Impact of recombinant *Drosophila* S2 cell population enrichment on expression of rabies virus glycoprotein

Nayara G. L. Santos · Mayra P. Rocca · Carlos A. Pereira ·
Daniella C. Ventini · Ana Lia P. Puglia · Soraia A. C. Jorge ·
Marcos A. N. Lemos · Renato M. Astray

Received: 11 January 2016 / Accepted: 12 May 2016 / Published online: 23 May 2016
© Springer Science+Business Media Dordrecht 2016

Abstract Recombinant *Drosophila* S2 cells have been used for the expression of many proteins of medical interest. However, membrane-attached glycoproteins, which commonly exhibit lower expression levels compared to soluble proteins, may require special procedures in order to attain high levels of expression. In this study, two S2 cell population enrichment methods (antibiotic and immunomagnetic selection) were evaluated for their ability to enhance expression of the membrane-anchored rabies virus glycoprotein (RVGP). Quantification of RVGP production and determination of its cDNA copy number in transformed cells showed that both enrichment methods increased RVGP expression without significantly affecting its gene copy number. More interestingly, RVGP mRNA levels measured after cycloheximide treatment were poorly correlated with glycoprotein levels. Both enrichment methods enhanced expression of RVGP by recombinant S2 cells, with the highest level of expression achieved using immunomagnetic selection.

Keywords S2 cells · Rabies virus glycoprotein · RT-qPCR · mRNA · Cycloheximide · Immunomagnetic selection

Abbreviations

RVGP	Rabies virus glycoprotein
RT-qPCR	Quantitative reverse transcriptase-polymerase chain reaction
X	Cell concentration (cell/mL)
S2 RVGP	S2 transfected with RVGP and selected by hygromycin resistance
S2 RVGP hy	S2 RVGP cultivated for more 28 days with 600 µg/mL of hygromycin
S2 RVGP imm	S2 RVGP submitted to immunomagnetic selection

Introduction

Many studies have been done with *Drosophila melanogaster* S2 cells that express high levels of recombinant proteins (de Jongh et al. 2013; Moraes et al. 2012). Although some studies have performed selection of S2 clones using approaches based on limit dilution with the use of feeder cells (Yang et al. 2012; Baum and Cherbas 2008), these methods are time-consuming and do not rapidly produce cell populations that are high producers of recombinant proteins,

---

N. G. L. Santos · M. P. Rocca · C. A. Pereira ·
D. C. Ventini · A. L. P. Puglia · S. A. C. Jorge ·
M. A. N. Lemos · R. M. Astray (✉)
Laboratório de Imunologia Viral, Instituto Butantan, Av.
Vital Brasil 1500, São Paulo 05503-900, Brazil
e-mail: renato.astray@butantan.gov.br

which is one of the main advantages of S2 cells over other stable expression systems in which clone isolation is often mandatory. In order to increase production of recombinant protein in S2 cells, some methods for population enrichment have been adopted, such as using high amounts of selection reagents (Lemos et al. 2009; Lee et al. 2009), or immunomagnetic selection (Brillet et al. 2008). Additionally, higher protein levels have been possible with the use of the histone deacetylase inhibitor sodium butyrate to enhance chromatin accessibility (Lemos et al. 2009). These methods are particularly important for the generation of cell populations with high expression of membrane proteins that are otherwise expressed at low levels (Smith 2011). To test two methods for enriching cell populations for high expression of recombinant rabies virus glycoprotein (RVGP) and to understand their mechanisms of action, the expression of RVGP was evaluated after culturing recombinant S2 cells in the presence of high levels of a selection reagent (hygromycin) or after immunomagnetic selection. Levels of correctly folded RVGP were assessed, as were RVGP mRNA levels and the copy number of RVGP cDNA in transformed cells. Results show that the immunomagnetic enrichment process produced higher levels of RVGP than the use of continuous antibiotic pressure. Copy number of RVGP DNA did not statistically differ among the studied cell populations, however RVGP mRNA expression exhibited significant differences. Altogether, our data show that the two enriching strategies have different mechanisms of enhancing expression of RVGP by S2 cell populations.

Materials and methods

Cell line, subpopulations, and culture

The original S2 *Drosophila melanogaster* recombinant cell line used in this work was characterized previously (Lemos et al. 2009; Ventini et al. 2010). Briefly, a single DNA vector containing the RVGP gene and a hygromycin resistance gene cloned under the control of *Drosophila* metallothionein and pCopia promoters, respectively, was transfected into S2 cells, and the S2 RVGP line was established after hygromycin selection (Lemos et al. 2009). After cultivating

the S2 RVGP population for 28 days under high concentrations of hygromycin (600 µg/mL), the S2 RVGP hy subpopulation was established. In addition, treatment with CuSO₄ for 24 h was used to induce RVGP expression in the S2 RVGP population before immunomagnetic selection was performed (as described below) to obtain the S2 RVGP imm subpopulation. See also the list of abbreviations for information.

Immunomagnetic selection of the S2 RVGP imm subpopulation

S2 RVGP cells were gently washed with sterile PBS to remove dead cells. Cells were resuspended in PEB (PBS, 0.5 % BSA, 2 mM EDTA) and cell concentration was determined. The volume equivalent to 3.4×10^7 was centrifuged at $400 \times g$ for 5 min. The cell pellet was suspended with anti-RVGP antibodies (monoclonal D1 antibodies, Institute Pasteur, Paris, France) diluted in 340 µL PEB (1:10) and incubated for 10 min at 4 °C. Cells were washed twice with 3 mL of PEB to remove antibody excess and centrifuged at $400 \times g$ for 10 min at 4 °C. The cell pellet was resuspended with 60 µL anti-mouse IgG magnetic micro beads (MACS 120 TM, Miltenyi Biotec, Bergisch Gladbach, Germany) in 240 µL PEB and incubated for 15 min at 4 °C. Cells were washed to remove unbound microbeads, suspended in 500 µL PEB, and applied to a column placed in a magnetic support. After washing out unbound cells with PEB buffer, bound cells were recovered by removing the column from the support and eluting it with 1 mL PEB directly into a recovery tube. Finally, cells were centrifuged at $400 \times g$ for 5 min at 4 °C, suspended with 1.5 mL of culture medium containing gentamicin sulfate (5.0 µg/mL) and incubated at 28 °C.

Cell culture and sampling

Each population was cultured in triplicate, in three independent studies. Cell culture was performed in suspension (100 rpm) with SF900 III medium (Life Technologies, Sao Paulo, Brazil) in Duran flasks (Schott, Iтуpeva, Brazil) at 28 °C for 96 h. Samples were obtained from cells growing under three experimental conditions, i.e., not induced, induced with CuSO₄ (700 µM), and following treatment with cycloheximide (35 µM).

Rabies glycoprotein analysis

For rabies glycoprotein analysis and quantification, samples of 10^6 cells were treated with lysis buffer (2 mM Tris, 25 mM NaCl, 5 mM KCl, 0.2 % Igepal) and analyzed by ELISA (Rabies Glycoprotein Enzyme Immunoassay, Institut Pasteur), comparatively to a standard curve composed of rabies glycoprotein purified from virus particles, as previously described (Perrin et al. 1996; Astray et al. 2008), or treated with RIPA lysis buffer (10 mM Tris, 140 mM NaCl, 1 % Igepal, 0.5 % Deoxycholate, 0.1 % SDS, 1 mM PMSF, pH 8.0) before performing 12 % SDS-PAGE under non-denaturing conditions (LDS loading buffer, Thermo, Sao Paulo, Brazil). Proteins were transferred to nitrocellulose membrane (100 V for 1 hour), membrane was blocked with low-fat milk and treated with RV1C5 anti-RVGP antibodies (Lifespan Biosciences, Sao Paulo, Brazil) and anti-mouse HRP conjugated antibodies (Thermo). Membranes were revealed with ECL SuperSignal West pico kit (Thermo). For rabies virus glycoprotein detection on the cell membranes, we used an immunofluorescence assay. Cells were grown on coverslips and were induced for RVGP expression for 24 h. The culture medium was discarded and coverslips were washed twice with PBS. Cells were fixed with methanol/acetone 1:1 and incubated with RV1C5 anti-RVGP antibodies (Lifespan Biosciences). After removing the first antibody, cells were labelled with fluorescein conjugated anti-mouse antibodies (Thermo). Samples were visualized in a fluorescence microscope (Olympus BX21) and photomicrographs acquired and analyzed with Cell Sens^{TM} software (Olympus, Sao Paulo, Brazil).

RVGP cDNA and RNA quantification

Samples of 3×10^6 cells were kept at -80°C until analysis. Cell samples were thawed and DNA or RNA was extracted with Purelink^{TM} columns (ThermoScientific). After extraction RNA was quantified by Quant-it^{TM} RNA assay kit (Thermo) that allows individual quantification of total RNA in DNA and RNA mixed samples. For removal of residual DNA, 1.2 μg RNA was treated with 2 U of RNase-free DNase I (Promega, Sao Paulo, Brazil) at 37°C for 30 min, in the presence of RNase inhibitor (RNase-Out^{TM}, Thermo). After the incubation time, the DNase

was inactivated with the enzymatic inhibitor EGTA at 65°C for 10 min. A total of 200 ng of DNA-free RNA was then reverse transcribed with gene specific reverse primers for RVGP or for *Drosophila* Alpha-Tubulin, RVGPr3 (5'-AGCCGCAAGTCTCACTCCC-3') or S2Tub-R (5'-AGCAGGCGTTTCCAATCTG), respectively, and Superscript II^{TM} reverse transcriptase (Thermo). Obtained cDNAs were quantified by qPCR using primers for RVGP or Alpha Tubulin: RVGP9 (5'-TGACTACCACTGGCTTCG), RVGPr1 (5'-TGT AATCGTGGTTAGTGGAGC); or S2Tub-F (5'- TGT CGCGTGTGAAACACTTC) and S2Tub-R (5'- AGC AGGCGTTTCCAATCTG) (Ponton et al. 2011). The RT-qPCR was performed according to published guidelines (Nolan et al. 2006). The reaction standardization was previously published (Astray et al. 2013). Briefly, by evaluating the absence of dimers, lowest Ct, highest end point fluorescence, and absence of signal in the negative controls, the primer concentration was determined as 200 nM for both targets and the best cDNA concentration as 7.2 ng/mL (the same amount was also used for RVGP cDNA amplification). Relative quantification (R) of RVGP cDNA and RVGP mRNA was determined by using a mathematical model (Pfaffl 2001). The amplification efficiencies were obtained from the regression line of Ct to log of tenfold dilutions, being 2.06 for RVGP and 1.72 for tubulin. The RT-qPCR was established on the StepOne4^{TM} Thermal Cycler (Thermo). The reactions were performed in triplicates using Kapa Syber^{TM} green kit (Kapa Biosystems, Wilmington, MA, USA) with 90 ng of cDNA in a final volume of 12.5 μL in MicroAmp^{TM} optical microplates (Thermo). Amplifications were performed using the following temperature profile: 50°C for 2 min, 95°C for 2 min and (95°C for 15 s, 55°C for 30 s, 72°C for 30 s) $\times$ 34. Melting curves were measured from 60°C to 90°C . Results were expressed as fold increase on cDNA or RNA amounts relative to the RVGP cDNA or mRNA amount present in the S2 RVGP sample after 24 h of CuSO_4 induction (calibrator).

Results

The procedures of selective pressure with hygromycin and immunomagnetic selection generated the S2 RVGP hy and S2 RVGP imm subpopulations, respectively. These cell subpopulations presented growth

curves very similar to that of the original population (S2 RVGP), even after the induction of expression, indicating that the selection procedures did not significantly change cell growth. However, RVGP expression was increased by 80 % in S2 RVGP hy and by 120 % in S2 RVGP imm (Fig. 1). To evaluate the quantity of mature RVGP present on cell membranes and the proportion of cells expressing the protein, cells were grown on coverslips and were labelled with anti-RVGP antibodies (Fig. 2). The S2 RVGP hy population exhibited only 3 % more fluorescent cells than S2 RVGP, but the intensity of fluorescence was higher, due to higher expression levels per cell unit (Fig. 1). On the other hand, S2 RVGP imm exhibited 20 % more fluorescent cells than S2 RVGP, indicating an increase in the proportion of cells expressing RVGP at detectable levels. To understand the events underlying cell population enrichment, the number of RVGP gene copies per cell was assessed by qPCR (Fig. 3). We observed no statistical difference in gene copy number between cell populations, indicating that the increase in RVGP expression was not a result of changes in gene copy number per cell, as we could expect after performing selective pressure and/or immunomagnetic selection procedures. As no evidence of gene copy number differences was found, the amount of RVGP mRNA was quantified after induction of expression. In order to evaluate the amount of RNA

actually transcribed after induction, cycloheximide, a translation inhibitor, was added to cell cultures to stop translation activity. We observed a large difference between the amount of RNA found before and after adding cycloheximide in the three populations (Fig. 4). RVGP mRNA levels were similar in cell populations analyzed before translation inhibition. However, after adding cycloheximide to the cultures, RNA accumulated without being translated and reached high levels, especially in the S2 RVGP hy population.

As the amount of RVGP mRNA was not directly correlated with the amount of RVGP detected in cell populations, possible differences in RVGP characteristics between populations could be a matter for the quantification strategy, as the ELISA test used detects only trimeric and correctly matured RVGP. The differences in RVGP monomer size produced by each population were investigated by Western blotting (Fig. 5). Samples of each cell population exhibited a band of around 58 kDa. This molecular weight was expected for RVGP, based on the glycosylation pattern of insect cells and on results of SDS-PAGE under non-denaturing conditions. The slightly smaller RVGP bands probably correspond to different glycoforms. In S2 RVGP hy, there were two more bands of approximately 25 and 35 kDa, which probably correspond to RVGP degradation products.

Fig. 1 Kinetics of S2 cell growth and RVGP expression. S2 cells were cultured in shaken flasks at 28 °C and the cell concentration was periodically determined by hemocytometer counting: S2 RVGP (open diamond); S2 RVGP imm (open

circle); S2 RVGP hy (open triangle). RVGP expression was induced with 700 µM CuSO₄ at 24 h after inoculum. Columns show the RVGP expression obtained at 96 h of culture, analyzed by ELISA after cell lysis and RVGP solubilization

Fig. 2 Immunofluorescence of S2 cells expressing RVGP. S2 cells were cultured on coverslips in 6-well plates. After 48 h of CuSO_4 induction, cells expressing RVGP were fixed and labeled with anti-RVGP antibodies, followed by detection using fluorescein conjugated anti-antibodies (*green dots*). Images were acquired in a fluorescence microscope ($\times 200$), and counts were made by placing a digital grid on the photomicrographs. S2 RVGP, 27 % fluorescent cells (**a**); S2 RVGP imm, 47 %

fluorescent cells (**b**); S2 RVGP hy, 30 % fluorescent cells (**c**). Relative fluorescence units were defined for each sample as the ratio between mean fluorescence intensity of fluorescein (*green channel*) and mean fluorescence intensity of Evans blue counterstain (*red channel*). Fluorescence was determined as 1.43, 1.40 and 1.53 units for S2 RVGP, S2 RVGP imm and S2 RVGP Hy, respectively. (Color figure online)

Fig. 3 Copy number of RVGP cDNA in S2 cell populations, expressed relative to that of the S2 RVGP cell population. DNA was extracted and used for RVGP cDNA quantification by qPCR. Data represent the average of three different experiments $\pm$ standard deviation. There were no statistically significant differences between group means as determined by one-way ANOVA ($p = 0.84$)

Fig. 4 Intra- and interpopulation analysis of relative quantity of RVGP mRNA in S2 cells. Samples of induced S2 cells were analyzed before (*grey columns*) and 3 h after (*dashed columns*) cycloheximide treatment. RVGP mRNA was determined by RT-qPCR, and values are relative to that of control S2 RVGP cells. Recombinant cells were induced with CuSO_4 for 24 h prior to analysis. Data represent the average of three different experiments $\pm$ standard deviation. Relative RVGP mRNA amounts after cycloheximide treatment are statistically different ($p < 0.05$)

Discussion

Improving protein expression levels is an important step in the process of establishing a stable recombinant cell line. In this study, two S2 cell population enrichment methods were evaluated with regard to expression of the membrane-anchored rabies virus

glycoprotein. Maintenance of selective pressure by hygromycin and immunomagnetic selection were evaluated with the goal of increasing the level of expression of RVGP in S2 recombinant cell populations. Both strategies generated S2 populations with increased RVGP expression per cell unit compared to

Fig. 5 Western blotting analysis of RVGP produced by each S2 population. A total of 10^6 recombinant S2 cells were sampled 24 h after induction, lysed with RIPA buffer, separated by 12 % SDS-PAGE under non-reducing conditions, transferred to nitrocellulose membranes, probed with anti-RVGP antibodies, labeled with HRP conjugated anti-antibodies, and revealed by the ECL method. Chemiluminescent bands correspond to RVGP from S2 RVGP imm, S2 RVGP and S2 RVGP hy

that of the original S2 RVGP population. Because no change in growth pattern was observed after enrichment, it was also obtained an increase in volumetric productivity.

The reason for increased expression of the recombinant protein by both S2 RVGP hy and S2 RVGP imm was further investigated. First, the relative amount of RVGP cDNA in each cell population was quantified by qPCR. Results showed that RVGP gene copy number did not differ statistically between the populations, ruling out the possibility that variation in gene copy number contributed to the different levels of expression, an effect that has been previously reported (Deml et al. 1999). In addition, only small differences were found between populations when RVGP mRNA molecules were quantified in samples after induction of expression without the presence of cycloheximide. However, both enriched populations had levels of RVGP mRNA that were higher than that of the original population when the mRNA was quantified in cell samples from cultures treated with cycloheximide for translation inhibition. The difference in the amount

of messenger RNA accumulated in cells under hygromycin selective pressure indicate that for these cells, transcription activity was higher than that of the original population, which may explain the higher RVGP expression observed. One possible explanation for this higher expression of RVGP mRNA is that since the vector transfected into these cells contained both the hygromycin resistance gene and the expression cassette, the enriched cell population may have been selected because it integrated the vector in genome regions that are highly expressed, resulting in both high antibiotic resistance and high RVGP expression. Interestingly, the higher amounts of RVGP mRNA were not sufficient to make these cells high producers of mature RVGP, as S2 RVGP imm produced 40 % more trimeric RVGP. A limited analysis of protein characteristics by Western blotting showed that the RVGP from S2 RVGP hy exhibited forms with low molecular weight, which likely correspond to degradation products. It is known that cell translation and post-translation machinery have limited processing capacity (Palomares et al. 2004), and partially matured forms of proteins can be degraded by proteasome activity. It has previously been reported that an increase of RVGP expression in S2 recombinant cells occurred just after a drop in RVGP mRNA levels (Astray et al. 2013). Here we show that high production of RVGP mRNA does not necessarily result in high protein production. Similar results were found when the expression of monoclonal antibodies by Chinese hamster ovary cells was correlated to mRNA levels (O'Callaghan et al. 2010), and mathematical modelling has shown that the multiple parameters involved in the expression of mRNA and proteins (e.g., competition between mRNA for limited ribosomes) do not allow straightforward prediction of protein levels based on mRNA levels (Mehra et al. 2003). Together, these results show that mRNA expression should be carefully taken into consideration when the objective is to evaluate the expression of a recombinant protein, especially with regard to the decision of which clone or population is the best producer. For the enrichment of recombinant S2 cell populations, the utilization of the immunomagnetic approach has been shown to be very useful (Brillet et al. 2008). In the present study, expression of RVGP was 120 % higher in the immunomagnetic selected population than in the original one, with the former exhibiting a higher proportion of cells expressing

detectable levels of RVGP as well as production of glycoprotein with the expected characteristics and an absence of degradation products. Actually, the method was capable of sorting a recombinant population based on the amount of antibody adsorbed to the cell surface, which was proportional to the amount of RVGP expressed. The cells showing no RVGP or just low levels of recombinant expression were eluted separately. Thus, the immunomagnetic approach is recommended for enhancing recombinant expression in S2 cells after the population is established by antibiotic selective pressure.

Acknowledgments This work was financially supported by grants from FAPESP (2012/24647-0), CNPq (402439/2013-9) and Butantan Foundation. C.A. Pereira is recipient of a CNPq 1A senior fellowship.

References

- Astray RM, Augusto EF, Yokomizo AY et al (2008) Analytical approach for the extraction of recombinant membrane viral glycoprotein from stably transfected *Drosophila melanogaster* cells. *Biotechnol J* 3:98–103
- Astray RM, Jorge SAC, Lemos MA et al (2013) Kinetic studies of recombinant rabies virus glycoprotein (RVGP) cDNA transcription and mRNA translation in *Drosophila melanogaster* S2 cell populations. *Cytotechnology* 65:829–838
- Baum B, Cherbas L (2008) *Drosophila* cell lines as model systems and as an experimental tool. In: Dahmann C (ed) *Methods in molecular biology: Drosophila: methods and protocols*. Humana Press, Totowa, 420:391–494
- Brillet K, Perret G, Klein V et al (2008) Using EGFP fusions to monitor the functional expression of GPCRs in the *Drosophila* Schneider 2 Cells. *Cytotechnology* 57:101–109
- De Jongh WA, Salgueiro S, Dyring C (2013) The use of *Drosophila* S2 cells in R&D and bioprocessing. *Pharm Bioprocess* 1:197–213
- DemL L, Wolf H, Wagner R (1999) High level expression of hepatitis B virus surface antigen in stably transfected *Drosophila* Schneider-2 cells. *J Virol Methods* 79:191–203
- Lee JM, Lee HH, Hwang-Bo J et al (2009) Expression and immunogenicity of recombinant polypeptide VP1 of human hepatitis A virus in stably transformed fruitfly (*Drosophila melanogaster*) Schneider 2 cells. *Biotechnol Appl Biochem* 53:101–109
- Lemos MAN, Santos AS, Astray RM et al (2009) Rabies virus glycoprotein expression in *Drosophila* S2 cells. I. Design of expression/selection vectors, subpopulations selection and influence of sodium butyrate and culture medium on protein expression. *J Biotechnol* 143:103–110
- Mehra A, Lee KH, Hatzimanikatis V (2003) Insights into the relation between mRNA and protein expression patterns: I: theoretical considerations. *Biotechnol Bioeng* 84:822–833
- Moraes AM, Jorge SAC, Astray RM et al (2012) *Drosophila melanogaster* S2 cells for expression of heterologous genes: From gene cloning to bioprocess development. *Biotechnol Adv* 30:613–628
- Nolan T, Hands RE, Bustin SA (2006) Quantification of mRNA using real-time RT-PCR. *Nat Prot* 3:1559–1582
- O'Callaghan PM, McLeod J, Pybus LP et al (2010) Cell line-specific control of recombinant monoclonal antibody production by CHO cells. *Biotechnol Bioeng* 106:938–951
- Palomares LA, Estrada-Moncada S, Ramírez OT (2004) Production of recombinant proteins: challenges and solutions. In: Balbás P, Lorence A (eds) *Recombinant Gene Expression: reviews and protocols*. Humana Press, Totowa, pp 15–52
- Perrin P, Lafon M, Sureau P (1996) Enzyme linked immunosorbent assay (ELISA) for the determination of glycoprotein content of rabies vaccines. In: Meslin FX, Kaplan MM, Koprowski H (eds) *Laboratory techniques in rabies*. WHO, Geneva, pp 383–388
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:2002–2007
- Ponton F, Chapuis MP, Pernice M et al (2011) Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in *Drosophila melanogaster*. *J Insect Physiol* 57:840–850
- Smith SM (2011) Strategies for the purification of membrane proteins. In: Walls D, Loughran ST (eds) *Protein Chromatography: methods and protocols*. Humana Press, Totowa, pp 485–496
- Ventini DC, Astray RM, Lemos MA et al (2010) Recombinant rabies virus glycoprotein synthesis in bioreactor by transfected *Drosophila melanogaster* S2 cells carrying a constitutive or an inducible promoter. *J Biotechnol* 146:169–172
- Yang L, Song Y, Li X et al (2012) HIV-1 virus-like particles produced by stably transfected *Drosophila* S2 cells: a desirable vaccine component. *J Virol* 86:7662–7676