Recombinant occlusion bodies of baculovirus as carriers of a non-structural protein of foot-and-mouth disease virus

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Abstract

Here, we developed a diagnostic ELISA for foot-and-mouth disease using recombinant occlusion bodies (rOBs) of baculovirus. We fused $\Delta 3AB_{1-3}$, a polypeptide derived from non-structural proteins of foot-and-mouth disease virus, to polyhedrin (POLH), the major constituent of OBs, under *polh* promoter. To further assess the most convenient strategy to improve yields, we designed two recombinant baculoviruses, vPOLH and vPOLH_{E44G}. These carried the sequence of the fusion protein POLH- $\Delta 3AB_{1-3}$ with an additional copy in cis of *polh* or *polh*_{E44G}, respectively, under *p10* promoter. Our results show that both viruses expressed POLH- $\Delta 3AB_{1-3}$, which was detected by western blot in purified rOBs with anti-POLH and anti-3AB1 antibodies. We also found that vPOLH_{E44G} produced larger polyhedra and a significant increase of antigen yield ($p < 0.01$). Furthermore, the chimeric protein POLH- $\Delta 3AB_{1-3}$ was recognized by sera from experimentally infected animals, showing that translational fusion to POLH does not alter the antigenicity of $\Delta 3AB_{1-3}$. Finally, the rOBs were successfully used in an ELISA test to differentiate infected from vaccinated animals. Taken together, these results demonstrate the great potential of rOBs to develop diagnostic schemes adaptable to animal infectious diseases.

Keywords Occlusion bodies · Polyhedrin · Baculovirus · FMDV · DIVA diagnostic

Introduction

The baculovirus expression vector system (BEVS) has extensively been used for the production of heterologous proteins. In fact, several biotechnological products based on this system are available on the market (van Oers et al. 2015). However, downstream processing has remained a bottleneck for cost-effective protein expression. To overcome these limitations, a BEVS-based novel strategy was developed to improve the production and purification of proteins

(Je et al. 2003). It is based on the advantageous properties of baculovirus occlusion bodies (OBs) or polyhedra for protein expression, obtaining recombinant occlusion bodies (rOBs). The main advantages are the high level of rOB production, their ease of purification and their capability of long-term conservation without refrigeration. The latter is due to the properties of the protective matrix of OBs which ensures that occluded virions remain infectious indefinitely (Rohrmann 2013). At the same time, rOBs become a safe technology because occlusion-derived baculovirus do not infect mammals nor induce immune responses (Molina et al. 2016).

The first studies of baculovirus OBs as carriers of recombinant proteins were performed by coupling reporter-enhanced green fluorescent protein (EGFP) to the C-terminus of polyhedrin (POLH), the major component of baculovirus polyhedra. But, further contributions showed that to improve final fusion protein yield and its incorporation into rOBs, co-expression of additional copies of POLH in *cis* or in *trans* is needed (Je et al. 2003; Sampieri et al. 2015). Then, several approaches have been developed for the construction of rOBs (López et al. 2018a). More recently, our group demonstrated that rOBs are useful carriers of antigens for the development of an ELISA-based diagnostic of bovine

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babesiosis (López et al. 2018b). Also, we showed that *trans*-complementation of POLH_{E44G}, a polyhedrin variant previously characterized by our group (López et al. 2011), increases rOBs yields (López et al. 2018b). Besides, OBs made of POLH_{E44G} have a diminished ability to incorporate occluded virions (López et al. 2011). Altogether, these results suggest a great potential for the design of updated diagnostic systems for relevant animal infectious diseases.

Foot-and-mouth disease (FMD) is a severe disease that affects both domestic and non-domestic cloven-hoofed animals, with special interest in livestock such as cattle, sheep, goats and pigs. An outbreak of this disease would lead to huge losses and for FMD-free countries its reintroduction poses a serious threat. Hence, for frequent animal testing, it is required a low-cost, sensitive assay that can also be stored in rural facilities at room temperature for long periods of time. Commercial diagnostic kits are based in non-structural proteins (NSPs) of foot-and-mouth disease virus (FMDV), the etiological agent of FMD (Ma et al. 2011). This diagnostic method differentiates infected from vaccinated animals (DIVA). It is based on the fact that inactivated virus-based vaccines can generate antibodies only against structural proteins, due to its inability to replicate in the animal. On the contrary, animals infected with replicative FMDV generate antibodies against all viral proteins. Nonetheless, most of the available diagnostic commercial alternatives are based on the expression of NSPs in prokaryotic systems (Ma et al. 2011). Thus, it leads to unspecific reactivity of bovine sera due to bacterial contaminants. The aforementioned characteristics and advantages of the rOBs of *Autographa californica* nucleopolyhedrovirus (AcMNPV) drove us to hypothesize that a new diagnostic scheme for FMD based on rOBs carrying NSPs can be designed, as an update of current methods for diagnosis. Moreover, to obtain an improved system, we aimed to compare *cis*-complementation of POLH_{E44G} against conventional POLH complementation. In this work, we proved that *cis*-complementation of POLH_{E44G} increased yields and that rOBs carrying epitopes of NSPs of FMDV retained their antigenic properties when coupled to POLH.

Methods

Cells, virus, antibodies and reagents

Sf9 cells were maintained at 27 °C in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (Inter-negocios) and antibiotic/antimycotic (GIBCO). Wild-type (wt) AcMNPV was obtained from Pharmingen (BD Biosciences). Antibodies used include polyclonal anti-POLH (GeneTex) and monoclonal anti-3AB1 (Trotta 2009). Secondary antibodies were horseradish peroxidase-coupled (HRP) anti-mouse (Santa Cruz), anti-chicken (Bethyl) and

anti-bovine (KPL). For ELISA, the antigen was quantified with Pierce™ BCA Protein Assay (Thermo Scientific™) and the colorimetric reaction was conducted with TMB-based BD OptEIA™ (BD Biosciences). Procedures made use of protocols recommended by the manufacturer.

Generation of the recombinant baculoviruses

The recombinant baculoviruses were generated following the Bac-to-Bac® system's recommended procedures. Briefly, *polh*, *polh*_{E44G} and $\Delta 3AB_{1-3}$ were amplified by PCR with primers listed in Table S1. PCR products were cloned into donor plasmid pFastBac™ Dual (Thermo Scientific™). First, *polh* amplified from genomic DNA of wt AcMNPV was cloned in frame between *Sall* and *SpeI* restriction sites, upstream *polh* promoter, to generate pFBD*polh*. The nucleotide sequence encoding amino acids 1517–1650 from FMDV A/Arg/01 (accession number KY404935.1) was cloned into pFBD*polh* between *SpeI* and *NotI* sites, resulting in pFBD*polh*- $\Delta 3AB_{1-3}$. Finally, wt *polh* sequence or *polh*_{E44G} amplified from p_{XXL}POLH_{E44G} plasmid (López et al. 2011) were cloned into *XhoI* restriction site, under *p10* promoter, to obtain pFBD*polh/polh*- $\Delta 3AB_{1-3}$ and pFBD*polh*_{E44G}/*polh*- $\Delta 3AB_{1-3}$, respectively. Constructions were sequenced to confirm their identity and then transposed into baculovirus bacmid in DH10Bac™ *Escherichia coli* strain. Purified bacmids were transfected into Sf9 cells using Cellfectin® II reagent (Invitrogen), following manufacturer's suggestions. Recombinant baculoviruses vPOLH and vPOLH_{E44G} were harvested and tittered by end-point dilution technique (O'Reilly et al. 1994).

Purification of rOBs

Sf9 cells were infected with wt AcMNPV, vPOLH or vPOLH_{E44G} at multiplicity of infection (MOI) of 5. After 5 days post-infection (dpi), infected cells were harvested and sonicated with 30-s pulses of 35% of amplitude in a VCX 500 (Sonics) sonicator. The sonicated cells were centrifuged at 2500×g for 15 min and pellet was washed with 0.5% SDS and then with 0.5 M NaCl. The purified rOBs were resuspended with bidistilled water.

Western blot and protein quantification assays

Aliquots of rOBs were dissolved in 0.1 M Na₂CO₃ and then resolved in 12% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane for western blot analysis with the antibodies mentioned above. The yields of total fusion protein were measured from 2.5×10^6 Sf9 cells infected with vPOLH or vPOLH_{E44G} ($n = 4$). Briefly, total purified rOBs and serial dilutions of BSA were resolved in 12% SDS-PAGE and then Coomassie blue stained. The gels were

photographed, converted to 8-bit images and the density of pixels was measured with ImageJ software (NIH).

Bovine serum samples

For western blot analysis, nine positive sera from infected cattle provided by the Food and Agriculture Organization (FAO) and the National Sanitary Authority in Argentina (SENASA) or obtained from a well-characterized bovine serum panel from the Instituto Nacional de Tecnología Agropecuaria (INTA) were employed. Additionally, a total of 24 sera were used to analyze the capacity of POLH- $\Delta 3AB_{1-3}$ to differentiate infected from vaccinated animals in an ELISA test. The sera from non-exposed cattle obtained from the FMD-free region of Argentina were used as negatives. Seroconversion in the vaccinated animals was confirmed as described previously (Jaworski et al. 2011). Sera from cattle infected experimentally with FMDV O1 Campos at 21 dpi were considered positives. All infected animals developed clinical signs and the infection was confirmed by RT-PCR and virus isolation by Jaworski et al. (2011).

ELISA

Immulon™ 1 B 96-well plates (Thermo Scientific™) were coated in PBS with 20 ng per well of rOB samples previously dissolved as described above. The wells were blocked with PBS 0.05% Tween-20 (PBS-T) and 5% skimmed milk for 1 h at 37 °C. Bovine sera from positive, vaccinated or negative animals were 50-fold diluted in PBS-T and 3% skimmed milk and incubated for 1 h at 37 °C. Between each step, several washes with PBS-T were made. Then, each well was incubated with a 1:1000 dilution of the secondary antibody for 1 h at 37 °C. Following the development of the reaction, the OD was measured and the cut-off value was defined as the mean of the negative samples + 3 standard deviations.

Statistical analysis

Statistical differences were estimated with two-tailed *t* tests with *p* value threshold of *p* < 0.01.

Results and discussion

To design the optimum antigenic sequence to incorporate into baculovirus OBs, we analyzed NSP candidates of FMDV (Fig. S1a). 3A and 3B proteins were selected due to their antigenic properties (Höhlich et al. 2003; López et al. 2005; Jaworski et al. 2011). Then, we conducted a thorough sequence analysis to determine the suitability of these polypeptides to be fused to POLH. The NSP 3A has

a hydrophobic domain of 20 amino acids in the N-terminal region that is involved in an homodimer formation and the association with membranes (González-Magaldi et al. 2012, 2014). Even when fused to 3B, 3A does not lose the capability of interacting in vitro with cellular membranes (González-Magaldi et al. 2014). As previously shown, membrane proteins are less likely to be incorporated into polyhedra (Ikeda et al. 2006). Therefore, we deleted 3A N-terminal region to eliminate its hydrophobic properties, though conserving the antigenic region (Fig. S1b, S1c). The resulting polypeptide, $\Delta 3AB_{1-3}$, consists of amino acids 1517–1650 from FMDV A/Arg/01 (accession number KY404935.1). Its coding sequence was cloned into pFastBac™ Dual plasmid downstream from POLH C-terminal sequence, under the control of *polh* promoter.

To assess which strategy is the most convenient to improve POLH- $\Delta 3AB_{1-3}$ yields, we designed two constructions (Fig. 1a). In addition to the fusion protein, these expressed under *p10* promoter an additional copy of POLH or POLH_{E44G}. After generation of recombinant baculoviruses with Bac-to-Bac® system, both viruses were compared and further characterized. As expected, vPOLH produced polyhedra with morphology similar to wild type, with a mean diameter of $1.5 \pm 0.5 \mu\text{m}$ (Fig. S2). At the same time, polyhedra produced by vPOLH_{E44G} were larger in size (Fig. 1b) with a mean diameter of $3.5 \pm 1.3 \mu\text{m}$ and diameters up to $7.5 \mu\text{m}$ (Fig. S2). This phenotype is in accordance with what was previously observed for this POLH variant (López et al. 2011). A statistically significant difference (*p* < 0.01) of polyhedron diameter was found between vPOLH and vPOLH_{E44G}, whereas polyhedra from vPOLH were no different from wt AcMNPV (Fig. S2).

The purified OBs from wt AcMNPV and rOBs were dissolved and analyzed by western blot. In rOBs from vPOLH and vPOLH_{E44G}, the anti-POLH antibody recognized two proteins, as expected (Fig. 1c). At approximately 29 kDa, a band was observed corresponding to POLH or POLH_{E44G}. It could also be observed a band with lower electrophoretic mobility at approximately 44 kDa corresponding to the fusion protein POLH- $\Delta 3AB_{1-3}$. This was confirmed with the anti-3AB1 monoclonal antibody that recognized the same band. As expected, no bands of the size of the fusion protein were detected in wt polyhedra (Fig. 1c). The relative intensity of the bands suggests a higher recovery of fusion protein from vPOLH_{E44G} virus. Therefore, to test whether vPOLH_{E44G} virus in fact produces greater yields of the antigen, we performed a quantitative assay. Given that rOBs are composed of both POLH or POLH_{E44G} and POLH- $\Delta 3AB_{1-3}$, a densitometric method was selected over a total protein quantification assay. First, we determined the optimal infection conditions (data not shown) and a MOI of 5 and harvesting at 5 dpi were selected for further quantification procedures. The mass

Fig. 1 **a** Diagram illustrating the constructions designed for the donor vectors. These carried the sequence of the fusion protein POLH-Δ3AB₁₋₃ and an additional copy of *polh* or *polh_{E44G}* for the obtention of vPOLH and vPOLH_{E44G} baculoviruses, respectively. **b** Sf9 cells infected with vPOLH and vPOLH_{E44G} baculoviruses, showing that polyhedra from vPOLH_{E44G} were larger in size. **c** Western blot analysis of dissolved rOBs isolated from wt AcMNPV, vPOLH and vPOLH_{E44G}. The fusion protein POLH-Δ3AB₁₋₃ obtained by each

of the recombinant baculoviruses was detected with anti-POLH and anti-3AB1 antibodies. No bands of fusion protein were identified in wt polyhedra, whereas POLH was detected with anti-POLH antibody. **d** Quantitative assay for the comparison of the relative amounts of recombinant protein obtained from vPOLH and vPOLH_{E44G} rOBs. The *cis*-complementation of POLH_{E44G} presented an increment of POLH-Δ3AB₁₋₃ yield, in comparison with POLH. Asterisk indicates a statistically significant difference with $p < 0.01$

of fusion protein from purified rOBs of three independent assays was estimated as described in “Methods” and further explained in (López et al. 2018b). As seen in the western blot, vPOLH_{E44G} produced the larger amount of fusion protein incorporated into polyhedra (Fig. 1d). Under the conditions tested, rOBs from vPOLH_{E44G} reached 3247 ± 433 ng of POLH-Δ3AB₁₋₃, whereas vPOLH produced rOBs containing 1732 ± 333 ng of the fusion protein. This increase was statistically significant ($p < 0.01$), proving that the *cis*-complementation of POLH_{E44G} constitutes an excellent strategy for rOB production. The advantages of using this variant of POLH might be due to the increase of polyhedra diameter, but to understand the mechanism that leads to this increment further testing is required. These results are consistent with our previous reports, where rOBs carrying POLH_{E44G} increased fusion protein yield with a *trans*-complementation approach

(López et al. 2018b). For POLH-Δ3AB₁₋₃, no considerable differences between *trans*- or *cis*-complementation with POLH_{E44G} were detected (data not shown). Thus, we achieved a simplification of the strategy maintaining the improved yields. For this reasons, rOBs from vPOLH_{E44G} were selected for subsequent serological methods.

Next, we tested if antibodies recognizing the fusion protein could be detected in sera derived from infected cattle. We evaluated nine sera comprised of one negative animal, one vaccinated and seven infected animals with different serotypes of FMDV. Sera from cattle infected with isolates studied presented antibodies against POLH-Δ3AB₁₋₃ (Fig. 2a). Certain differences in the intensity of the bands corresponding to the fusion protein were observed, in agreement with previous NSP-based tests (López et al. 2005; Gao et al. 2012). On the contrary, no signal was detected with negative and vaccinated sera. Thus, these results indicate

Fig. 2 **a** Immunoreactivity of POLH- Δ 3AB₁₋₃ detected by western blotting with sera from cattle infected with different serotypes of FMDV. Positive sera included isolates A/Arg/01, A Iran/96, O1 SKR/00, O1 Caseros/67, C3 Resende/55, C3 Arg/85, and Asia 1 Shamir. No immunoreactivity was detected in negative and vaccinated sera. **b** ELISA with dissolved rOBs against negative, vaccinated and infected cattle. The results presented are the mean of two replicates. The solid horizontal line indicates the cut-off value

that the translational fusion to POLH does not alter the antigenicity of Δ 3AB₁₋₃ under the conditions tested.

Finally, we conducted a preliminary assessment of the capability of rOBs to be used as antigens in the design of an ELISA-based DIVA test. For this purpose, we evaluated eight sera each of negative, vaccinated and positive animals (Fig. 2b). The cut-off value was arbitrary selected using the criterion described in “Methods”. In agreement with the results of the western blot analysis, 100% of positive animals and none of the negative and vaccinated animals were recognized by ELISA. Therefore, this serological method based on rOBs carrying FMDV epitopes can successfully differentiate infected from vaccinated animals. Moreover, sera from negative and vaccinated animals presented little or none background. This was because, unlike antigens obtained from prokaryotic systems, baculovirus-based expression systems do not present contaminants that need sophisticated purification protocols.

Even though successful attempts to express NSP fused to different proteins were made (Van Dreumel et al. 2015; Lotufo et al. 2017), the purification of soluble proteins from bacterial lysates has significant costs. With the system described here, the production and purification of antigen to

coat a 96-well plate has an approximate cost of US \$0.25. This is due to the ease of rOBs purification that, in contrast with conventional prokaryotic or baculovirus-based expression systems, does not require affinity chromatography steps nor electroelution techniques. Furthermore, a 96-well plate would be enough for the diagnosis with sera samples from up to 88 animals. Taken together, these findings highlight rOBs’ promising characteristics for the upstream and downstream processing of recombinant proteins with diagnostic purposes.

Author contributions MD performed experiments, analyzed the data and wrote the manuscript. MT analyzed the data. VA conceived and designed the experiments and analyzed the data. OT conceived and designed the experiments. MGL conceived and designed the experiments, and wrote the manuscript. All authors commented on the 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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