10* promoter, respectively. For this purpose, the EGFP (GenBank Accession No. NC_013179.1) sequence cloned into the pGEMT easy (Promega) plasmid was kindly provided by Dr Oscar Taboga (Centro de Investigaciones en Ciencias Veterinarias y Agronómicas, Instituto Nacional de Tecnología Agropecuaria, Buenos Aires, Argentina). Using this vector as a template, EGFP was amplified by PCR following the protocol described above using two specific primers, which added *Sma*I and *Nco*I restriction sites: 5′-GCCC GGG**ATG**GTGAGCAAGGGCGAGG-3′ (primer sense, *Sma*I site underline) and 5′-GCCATGGGTCAGTACAGCT CGTCCATGC-3′ (primer antisense, *Nco*I site underline). The PCR product was then cloned into the pFastBacDual vector (ThermoFisher) to generate the *EGFP-polh* vector. Then, the gpG_E cassette was purified and cloned using the *Eco*RI and *Xba*I sites of the *EGFP-polh* vector to generate the *EGFP-polh-gpG*$_E$ construction. The vector combining two promoters (*EGFP-polh-pSeL-gpG*$_E$) was constructed by modification of the *EGFP-polh-gpG*$_E$ vector. The *polh-pSel* promoter fragment was obtained from the *polh-pSeL-GFP* plasmid (Martínez-Solís et al. 2016) by digestion with *BstZ17*I and *Eco*RI enzymes. Then, the fragment was inserted into the *BstZ17*I and *Eco*RI sites of the *EGFP-polh-gpG*$_E$ vector, generating the *EGFP-polh-pSeL-gpG*$_E$ vector. All the DNA constructs were verified by Sanger sequencing.

Insect cell culture

The *Spodoptera frugiperda* (Sf9) insect cell line (Thermo Fisher Scientific) was maintained in suspension cultures in sterile Erlenmeyer and grown in Sf-900™ II SFM medium (Thermo Fisher Scientific) supplemented with 1% (V/V) antibiotic–antimycotic solution (Thermo Fisher Scientific) at 27 °C under continuous shaking at 100 rpm. Besides, the suspension volume did not exceed 10% of the total volume of the Erlenmeyer flask.

Virus production

The recombinant baculoviruses were obtained using the Bac to Bac® baculovirus expression system (Thermo Fisher Scientific) following the manufacturer's instructions. The *EGFP-polh-gpG*$_E$ and *EGFP-polh-pSeL-gpG*$_E$ vectors were transformed in chemically competent *E. coli* DH10Bac™ strain (Thermo Fisher Scientific) by heat shock to generate the recombinant bacmid by transposition. Then, the bacmids were purified and used to transfect 1 million Sf9 cells using Cellfectin II Reagent (Thermo Fisher Scientific). After a 3-day incubation at 27 °C, the cell culture supernatant was collected and centrifuged at 500 × *g* for 10 min. The transfection efficiency was determined by measuring EGFP expression by fluorescence under UV light. The recombinant AcMNPV baculoviruses polyhedrin-minus virus containing EGFP under the control of the *p10* promoter and the gpG_E sequence under the control of the *polh* or *polh-pSeL* promoters were named *Acpolh-G*$_E$ and *Acpolh-pSeL-G*$_E$, respectively (Fig. 1). Following three amplification steps, the virus titer was determined by a plaque assay (O´Reilly et al. 1994). The amplified virus stock was used for producing the recombinant protein in further experiments.

Insect cell infection

For the G_E expression assay, independent Sf9 suspension cultures in log phase at a cell density of 1×10^8 cells in 50 mL were infected with *Acpolh-pSeL-G*$_E$ or *Acpolh-G*$_E$ at a multiplicity of infection (MOI) of 0.1, 1, and 5. The infected suspension culture was incubated in an orbital shaker at 100 rpm in the dark at 27 °C for 4 days. To study the expression at different days post-infection (dpi), samples of 1 mL were collected each day. The culture supernatant was separated from the cell by centrifugation at 500×*g* for 10 min. The pellet and the supernatant were stored at − 20 °C until further experiments. For SDS-PAGE and western blot analysis, the culture supernatant was assessed without any extra treatment, while the cell pellet was treated as indicated in the following section to obtain total protein extract. An Sf9 suspension culture infected with baculovirus *AcMNPVHRPC*—previously constructed in our laboratory—was also included as a control (Targovnik et al. 2010).

Total protein extraction from infected cells

The cell pellet was resuspended in lysis buffer (50 mM Tris/HCl pH 8.0, 300 mM NaCl, and 1% [v/v] NP-40) with a protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, USA) by adding 100 µL of lysis buffer per 2×10^6 cells. After incubation at 4 °C for 30 min, the cellular debris was separated by centrifugation at 10,000×*g* at 4 °C for 10 min. The pellet was discarded, and the supernatant was stored at − 20 °C until further experiments.

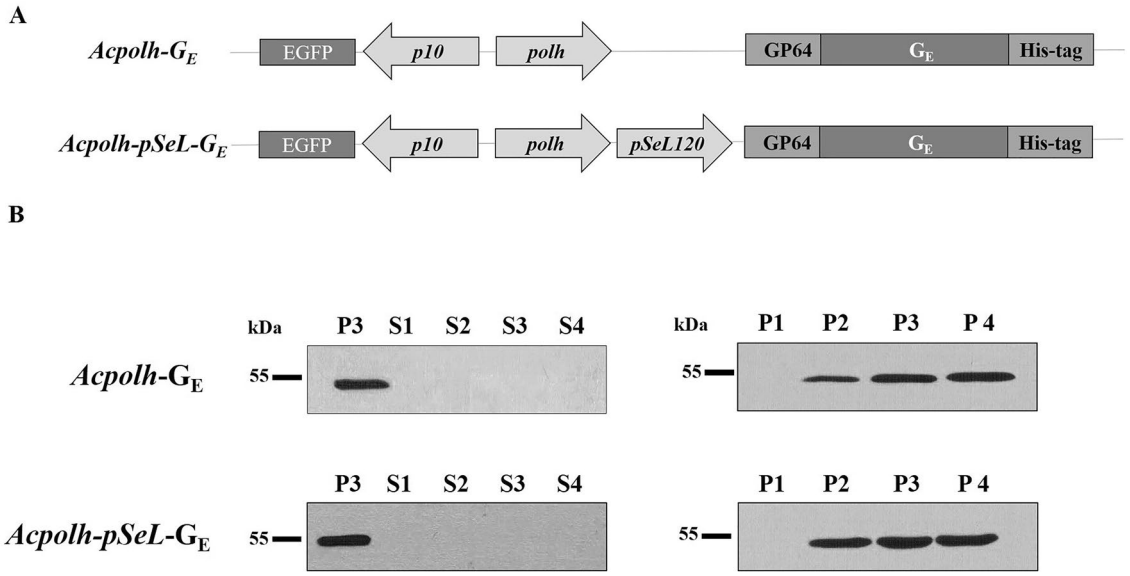

Fig. 1 **a** Recombinant baculovirus for the expression of G_E under the *polh* (*Acpolh-G*$_E$) and *polh-pSeL* (*Acpolh-pSeL-G*$_E$) promoters. GP64: viral secretion signal GP64; His-tag: six histidine tag. **b** Analysis of recombinant G_E localization in supernatants and cell pellets by western blot. Sf9 cells were infected with *Acpolh-G*$_E$ or *Acpolh-pSeL-G*$_E$ at MOI 5. At different days post-infection, the culture medium and infected Sf9 cells were harvested and analyzed. Western blot developed with mouse anti-His antibody. Line S1–S4: culture supernatant from 1 to 4 days post-infection; P1–P4: cell pellet from 1 to 4 days post-infection; *polh-G*$_E$: G_E expression under the *polh* promoter; *polh-pSeL-G*$_E$: G_E expression under the *polh-pSeL* promoter

Total protein measurement

Total protein concentration was determined by following the Bradford microassay protocol (Bradford 1976) with bovine serum albumin (BSA) as the standard, using the Quick Start™ Bradford reagent (Bio-Rad, Hercules, USA).

Electrophoretic analysis

The protein samples were resolved by SDS-PAGE on 12.5% or 15% polyacrylamide gels. The samples were heated at 100 °C for 5 min in sample buffer (125 mM Tris/HCl, pH 6.8, 4% [w/v] SDS, 20% [w/v] glycerol, 0.01% [w/v] bromophenol blue, and 10% [v/v] 2-mercaptoethanol). The resulting gels were stained with Coomassie Blue R-250. For western blot analysis, the gels were transferred onto a nitrocellulose membrane (GE Healthcare, Chicago, USA) and G_E was detected using a hyperimmune rabbit serum against rabies whole inactivated virus in a 1/1500 dilution as the primary antibody and horseradish peroxidase-conjugated mouse anti-rabbit IgG (H + L) (Sigma-Aldrich) in a 1/1500 dilution as the secondary antibody. Also, G_E was detected using a mouse anti-His antibody (BD Biosciences) in a 1/3000 dilution as the primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) (DAKO, Copenhagen, Denmark) in a 1/1000 dilution as the secondary antibody. The development was carried out with an enhanced chemiluminescence substrate (Thermo Fisher Scientific) and high-performance chemiluminescence film (Thermo Fisher Scientific).

G_E purification by immobilized metal ion affinity chromatography (IMAC)

The cell pellet from 50 mL of cell culture infected with *Acpolh-gpG*$_E$, *Acpolh-pSel-gpG*$_E$, or *AcMNPVHRPC* (baculovirus control) was resuspended and lysed in chromatographic equilibration buffer (50 mM Tris/HCl pH 8.0, 300 mM NaCl, 40 mM imidazol, and 10% (v/v) glycerol) with 1% (v/v) NP-40, protease inhibitor cocktail without EDTA (Sigma-Aldrich) and 100 U/mL deoxyribonuclease I (Sigma-Aldrich). Then, 1 mL of the buffer was used for 6.5 × 10^6 cells (Klaus et al. 2015). The lysates were incubated at 4 °C for 30 min and centrifuged at 10,000×*g* at 4 °C for 10 min. The soluble fraction was loaded on the 1 mL HisTrap column (GE Healthcare), equilibrated with the same buffer. Following a washing step with equilibration buffer containing 100 mM imidazol, an elution step was performed by increasing the imidazole concentration to 500 mM. The linear flow rate was 0.4 cm/min, and 1.5 mL fractions were collected. Protein separation was monitored by absorbance at 280 nm. All fractions were collected and analyzed by SDS-PAGE and western blot. The same process was used for Sf9 cells infected with the control virus. For image processing, the gels were scanned and then analyzed with the ImageJ software (National Institute of Health, USA). The

amount and purity of G_E were assessed by densitometric analysis of the band intensities from the SDS-PAGE and confirmed by ELISA and Bradford assay. The results are expressed as the mean ± standard deviation of at least three determinations from independent experiments.

Characterization of the expressed G_E by confocal microscopy

For confocal microscopy, 8×10^4 Sf9 cells were seeded into Lab-Tek Chambered coverglass dishes (Thermo Fisher Scientific) and infected with the *Acpolh-pSel-gpG_E* or *Acpolh-gpG_E* virus. The cells were incubated at 27 °C for 72 h, washed with PBS, and fixed in 4% paraformaldehyde (pH 7 in PBS) for 15 min. Then, the cells were washed twice with PBS-T (PBS-0.05% Tween) and permeabilized with 0.2% Triton X-100 for 30 min, or only washed twice with PBS. The permeabilized cells were subsequently washed twice with PBS-T and all the samples were treated with 1% BSA (Sigma-Aldrich) in PBS for 1 h. Next, the cells were incubated with a mouse anti-His antibody (1:100) (BD Biosciences) in 1% BSA for 60 min and washed twice with PBS-T to remove nonspecifically bound antibody. The cells were incubated with Cy™3 AffiniPure Donkey Anti-Mouse IgG (H + L) antibody (1:800) (Jackson ImmunoResearch, West Grove, USA) in 1% BSA for 60 min, washed twice with PBS-T, and incubated for 10 min with TO-PRO-3 Stain (1:300) (Thermo Fisher Scientific). Finally, the cells were washed three times with PBS-T and once with distilled water, before they were mounted in ProLong Gold antifade reagent (Thermo Fisher Scientific). Samples were visualized in a Leica TCS-SP5 spectral laser confocal microscope.

Quantification of G_E expression by sandwich ELISA assay

MaxiSorp microplates (Sigma-Aldrich) were coated with 100 µl of a mouse monoclonal anti-His antibody (1:1500) (BD Biosciences) in 1% non-fat dry milk overnight at 4 °C. The plates were washed with PBS-T and blocked with blocking buffer (PBS-T and 3% non-fat dry milk) for 1 h at RT. The plates were then washed with PBS-T and incubated with 100 µl of sample diluted 1/4 in sample diluent (same as blocking buffer) for 1 h at RT. Plates were then washed with PBS-T and incubated with 100 µl of hyperimmune rabbit serum against rabies whole inactivated virus diluted 1/500 in sample diluent for 1 h at RT. Next, the plates were washed with PBS-T and incubated with 100 µl horseradish peroxidase-conjugated mouse anti-rabbit IgG (H + L) (Sigma-Aldrich) in a 1/2000 dilution as the secondary antibody. The plates were washed again, and the colorimetric reaction was developed by adding 100 µL of TMB substrate (Thermo Fisher Scientific). After a 5-min incubation period at room temperature in the dark, color development was stopped by adding 50 µL of 4 N H_2SO_4. Optical densities (OD) were measured at 450 nm. The amount of recombinant G_E expressed in different experiments was estimated with a standard curve of G_E (6 to 0.2 µg/ml) previously purified from Sf9-infected cells and quantified by gel densitometry and Bradford Assay. A sample from Sf9 cells infected with baculovirus control was also included as a negative control. The results are expressed as the mean ± standard deviation of at least three determinations. The results were statistically analyzed by two-way ANOVA followed by Bonferroni's post hoc test using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, USA). A $p < 0.05$ was considered statistically significant.

Mass spectrometry analysis

A purified protein sample was separated by SDS-PAGE on 12% polyacrylamide gels and stained with colloidal Coomassie Blue. The band corresponding to the molecular weight of G_E was cut off and in-gel-digested with trypsin, and the peptides were separated by HPLC (Easy-nLC 1000, Thermo Fisher Scientific), using a reverse-phase column (EASY-Spray Column P/N ES80, Thermo Fisher Scientific) and analyzed by ESI-Orbitrap mass spectrometry (Q-Exactive, Thermo Fisher Scientific) with the software Proteome Discoverer version 1.4 (Thermo Fisher Scientific).

Glycosylation assay

The purified G_E was subjected to N-glycosidase F (Roche, Mannheim, Germany) digestion. For this purpose, 14 µg of protein was mixed with 80 µL denaturing buffer (SDS 2.5%, DTT 0.4 M). After heating at 100 °C for 10 min, the reaction buffer was added (0.05 M sodium phosphate buffer –PBS, pH 7.5, containing 1% (v/v) NP-40, Inhibitor cocktail protease and 3 U N-glycosidase F. As a control, 14 µg of denatured protein was incubated in the reaction buffer without enzyme. After incubation at 37 °C for 16 h, the sample was analyzed by SDS-PAGE and western blot, as described above.

Reactivity of RABV vaccinated llama sera to G_E protein by ELISA

Serum samples from vaccinated llamas were tested for the detection of anti-RABV Ig antibodies using MaxiSorp microplates (Sigma-Aldrich) coated with 0.5 µg of purified recombinant G_E. The plates were washed with PBS-T and blocked with blocking buffer (PBS-T and 3% non-fat dry milk) overnight at 4 °C. The plates were then washed with PBS-T and incubated with 100 µL of sera diluted 1/100 in sample diluent (same as blocking buffer) at 37 °C for 1 h.

The plates were then washed with PBS-T and incubated with 100 µl of horseradish peroxidase-conjugated rabbit anti-llamas Ig (Bethyl, Montgomery, USA) diluted 1/5000 in sample diluent for 1 h at 37 °C. The plates were washed again and the colorimetric reaction was developed by adding 100 µL of TMB substrate (Thermo Fisher Scientific). After a 10-min incubation period at room temperature in the dark, color development was stopped by adding 50 µL of 4 N H_2SO_4. Optical densities (OD) were measured at 450 nm. All determinations were performed in triplicate and the results are expressed as the mean ± standard deviation. The data obtained provided a cut-off value for further classification of each sample as positive/negative, for additional correlation with the vaccination status of each animal. The samples used in the analysis were from a llama serum bank belonging to Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. They were obtained during an antirabies vaccination campaign with a second-generation rabies vaccine (derived from the culture supernatant of infected BHK cells).

Results and discussion

Generation of the recombinant baculoviruses *Acpolh-gpG$_E$* and *Acpolh-pSeL-gpG$_E$*

The G_E sequence fused to the GP64 viral signal peptide and a His-tag (gpG_E) was cloned under the control of the *polh* promoter to obtain the *EGFP-polh-gpG$_E$* vector. The GP64 signal peptide would ensure post-translational modification and target the recombinant protein for secretion, whereas the addition of a C-terminal His-tag would facilitate the purification step of the glycoprotein by IMAC. In addition, the vector had the coding sequence of the EGFP protein cloned under the p10 promoter as a reporter gene to allow us to visualize the viral infection. To produce large quantities of G_E, the *polh* promoter was replaced with the *polh-pSeL* promoter to obtain the *polh-pSeL-gpG$_E$* vector (Supplementary Fig. S1). Plasmids were used to produce the corresponding bacmids. Transfection of the bacmids and amplification in Sf9 cells allowed us to obtain the *Acpolh-G$_E$* and the *Acpolh-pSeL-G$_E$* viruses (Fig. 1a).

G_E expression and localization in Sf9 cells

To determine G_E expression and localization in insect cell cultures, we infected Sf9 cells with *Acpolh-G$_E$* or *Acpolh-pSeL-G$_E$*. We analyzed the culture supernatant and cell pellets by western blot (Fig. 1b). The expression was evaluated up to 4 dpi to avoid high cell lysis that could affect the following purification step. Although the protein was expressed under the GP64 viral signal peptide, the system failed to secrete the protein. The protein was detected in the cell pellet (Fig. 1b). For this reason, the cells were lysed using a buffered detergent solution. The non-ionic detergent NP-40 contributed to the cell lysis without affecting the protein structure, and its presence was essential for the total solubilization of G_E. It was not necessary to add EDTA to stabilize membrane proteins in solution and dimethylsulfoxide to generate pores on the lipid membrane. Thus, G_E may be more weakly associated with the membrane than the full version.

The localization of G_E expressed under *polh* and *polh-pSeL* was confirmed by immunofluorescence assay developed with a specific antiserum against the His-tag. As shown in Fig. 2, G_E was associated with the cell membrane, although its transmembrane region was removed, whereas EGFP was located throughout the cytoplasm. In addition, TO-PRO 3 fluorescent dye stained the nucleus of infected Sf9 cells. The results indicate that the His-tag were correctly exposed, since the antibodies were able to detect them in the native protein inside the cells, an important fact for the following purification step by IMAC.

The insect cell machinery is often overwhelmed due to the production of high levels of recombinant protein (Dalton and Barton 2014). The problem is exacerbated by the shutdown of cell protein synthesis during the baculovirus infection (Urtasun et al. 2015). Thus, recombinant G_E might be trapped inside the cell due to insufficient cellular resources for their secretion into the culture medium (Steele et al. 2017). In addition, the translocation of G_E to the extracellular medium might be affected by its own physicochemical characteristics and by the absence of a proper protein trafficking system in the heterologous expression host (Ashikawa et al. 2011).

Previous studies have shown inefficient secretion of some other recombinant glycoproteins produced by BEVS (Klaus et al. 2015; Trabucchi et al. 2019). However, the fact that the expressed protein remained inside the cells did not affect its production and recovery.

Optimization of G_E expression and assessment of the potential improvement using the dual promoter

To determine the best conditions to produce the recombinant protein (MOI, dpi, promoter) and compare the *polh-pSeL* promoter activity with the standard *polh* promoter, we infected Sf9 cells with *Acpolh-G$_E$* or *Acpolh-pSeL-G$_E$* at MOIs of 0.1, 1, and 5, and analyzed the culture cell pellets at different dpi by sandwich ELISA (Fig. 3). The expression kinetics showed that the G_E expression increased gradually from 2 dpi, achieving a maximal expression level at 4 dpi for MOI 1 and 3 dpi for MOI 5. The expression level achieved at MOI 0.1 was lower than that of the other MOIs examined. The *polh-pSeL* promoter showed higher activity for the

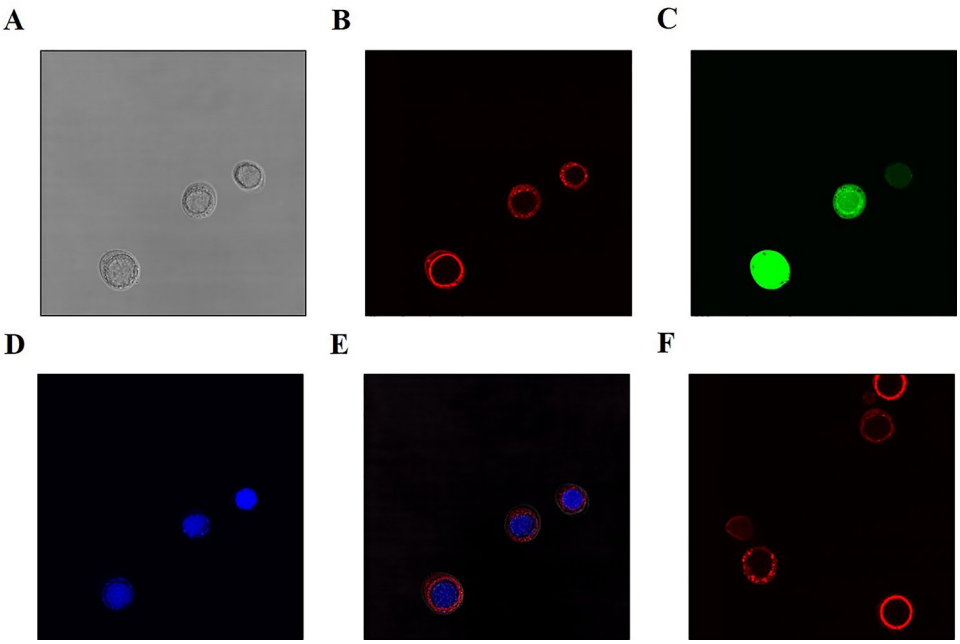

Fig. 2 Confocal microscopy analysis of G_E-expressing Sf9 cells. **a** Bright Field image of infected Sf9 cells. **b, f** Infected Sf9 cells expressing G_E incubated with a mouse anti-His monoclonal antibody and then with Cy™3 AffiniPure Donkey Anti-Mouse IgG (H + L) antibody. **c** Infected Sf9 cells expressing EGFP. **d** TO-PRO-3-stained cells. **e** Overlay of TO-PRO-3- and Cy™3-stained cells. **a**–**e**: Sf9 cells infected with *Acpolh-pSeL-G*$_E$; **f**: Sf9 cells infected with *Acpolh-G*$_E$

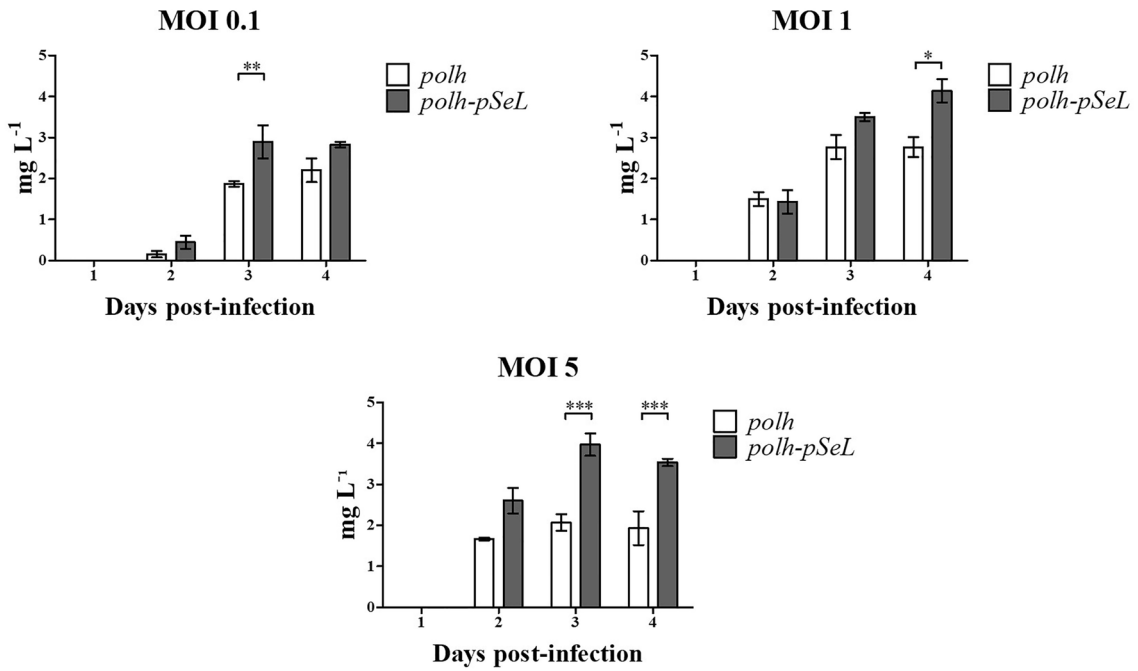

Fig. 3 Expression kinetics of G_E expressed under control of *polh* (white column) and *polh-pSeL* (gray column) promoters. Sf9 cells were infected at MOIs 0.1, 1, and 5 with *Acpolh-pSeL-G*$_E$ or *Acpolh-G*$_E$. At different days post-infection, the infected cells were harvested, lysed and analyzed by ELISA. Data are shown as mean ± standard deviation from three independent experiments. Columns with an asterisk were significantly different. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$

expression of G_E than the *polh* promoter alone in most conditions analyzed. The biggest difference in G_E expression was detected at MOI 5 and 3 dpi, where the yield achieved under the *polh-pSeL* promoter was 100% higher (4.0 ± 0.3 mg/L culture; 40 µg/2 × 10^7 cells) than that achieved with the *polh* promoter alone (2.0 ± 0.2 mg/L culture; 20 µg/2 × 10^7 cells). However, the same expression level reached under the *polh-pSeL* promoter at MOI 5 and 3 dpi was detected at lower MOI, often necessary for large-scale protein production. When the cell culture was infected at MOI 1, the expression level under the *polh-pSeL* was maximal at 4 dpi, reaching a yield of 4.1 ± 0.3 mg/L culture (41 µg/2 × 10^7 cells), the

expression being 46% higher than that achieved with the *polh* promoter alone (2.8 ± 0.2 mg/L culture; 28 µg/2 × 10^7 cells). After the expression, no significant difference in the final titre reached for *Acpolh-*G_E and *Acpolh-pSeL-*G_E was found (~1 × 10^8 UFP/ml), suggesting that the expression difference is not influenced by the virus replication. In addition, the yield of G_E achieved in Sf9 was higher than that reached for full-length RABV-G expression in High Five® (Ramya et al. 2011) cells under the *polh* promoter (1.78 µg/2 × 10^7 cells) using four times less virus.

Therefore, for further purification experiment, we selected the optimized culture parameters (MOI 1 and 4 dpi) and the *polh-pSeL* promoter to produce G_E in Sf9 cells.

The *polh-pSeL* promoter was developed to improve the expression of GFP in insect cells (Martínez-Solís et al. 2016). However, this novel promoter had not been assayed to produce other recombinant proteins and had not been tested in Sf9 insect cell lines. Thus, we describe, for the first time, the use of the *polh-pSeL* promoter to enhance the expression level of a protein of diagnostic interest in Sf9 insect cell line.

Purification of G_E from Sf9 cells

IMAC was performed to purify the G_E from lysates of Sf9 cells. The fact that the cells were cultured in a medium without fetal bovine serum facilitated the purification step, thus reducing the contaminants in the sample. After the sample was loaded into the column, G_E was adsorbed on the matrix with high affinity due to the presence of the exposed His-tag, confirmed previously by the immunofluorescence assay and western blot. Fortunately, the protein was recovered only in the elution fraction by addition of 500 mM imidazole (Fig. 4). Most contaminating proteins, including EGFP, appeared in the flow through (i.e., not adsorbed) and the addition of 40 mM imidazole to the equilibration buffer and the washing step with 100 mM imidazole enhanced the purity of G_E (Fig. 4a). Figure 4 shows the corresponding SDS-PAGE (Fig. 4a) and western blot pattern (Fig. 4b) of the final product compared to the starting material. The purified G_E was correctly detected by western blot using a hyperimmune rabbit serum against rabies whole inactivated virus. After the purification step, it was possible to obtain a 4.0 ± 0.1 mg/L culture with a yield of 95% and a purity of 87% (Table 1). In addition, the highest activity of the *polh-pSeL* versus *polh* promoter for the expression of G_E was also confirmed by gel densitometry after IMAC purification step (Fig. 4c).

Characterization of the purified G_E

The mass spectrometry analysis of the purified protein after trypsin treatment showed that the 15 peptides obtained perfectly matched those predicted for G_E, thus confirming the

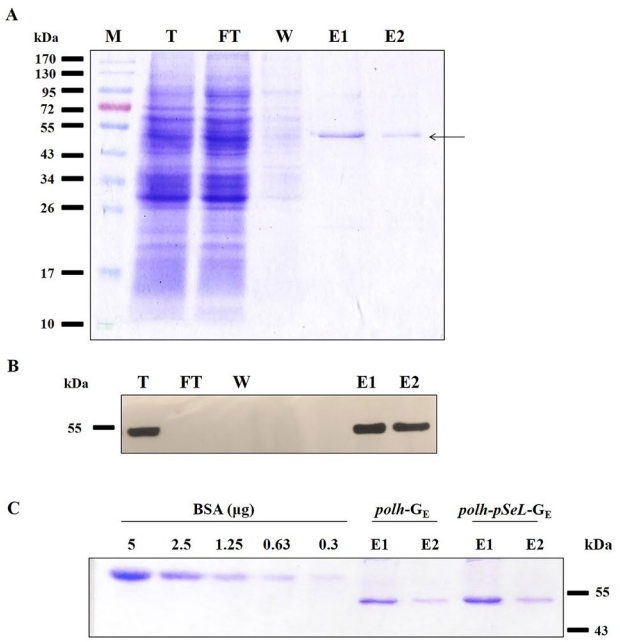

Fig. 4 IMAC purification of G_E expressed under the *polh-pSeL* (**a**–**c**) and *polh* (**c**) promoters. **a** SDS-PAGE analysis of fraction collected during purification process. **b** Western blotting analysis of fraction collected during purification process using rabbit anti-RABV polyclonal antibody. **c** SDS-PAGE analysis and quantification of purified G_E expressed under control of *polh* and *polh-pSeL* promoters at MOI 1 and harvested at 4 dpi. *Lane M* protein marker, *T* total protein extract, *FT* flow through, *W* washing step (equilibration buffer with 100 mM imidazole); *E1*, *E2*: IMAC fraction eluted by 500 mM imidazole

identity of the recombinant protein with a coverage of 49% (Supplementary Table 1).

To further characterize the purified G_E, it was treated with N-glycosidase F digestion (Fig. 5). The MW of the protein of around 53 kDa (Fig. 4a) was larger than sequential weight theoretically predicted of 50 kDa, taking into account the His-tag (Gasteiger et al. 2005). It has been reported that G_E has three potential N-glycosylation sites and that only two of them are efficiently glycosylated in virions (Shakin-Eshleman et al. 1992). After cleavage, the molecular weight of the glycoprotein decreased to about 50 kDa. Thus, this evidence suggests that the N-glycosylation is responsible for the differential size between the digest and undigested G_E.

Even though the secretion pathway could be functionally affected during baculovirus infection in insect cells, the BEVS is capable of producing glycosylated protein using very late baculoviral promoters such as *polh* (Metz et al. 2011; Targovnik et al. 2010). For instance, when the full-length RABV-G was expressed in High Five® cells under the *polh* promoter, it was possible to obtain a glycosylated protein anchored to the plasma membrane (Ramya et al. 2011). In addition, viral proteins are glycosylated by the host cell during the infection (Stiles and Wood 1983). To avoid

Table 1 Purification process of G_E from Sf9 cells expressed under *pol-pSeL* promoter by IMAC

Step	Volume (ml)	G_E (mg)	Total protein (mg)	Purity (%)	Yield (%)	Purification (Fold)
I	50	0.21	20.55	1.02	100	1
II	3	0.20	0.23	87	95	85

I crude extract before purification, *II* IMAC fraction eluted by 500 mM imidazole. G_E purification process determined by ELISA and Bradford assay

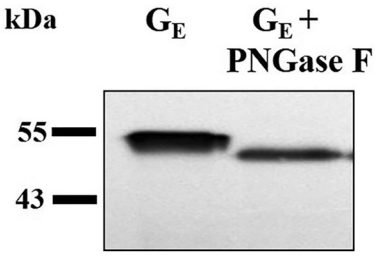

Fig. 5 G_E glycosylation analysis. N-glycosidase F-mediated in vitro deglycosylation of purified G_E. Western blot developed with mouse anti-His antibody. Line G_E: Purified G_E; G_E + PNGase F: Purified G_E treated with N-glycosidase

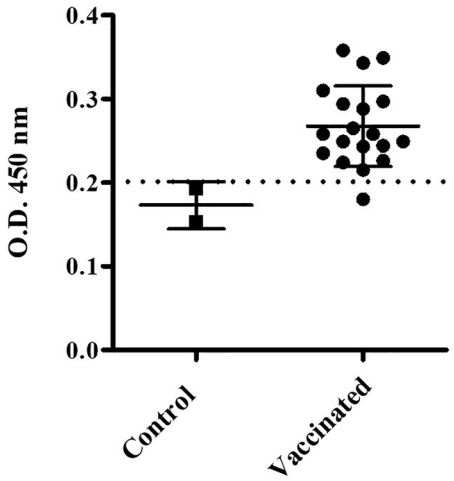

Fig. 6 Evaluation of the immunoreactivity of G_E to detect RABV antibody in serum samples from llamas. Control: serum from unvaccinated llamas; Vaccinated: serum from llamas vaccinated with a second-generation RABV vaccine

further cell damage and ensure higher quality post-translational modification, protein expression is usually restricted to more o less 3 dpi (Steele et al. 2017). Different degrees of success in glycosylation have been achieved depending on the protein expressed (Toth et al. 2011). In the present work, the results suggest that G_E moved successfully through the secretory pathway undergoing the expected glycosylation and were then anchored to plasma membrane for the possible reasons described above.

Use of G_E to detect anti-RABV Ig in serum samples from vaccinated llamas

The breeding of South American camelids has become an extended practice. In South America, llamas have been considered one of the most outstanding breed animals for the last 30–40 years. This species has been reported to suffer from many infectious diseases such as rabies, which causes considerable economic loss (Friedrich et al. 2014). For this reason, we finally studied the use of the recombinant G_E to detect anti-RABV Ig in serum samples from vaccinated llamas. Therefore, multiwell plates were coated with 0.5 µg of purified G_E and the serum from llamas vaccinated or not with different RABV vaccines was tested. As a control, the elution purification fraction from Sf9 cells infected with control baculovirus was used to coat another plate and evaluate whether the contaminant that remained in the purified G_E reacted with the Ig from RABV. Fortunately, only when the plates were coated with G_E, the reaction was specific, and this test was able to detect Ig from RABV, correctly differentiating the vaccinated animals (Fig. 6). This suggests that G_E was correctly folded and enough to detect Ig in serum samples and that the kind of glycan structure typical of insects did not affect its antigenicity. In addition, the purity achieved by IMAC was adequate to develop the assay. Therefore, 1 L of cell culture was enough to produce around 83 plates of 96 wells. The assay developed in this work was suitable for a fast evaluation of the effect of vaccination in animals.

Conclusions

We developed a complete process for producing large amounts of the G_E in Sf9 cells. Although the system failed to secrete this protein, it was possible to recover it from inside the cell and purify it in only one step by IMAC. In addition, we validated the new chimeric *polh-pSeL* promoter by expressing a protein of diagnostic interest. The G_E yield obtained using the *polh-pSeL* promoter was significantly higher than that obtained using the standard strong promoter *polh*. This has opened the possibility to extend the use of this novel promoter for expressing commercially relevant

proteins in the future using the BEVS. The results allow us to conclude that the platform is an interesting alternative engineering approach to produce this recombinant protein as an antigen for the future development of a diagnostic test for rabies.

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Author contributions AMT, SH, AF, and MVM designed the experiments. AMT and MMS constructed the baculovirus vectors. AMT and GJM performed the expression experiments. AMT and LFB contributed to downstream processing. IS and MBA performed the characterization experiments. AF contributed to the development of the ELISA. VA and MGL performed the confocal analysis. AMT, AF, SH, and MVM analyzed the data. All authors discussed the results and agreed upon the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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