Arch Virol (2016) 161:1769–1782
DOI 10.1007/s00705-016-2835-6

ORIGINAL ARTICLE

Bovine viral diarrhea virus structural protein E2 as a complement regulatory protein

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Received: 14 November 2015 / Accepted: 17 March 2016 / Published online: 1 April 2016

Abstract Bovine viral diarrhea virus (BVDV) is a member of the genus *Pestivirus*, family *Flaviviridae*, and is one of the most widely distributed viruses in cattle worldwide. Approximately 60 % of cattle in endemic areas without control measures are infected with BVDV during their lifetime. This wide prevalence of BVDV in cattle populations results in significant economic losses. BVDV is capable of establishing persistent infections in its host due to its ability to infect fetuses, causing immune tolerance. However, this cannot explain how the virus evades the innate immune system. The objective of the present work was to test the potential activity of E2 as a complement regulatory protein. E2 glycoprotein, produced both in soluble and transmembrane forms in stable CHO-K1 cell lines, was able to reduce complement-mediated cell lysis up to 40 % and complement-mediated DNA fragmentation by 50 %, in comparison with cell lines not expressing the glycoprotein. This work provides the first evidence of E2 as a complement regulatory protein and, thus, the finding of a mechanism of immune evasion by BVDV. Furthermore, it is postulated that E2 acts as a self-associated molecular pattern (SAMP), enabling the virus to avoid being targeted by the immune system and to be recognized as self.

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Introduction

Bovine viral diarrhea virus (BVDV) is a member of the genus *Pestivirus*, family *Flaviviridae*. This virus family comprises many human pathogens of current relevance, such as dengue virus (DENV), West Nile virus (WNV), yellow fever virus (YFV) and hepatitis C virus (HCV). Because of logistical difficulties and the absence of effective vaccines in many cases, BVDV has been proposed as a surrogate model for studying the biology of members of this virus family [1].

BVDV infects animals of the order *Artiodactyla*, which includes cattle, sheep, camelids, antelopes and deer. It is now recognized that BVDV is one of the most widely distributed viruses in cattle worldwide. Approximately 60 % of cattle in endemic areas without control measures are infected with BVDV during their lifetime [2]. This wide prevalence of BVDV infection in cattle populations results in significant economic losses [3, 4].

BVDV elicits two types of infection: transient and persistent infection. Each one is caused by a different virus biotype. Transient or acute infection, associated predominantly with the cytopathic biotype (CP), provokes a wide spectrum of effects ranging from mild to moderate, such as diarrhea and respiratory symptoms, to more severe, such as embryonic death, abortion and congenital malformations in cases of infections of pregnant cattle. On the other hand, persistent infection is achieved when the non-cytopathic biotype (NCP) infects cattle during the 2- to 4-month period of pregnancy. Persistence occurs as a consequence of the calf becoming immunotolerant to BVDV and recognizing it as self. Persistently infected (PI) animals are the main reservoir of virus in nature and the main source of infection in cattle populations [2]. In

all other cases, NCP BVDV induces acute disease in cattle. Clinical presentation might differ depending on whether the animal is infected with type I or type II BVDV.

It is well known that viruses capable of infecting and persisting in their hosts for long periods of time, and even for a lifetime, have evolved sophisticated mechanisms to evade the host immune system. Historically, research has focused on mechanisms designed for evading the adaptive immune system [5–8]. BVDV presents the peculiarity of achieving evasion from the adaptive immune system by its capacity to infect fetuses. However, this mechanism does not explain how BVDV evades the innate immune system (e.g., the complement system). In order to persist in its host during its entire lifetime, BVDV must possess mechanisms that allow it not to be detected and destroyed by the surveillance of the innate immune system.

E2 is the main target of neutralizing antibodies. E2 has proven to be an excellent antigen candidate for developing a subunit vaccine against BVDV [9–14]. It is believed that its efficacy is due to the fact that E2 is the protein responsible for virus binding to its cellular receptors CD46 [15] and LDL-R [15–17]. However, it was recently reported that E2 is able to bind properdin [18], a pattern-recognition molecule involved in the activation of the complement system [19].

Despite being discovered a century ago, only in the last two decades has there been a growing awareness of the critical importance of the complement system in the defense against pathogens. This fact is in part explained by the discovery of many and diverse strategies developed by viruses to evade this system [20–23]. One of the most important of these strategies consists of the expression of complement regulatory proteins (Cregs). Cregs are now considered excellent candidates for rational design of vaccines, since blocking them renders the virus susceptible to complement lytic activity [24].

In view of the above, it was hypothesized that the E2 glycoprotein could be involved in protecting BVDV against the immune system and contribute to its persistence in its natural host. The objective of the present work was to test the potential activity of E2 as a complement regulatory protein. Two experimental approaches were used to evaluate the capacity of the protein to inhibit complement-mediated cell lysis and DNA fragmentation, two distinctive and immediate effects of complement on target cells. The results obtained in this work show that E2 is capable of inhibiting complement-mediated cell lysis and complement-mediated DNA fragmentation. This work provides the first evidence of E2 as a complement regulatory protein and, thus, the finding of a mechanism of evasion of the immune system by BVDV.

Materials and methods

Bioinformatic analysis and recombinant protein expression strategy

The E2 coding sequence from BVDV type I, NADL strain (accession number NC_001461.1, GI: 9626649) was used in this work. This sequence was studied by bioinformatic analysis in order to detect regulatory domains important for protein synthesis. Probable signal peptides were identified using SignalIP [25], transmembrane regions and their topology were predicted using SOSUI [26], and potential N-glycosylation sites were identified using NetNGlyc [27].

Starting from the known amino acid sequence of E2, the corresponding coding region was localized in the BVDV genome at positions 2462 to 3583. The sequence was subsequently translated and bioinformatically analyzed. This sequence contained four N-glycosylation sites at positions 117, 186, 230 and 298 of the mature protein. It also possessed a transmembrane domain between positions 343 and 365. However, this sequence did not contain a signal peptide. However, when the sequence immediately preceding E2 in the BVDV polyprotein, consisting of 16 amino acids and starting with a methionine, was included in the analysis, a potential signal peptide was detected. The most likely cleavage site of this predicted signal peptide was immediately before the histidine that marks the beginning of the mature protein. Therefore, it was assumed that the E2 coding region actually begins at position 2414 of the BVDV genome. The complete bioinformatic analysis is summarized in Figure 1.

Genetic engineering and cloning

DNA fragments corresponding to the region encoding the E2 transmembrane protein (E2, position 2414 to 3583 in the BVDV genome) and a truncated version of E2 without its transmembrane domain (E2T, position 2414 to 3487) were obtained by PCR from a cDNA clone of the complete

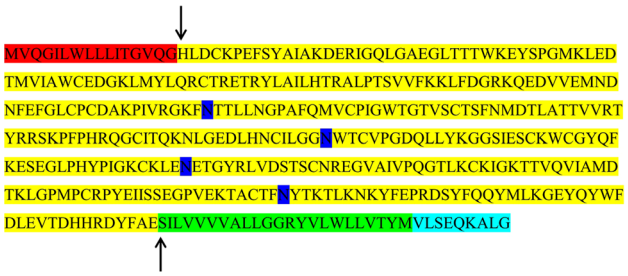

Fig. 1 Bioinformatic analysis. Signal peptide, red; extracellular domain, yellow; transmembrane domain, green; intracellular domain, light blue; N-glycosylation sites, blue. Arrows indicate signal peptide cleavage site and the end of truncated E2 (E2T) (color figure online)

BVDV genome (type I, NADL strain) and cloned into pGEM-T Easy Vector (Promega). Reverse primers included a coding sequence for a C-terminal His_6 tag. A number of positive clones were sequenced and analyzed using ClustalX [28]. Only clones without mutations or with silent mutations were used in the subsequent steps. Selected clones were subcloned into the eukaryotic expression vector pcDNA 3.1 (Invitrogen).

Transfection of CHO-K1 cells and generation of a stable cell line

CHO-K1 cells were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS). Cells were transfected with the expression vectors using cationic lipids (Lipofectamine 2000, Invitrogen). Transfected cells were subsequently cultured in the presence of selective antibiotics (G418, 700 μg/ml; Invitrogen) for several days. After the selection process, individual recombinant clones were isolated by limiting dilution in 96-well plates. Positive clones producing recombinant E2 were detected by Western blot using a mouse monoclonal antibody against E2 and a horseradish-peroxidase-conjugated anti-mouse IgG antibody and visualized by chemiluminescence (PerkinElmer). Positive clones were used for a second limiting dilution process, including verification of protein expression, and then amplified and frozen in liquid nitrogen for future use. A stable cell line expressing the complete E2 protein with the transmembrane domain was generated previously [10].

Optimization of protein expression and purification

Stable cell lines were cultured under different growth conditions in order to optimize protein expression. DMSO (2 %) and sodium butyrate (2 mM) were used as enhancers of recombinant expression because they are known to extend the exponential phase by inducing G1-phase cell arrest [29–33]. Protein levels in cell culture supernatants were screened by ELISA. Briefly, 96-well plates (Maxisorp, NUNC) were coated with a 1:100 dilution of an anti-E2 mouse monoclonal antibody (2.9H). Samples were added and incubated for 1 h at 37 °C. Subsequently, a 1:2000 dilution of a rabbit polyclonal serum and a 1:250 dilution of a rabbit anti-IgG monoclonal antibody were added successively. The reaction was visualized using ABTS (Sigma), and the absorbance was measured at 405 nm in a spectrophotometer (Multiskan EX, MTX Lab Systems).

Protein purification was carried out by immobilized metal affinity chromatography (IMAC). Supernatants from stable cell lines were collected after 7-8 days of culture, centrifuged for 30 min at 2,000×*g* and used for protein purification. PMSF and leupeptin were added as protease inhibitors. A 10× binding/washing (BW) buffer (3 M NaCl, 0.5 M $NaHPO_4$) and 13 mM imidazole were added to cell cultures in order to increase the ionic strength and reduce nonspecific protein binding. Ni-NTA (QIAGEN) was used at a ratio of 1.67 ml of resin per L supernatant. Supernatants were incubated overnight with shaking at 4 °C. The resin was washed with 1× BW buffer containing 13 mM imidazole until the absorbance at 280 nm was negligible. Protein was eluted from the resin with BW buffer 1× containing 200 mM imidazole. Eluates were washed with PBS and concentrated using Amicon-Ultra 4 centrifugal filter devices (Millipore). Purity and protein concentrations were determined by SDS-PAGE and Coomasie blue staining using a BSA standard curve. Gel analysis was performed using ImageJ [34]. Purified proteins were stored at –70 °C.

E2 surface binding to peripheral blood mononuclear cells (PBMCs)

Bovine PBMCs were isolated from heparinized blood using Ficoll-Paque PLUS (Amersham). In each assay, 5×10^5 cells were incubated with 1) recombinant protein (0.5 μg, 30 min at 4 °C); 2) anti-E2 monoclonal antibody (30 min. at 4 °C); 3) PE-labeled rat anti-mouse IgG (30 min at 4 °C). Analysis was performed using a FACScan flow cytometer (BD Biosciences). Typically, 10,000 events were acquired per assay.

Complement-mediated cell lysis

Rationale

The most commonly used methods to measure complement-mediated cell lysis rely on using vital stains that detect membrane damage. However, these methods do not provide direct evidence of cell lysis. Another method that is not so widely used involves measuring malate dehydrogenase (MDH) enzyme activity released from red blood cells. The problem with this method is that red blood cells are much more susceptible than nucleated cells to complement-mediated cells lysis.

Beta-galactosidase is an enzyme widely used as a reporter of cellular events or changes in expression levels. It is a tetrameric and voluminous protein with approximate dimensions of 135 Å in width and 175 Å in length and therefore cannot pass through **?membrane attack complexes?** (MACs). As a consequence, the only possible reason for finding it in cell culture supernatants is cell lysis. On the other hand, CHO cells are particularly suitable for

studies of complement activity, due to their incapability of activating or expressing membrane regulatory proteins that could interfere with the complement system from other species [35]. Thus, it was developed as an experimental system to measure complement-mediated cell lysis, combining the two aforementioned aspects by generating a stable CHO-K1 cell line for the constitutive cytoplasmic expression of beta-galactosidase.

The experimental design consisted of the following steps: (1) cell sensitization by anti-CHO polyclonal antibodies, (2) addition of bovine serum as a source of complement, and (3) quantification of cell lysis by measuring beta-galactosidase activity in cell culture supernatants.

Protocol

A stable CHO-K1 cell line constitutively expressing beta-galactosidase, was seeded at an initial density of 10^5 cells/ml in 96-well plates and grown to confluency. Cells were first sensitized with anti-CHO polyclonal antibodies for 30 min at 4 °C. For that purpose, an inactivated bovine serum generated by immunization of cattle with CHO-K1 cells was used at a 1:100 dilution in RPMI-1640. Cell monolayers were then treated with bovine serum, as a source of complement, for 1 h at 37 °C. This serum was prepared at a concentration of 20 % in GVB^{++} buffer (142 mM NaCl, 5 mM sodium diethylbarbiturate, 0.1 % gelatin, 0.15 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4). At this step, different concentrations of purified E2 (0-50 μg/ml) were added. Finally, cell culture supernatants were transferred to a new plate, and beta-galactosidase activity was measured colorimetrically using ONPG (β-Gal Assay Kit, Invitrogen). Absorbance was measured at 405 nm in a spectrophotometer (Multiskan EX, MTX Lab Systems). Intact cell cultures and cells lysed with detergent (1 % Triton X-100) were used as negative and positive controls, respectively.

Complement-mediated DNA fragmentation

Rationale

Complement-mediated DNA fragmentation is a relatively fast event that can be detected as early as 30 min after treatment with complement [36, 37]. Moreover, fragmented DNA can pass through nuclear pores and MACs and be released subsequently into the medium [38]. This is the basis for quantification of apoptosis by detection of the sub-G1 region, which entails DNA loss as a consequence of membrane permeabilization during the procedure of staining with propidium iodide [39]. Therefore, the possibility of measuring complement-mediated DNA fragmentation immediately after treatment with complement by DNA content analysis was tested. In order to develop a more precise and exhaustive method, DNA content analysis was coupled to high-content flow cytometry data analysis (HC-FCM) [40]. Along with greater precision, this approach makes it possible to obtain more information and to study other relevant cell state parameters, such as changes in the cell cycle.

Protocol

Stable CHO-K1 cell lines expressing beta-galactosidase (control) and full-length (transmembrane) E2 glycoprotein (experimental) (5×10^5 cells/experiment) were sensitized with different dilutions of a bovine anti-CHO polyclonal serum for 30 min at 4 °C. Afterwards, cells were washed twice and incubated with different concentrations of complement (bovine serum prepared in GVB^{++} buffer) for 1 h at 37 °C. Cells were washed again and stained with propidium iodide (PI buffer: 100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1 % Nonidet P-40) for 30 min at room temperature. Samples were evaluated by flow cytometry (FACScan, BD Biosciences), acquiring 10,000 events in each case.

Analysis

Bioinformatic analysis was performed using high-content flow cytometry data (HC-FCM). Cytometric fingerprinting was conducted using the package FlowFP [41–43], which belongs to the Bioconductor project. Cell cycle analysis was performed using FlowJo (Tree Star, Inc.). A Watson model was used, and G2=2×G1 and a G1 fixed value were assigned as constraints.

Cytometric fingerprinting (CF) consists of the recursive binning of the phenotypic space, based on the choice of a given number of parameters and on the direction of maximum variability [41–43]. The vector that specifies the number of events in each bin represents the fingerprint of the sample. Cytometric fingerprinting presents various advantages for its use in this work. Firstly, it does not assume any type of distribution model. This allows detection of rare and unusual events that are lost in a treatment based on Gaussian models. This would be the case if events at the sub-G1 region appear. Secondly, CF enables the comparison of many samples and their analysis with a unique statistical model.

The binning model was generated using the samples corresponding to LACZ. The model was constructed with the sum of all samples, which allowed a suitable model to be developed that was capable of discriminating between the different variations in the cell population as a consequence of complement activity. The chosen parameters for the binning process were cell size and DNA content, which were the most variable parameters in the entire sample. A

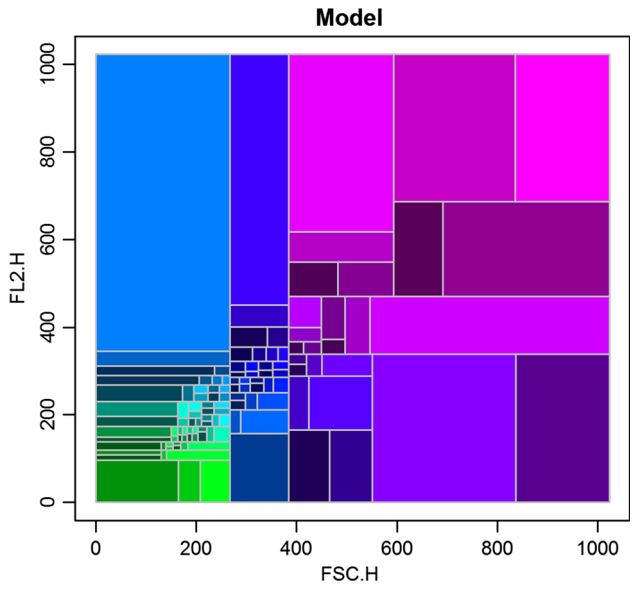

Fig. 2 Cytometric fingerprinting: binning model. The binning model was generated using the samples corresponding to LACZ, using the function flowFPModel from the package flowFP. The chosen parameters were cell size (FSC.H) and DNA content (FL2.H). The recursion level was set to 7, which determines a model with 128 bins

recursion level of 7 was used, resulting in a model with 128 bins (Fig. 2).

Data analysis, statistical analysis and data visualization

Data analysis, statistical analysis and data visualization were carried out using the programming language R [44–46]. Many R packages were used in this work, especially several included in the Bioconductor project [47]. For instance, flow cytometry data analysis and visualization were carried out using flowCore [48] and flowViz [49], respectively. On the other hand, data visualization was conducted mainly using lattice [50] and pheatmap [51]. Statistical analysis was carried out by analysis of variance (ANOVA) and the Tukey test, using $P < 0.05$ as evidence of significant differences.

Results

Biochemical and biological characterization

A truncated form of the E2 glycoprotein (amino acids 1-342) was produced in a stable CHO-K1 cell line, collected from the supernatant and purified by affinity chromatography (IMAC). The protein obtained by this procedure was of the expected size of around 50 kDa (Fig. 3A, left), and was antigenically active as evidenced by Western blot (Fig. 3A, right).

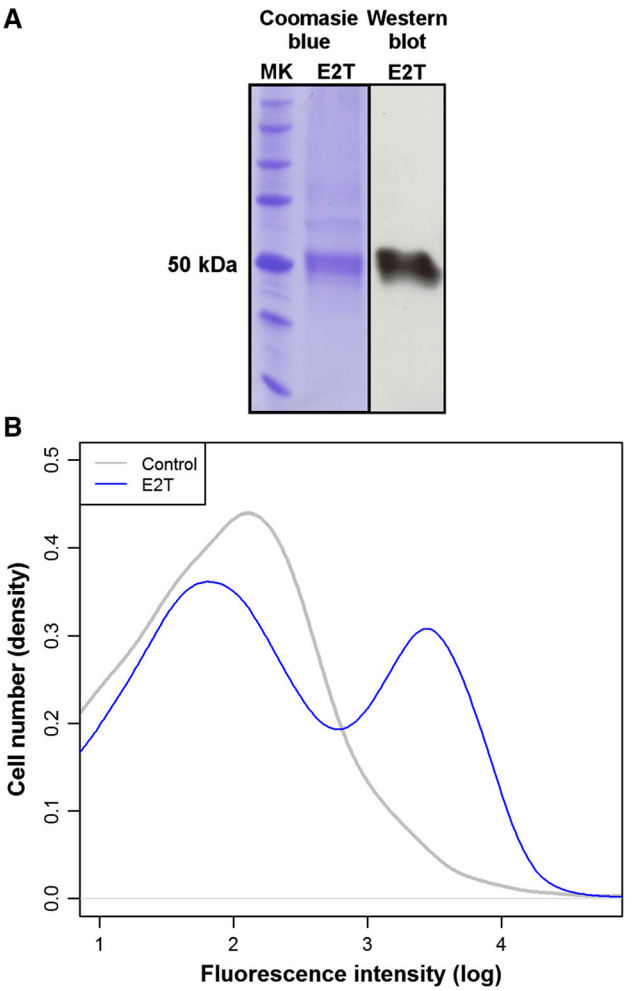

Fig. 3 Production, purification and characterization of a secreted form of E2. (A) Cell culture supernatants from a CHO-K1 stable cell line producing truncated E2 (E2T) were collected. E2T was purified by affinity chromatography (IMAC) and concentrated by filter centrifugation. Aliquots were then analyzed by SDS-PAGE, and the gel was stained with Coomasie blue. A band of the expected size of 50 kDa was obtained at high purity (left). The identity of the protein and its antigenicity were confirmed by Western blot, using a monoclonal antibody against E2 from BVDV (right). (B) The E2 glycoprotein was capable of binding to the surface of bovine PBMCs, as demonstrated by flow cytometry analysis

To verify the biological activity of the product, bovine peripheral blood mononuclear cells (PBMCs) were incubated with the protein and labeled for surface binding analysis by flow cytometry. As expected, E2 glycoprotein was capable of binding to the surface of these cells, most probably through its known receptors CD46 and LDL-R (Fig. 3B).

Complement-mediated cell lysis

First, the sensitivity and specificity of the assay for measuring complement activity through its lytic function on

target cells were tested. For that purpose, the ability of complement to induce cell lysis in the presence of different sensitizing antibodies and bovine serum concentrations was evaluated by measuring beta-galactosidase activity in cell culture supernatants.

Cell lysis, measured by beta-galactosidase release into cell culture supernatants, was directly proportional to the concentrations of sensitizing antibodies and bovine serum (Fig. 4A). Sensitizing antibodies highly potentiated complement lytic activity, which began to be detected at concentrations above 10 %. A more detailed analysis of the data showed an abrupt change in complement activity at absorbance values between 0.25 and 0.3, which is shown in Figure 4B as a change in color from blue to yellow. The values above this level clearly indicated complement lytic activity, whereas its activity was negligible at lower levels. This value was therefore established as the threshold at which complement-mediated cell lysis began. Concentrations above this threshold were regarded as "lytic levels", and concentrations below it as "sub-lytic levels". The results were consistent and indicative of the capacity of the assay to quantify complement lytic activity in a precise and sensitive way.

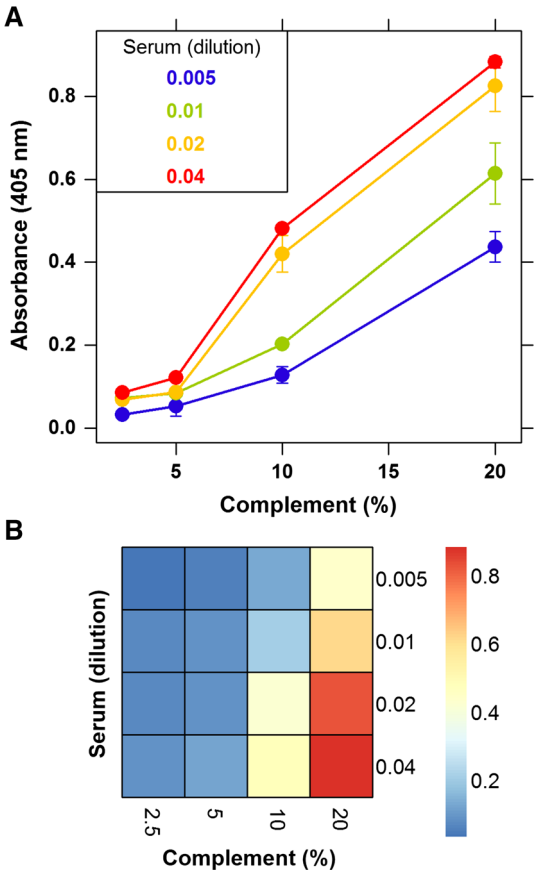

Fig. 4 Complement-mediated cell lysis: testing the assay. (A) Cells from the stable LACZ cell line were seeded in 96-well plates and incubated successively with various concentrations of sensitizing serum (30 min, 4 °C) and complement (1 h, 37 °C). Subsequently, cell culture supernatants were transferred to a new plate. ONPG was added, and absorbance was measured at 405 nm. The results represent the mean and standard error of three independent experiments. (B) Mean results obtained in A visualized as a heatmap

A potential role of E2 as a complement regulatory protein was evaluated. This was accomplished by adding various concentrations of truncated E2 (without its transmembrane domain) to this assay. The results are summarized in Table 1. E2 was capable of inhibiting complement-mediated cell lysis up to 40 %. This level of inhibition was reached at a concentration of 20 μg/ml. No further inhibition was detected at a higher protein concentration.

Complement-mediated DNA fragmentation

A stable LACZ cell line was treated with various concentrations of serum and complement, and cell DNA content was analyzed by propidium iodide staining and flow cytometry.

Treatment with complement caused the appearance of the sub-G1 region in a dose-dependent manner (Fig. 5A). The increase in intensity of treatment, visualized as a shift from light green to dark blue in the corresponding curves, was accompanied by the appearance of cells with lower fluorescence intensities. Moreover, the curve peaks, initially located at the G1 region, were displaced to the sub-G1 region. The same effect was evident with the G2 peak, which experienced a displacement to the S region.

The same methodology was also applied to a stable CHO-K1 cell line expressing a full-length (transmembrane) E2 glycoprotein. The treatment with various serum and complement concentrations affected the DNA content distribution in the cell population. However, the effect was less marked than in the previous case, as evidenced by a higher superposition of the distribution curves (Fig. 5B).

Table 1 Complement-mediated cell lysis: inhibition by E2

E2T (μg/ml)	Inhibition (%)
0	0.00±9.58
10	13.77±0.83
20	**41.16±5.65***
50	33.18±1.70

The experiment was carried out exactly as in Figure 3, except for the addition of varying concentrations of purified E2T at the moment of incubation with complement. The sensitizing serum and complement concentrations used were 0.01 (dilution 1/100) and 20 %, respectively. Results represent the mean and standard error of three independent experiments. The asterisk and bold type indicate statistical significance from the control ($P < 0.01$) and the previous concentration ($P < 0.005$), obtained by ANOVA. There is no statistical significance between the two highest concentrations

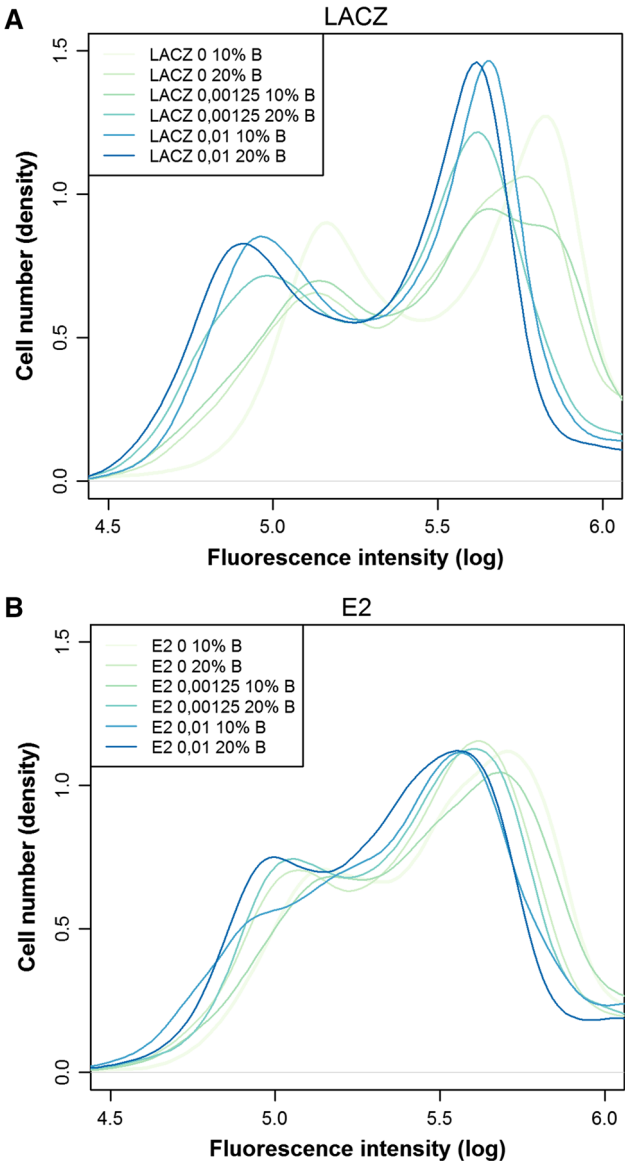

Fig. 5 Complement-mediated DNA fragmentation. Cells from the stable CHO-K1 cell lines LACZ (A) and E2 (B) were sensitized with various dilutions of anti-CHO polyclonal serum for 30 min at 4 °C. Then, cells were incubated with various concentrations of complement for 1 h at 37 °C. Cells were washed and stained with PI buffer for 30 min at room temperature, and the samples were examined by flow cytometry. Density curves corresponding to cell DNA content after treatment (logarithmic scale) are shown. Colors shift from light green to dark blue in accordance to an increase in the intensity of treatment. Example of nomenclature: LACZ (cell line) 0.01 (dilution of sensitizing serum) 20 % (complement concentration) B (series visualized). The results obtained with the series A were very similar (data not shown) (color figure online)

High-content flow cytometry data analysis

The results were then analyzed by high-content flow cytometry analysis. For that purpose, the methodology called cytometric fingerprinting (CF) was used.

CF analysis applied to LACZ and E2 cells are shown in Fig. 6A and B, respectively. In each case, the data are shown as a histogram with the superposition of the fingerprints of the different treatments, that is, as the number of events present in each bin. The bins in which the number of events increased as a consequence of the treatment were selected and located in the corresponding dot plot. The process was repeated with the bins in which the number of events decreased. The first ones were clearly located in the sub-G1 and S regions, and the second ones were located at the G2 region.

Next, bins corresponding to the different cell cycle phases were grouped, and the number of events in each group was computed according to the treatment (Fig. 7). This analysis revealed an evident correlation between the intensity of treatment and the increase (sub-G1 and S regions) or decrease (G2 region) in the number of events (Table 2), but more importantly, this correlation was much more noticeable in LACZ cells. In contrast, E2 was more stable, resilient and less disturbed after the varying treatments with complement. In fact, whereas, in the first case, the sub-G1 region increased up to 16 %, in the second case, it increased only to 8 % (statistical significance found between cell lines, $P < 0.02$, Table 2).

The increase in the S region may be explained in two different ways. One possibility is that complement may have induced cell cycle arrest at the step from S phase to G2, with a subsequent increase in S and decrease in G2. Another possibility is that it may be the result of the appearance of a sub-G2 region, that is, DNA fragmentation and loss by cells in the G2 phase [39]. The similarity between the curves sub-G1 and sub-G2 with respect to shape and displacement, resulting from complement activity (see Fig. 5A), compared to their initial G1 and G2 curves, suggests that this would be the case. However, due to the impossibility of the chosen methodology to discriminate between one case and the other, the sub-G2 region was not used for the calculation of DNA fragmentation.

Finally, the experiment was analyzed using the Cell Cycle platform in the program FlowJo. The mean values of the percentages obtained for each cell cycle phase were computed and visualized as a heatmap (Fig. 8A). In this manner, it was possible to detect an important increase in the number of cells in S phase in LACZ cells, as a consequence of complement activity. More importantly, the increase in the percentage of cells in sub-G1 was more noticeable than in E2. When the percentage of cells at sub-G1 after treatment with 10 % complement was plotted (Fig. 8B), it was found that E2 was capable of protecting cells from complement-mediated DNA fragmentation, reducing it from 13 to 9 % ($P < 0.05$). The reason for choosing this complement concentration

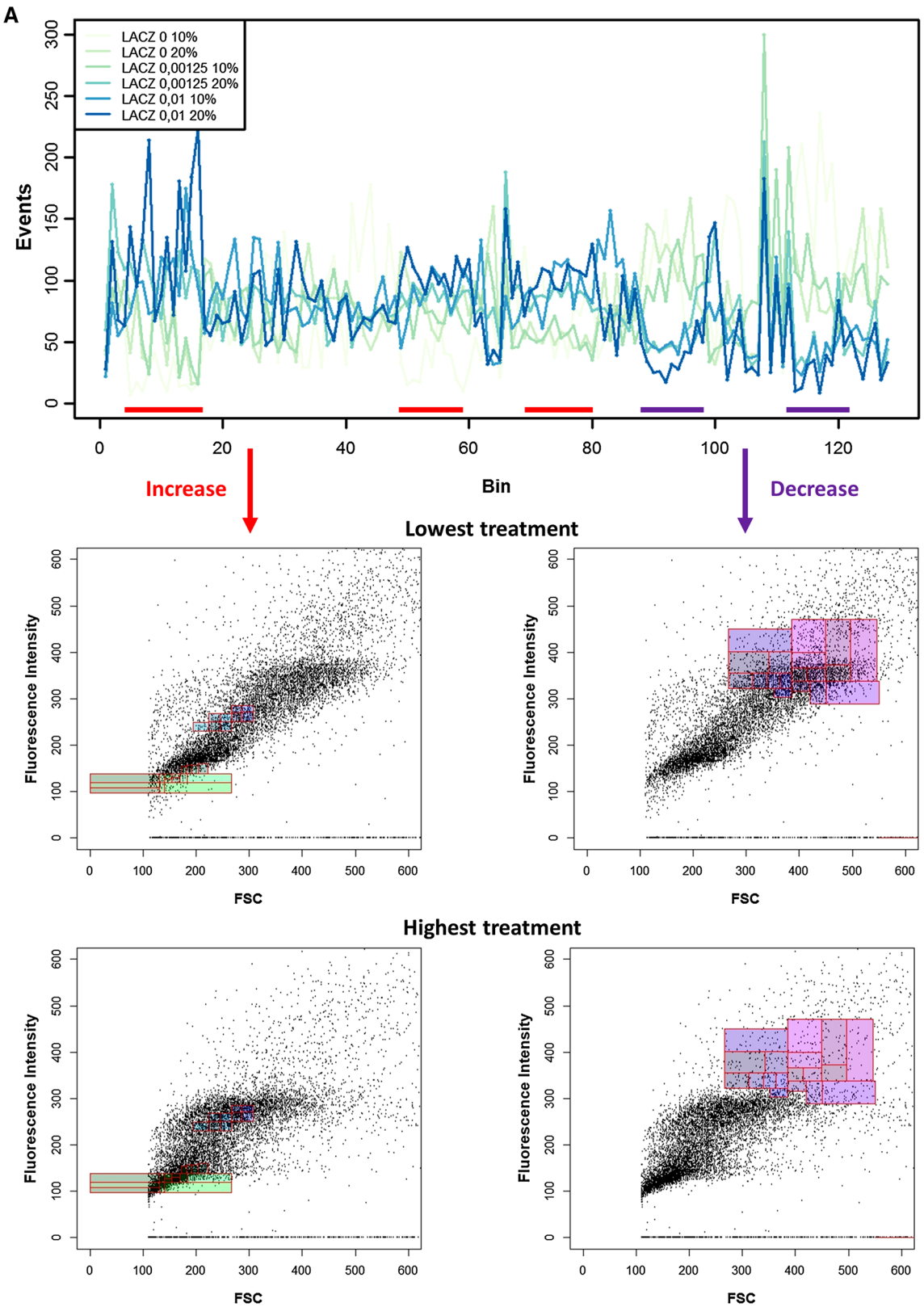

Fig. 6 Complement-mediated DNA fragmentation: cytometric fingerprinting. Samples corresponding to LACZ (A) and E2 (B) were analyzed with the package flowFP. Fingerprints were plotted superimposed in a histogram. Bins that showed clear changes as a consequence of treatment were selected and located on the corresponding dot plots. Bins that manifested an increase (red bars) were located in the sub-G1 and S regions, while those that showed a decrease (violet bars) were located at the G2 region (color figure online)

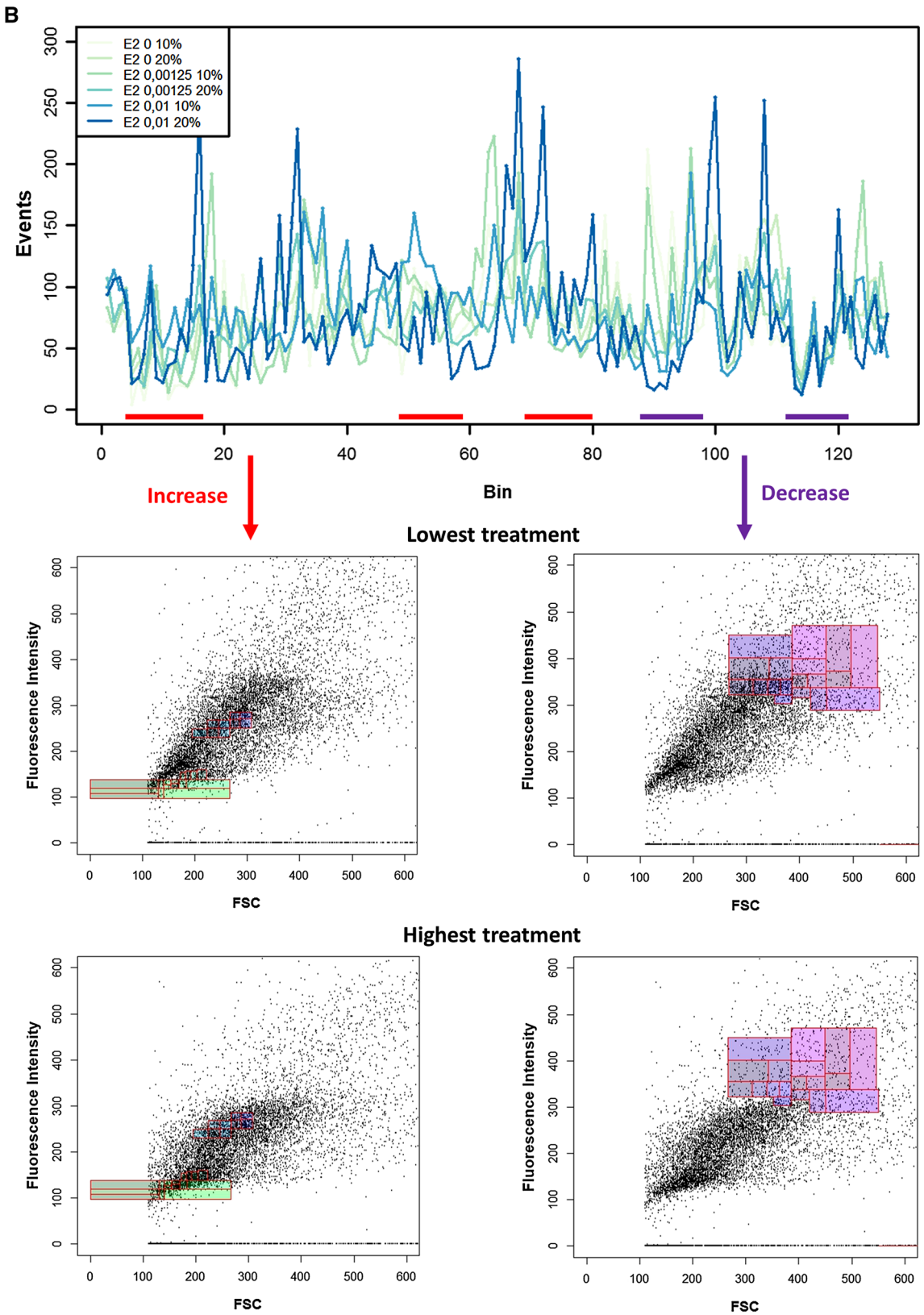

Fig. 6 continued

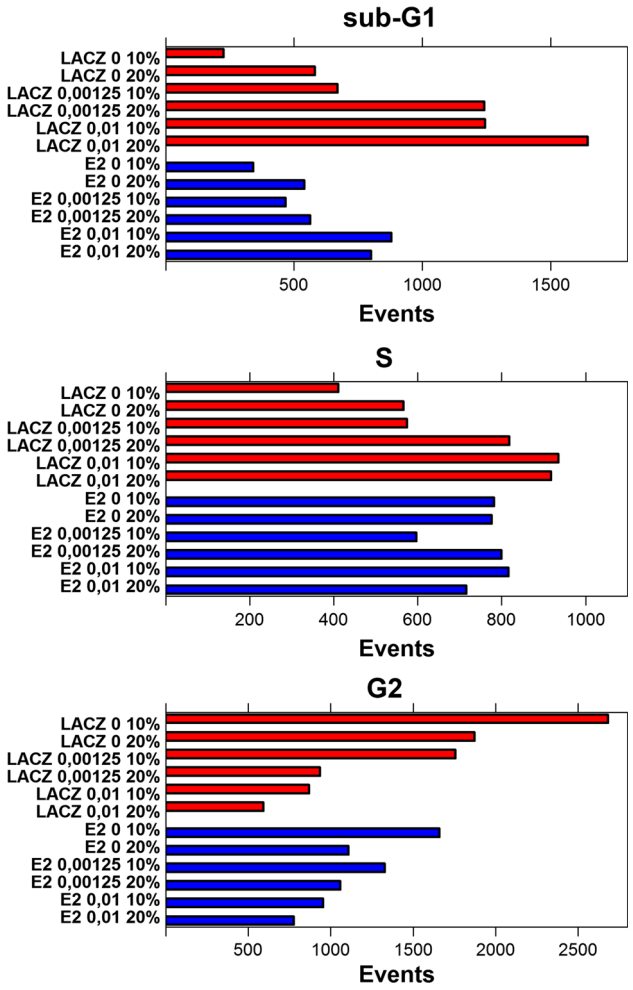

Fig. 7 Cytometric fingerprinting: data analysis. Bins corresponding to the different cell cycle phases were grouped, and the number of events in each group was calculated and plotted according to treatment. The number of total events in all of the samples was the same (10,000 events)

Table 2 Statistical significance of the results obtained in each cell cycle phase, according to cell line and treatment

Cell cycle phase	Cell line	Treatment
subG1	**0.020**	**0.0005**
S	0.581	**0.036**
G2	0.133	**0.0006**

Significant *P*-values, obtained by ANOVA, are given in bold

(10 %) was that the resulting treatments were sublytic. With this, it was intended to show that complement-mediated DNA fragmentation occurred at concentrations that normally were not sufficient to lyse cells. Many explanations have been proposed to explain this phenomenon. Some authors hold that the entry of DNases through MACs from the extracellular medium is responsible for

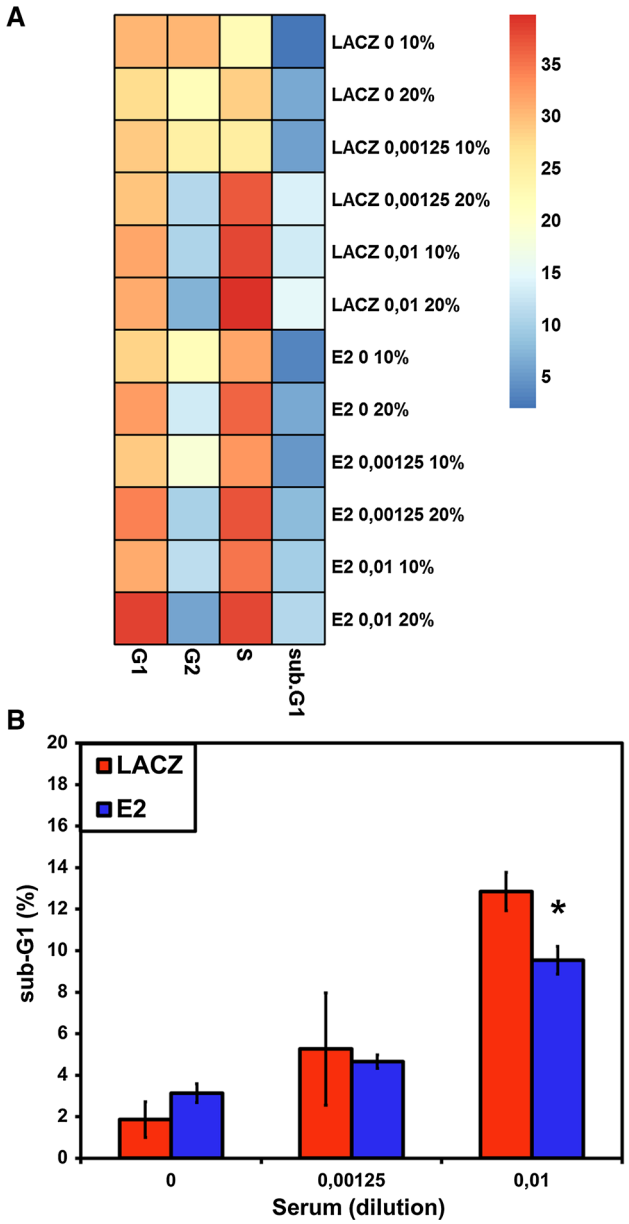

Fig. 8 Complement-mediated DNA fragmentation: cell cycle analysis. Samples were analyzed using the Cell Cycle platform of FlowJo. A Watson model was used, and G2=2 × G1 and a G1 fixed value were assigned as constraints. (A) Heatmap plotting the mean values of the percentage of cells in each phase of the cell cycle. (B) Percentage of cells in the sub-G1 region as a consequence of treatment with different serum dilutions and 10 % complement. Results represent the mean and standard error of two independent experiments. Statistical significance ($P < 0.05$) between LACZ and E2 is denoted by an asterisk (*)

this effect [37]. Other authors think that the reason is the activation of the mitochondrial pathway of apoptosis, caused by the rapid loss of inner mitochondrial membrane potential produced by massive Ca^{++} influx through MACs [52].

Discussion

E2: first Creg of the genus *Pestivirus*, second Creg of the family *Flaviviridae*

The research carried out in this work demonstrates, for the first time, the participation of the glycoprotein E2 from BVDV in complement regulation. E2 was capable of inhibiting complement-mediated cell lysis and complement-mediated DNA fragmentation.

E2 is the first Creg to be discovered in a member of the genus *Pestivirus*. Until now, the only known complement regulatory protein of a member of the family *Flaviviridae* was NS1, a protein found in members of the genus *Flavivirus*, such as West Nile virus (WNV), dengue virus (DENV) and yellow fever virus (YFV) [53, 54]. NS1 is a secreted non-structural glycoprotein that is not produced by members of the genus *Pestivirus* and does not share any relevant sequence homology to the E2 glycoprotein (data not shown). Moreover, NS1 does not share any sequence homology to proteins with CCP modules, the most usual structural domains in Cregs, nor does it have a direct cofactor activity [54]. The mechanism of action of this protein is very different from that of most Cregs, which bind C3b and C4b. NS1 from WNV binds to factor H, which then leads to C3b inactivation by factor I [54]. NS1 from DENV binds to C1s, leading to C4 cleavage to C4a and C4b [53]. NS1 from WNV and YFV is also expected to use this latter mechanism.

Mechanism of action of E2 as complement regulatory protein

E2 does not appear to use either of the mechanisms proposed for NS1, since it is capable of regulating complement activity both in soluble and membrane-bound form. In general, the Cregs that are able to accomplish this are those with a direct inhibitory activity, which is not the case for either mechanism proposed for NS1. The complement regulatory activities of Cregs are basically two: cofactor activity and decay accelerating activity [55, 56]. In the first case, the Creg binds to C3b and C4b and acts as a cofactor for their factor-I-mediated degradation. This is the activity exerted, for instance, by CD46. In the second case, the Creg dissociates C3b and C4b from the protease component of C3 convertase (C2a or Bb).

Recently, it has been shown that E2 glycoproteins from CSFV and BVDV are capable of binding complement factor properdin (CFP) [18]. Properdin is a complement protein that stabilizes the C3 convertase, which results in an increased half-life of the complex [19]. It is therefore possible that E2 binds and inactivates properdin, inducing the disintegration of the C3 convertase, thus blocking the formation of the MAC. In this way, E2 would exert an indirect decay-accelerating effect. However, other interactions and effects of E2 on other complement proteins cannot be ruled out.

Efficiency of E2 as complement regulatory protein

In this work, E2 was capable of inhibiting complement-mediated cell lysis by approximately 40 % at a concentration of 20 μg/ml (0.4 μM). Are these results comparable to the values obtained with other Cregs? Porcine CD46 was found to inhibit complement-mediated rabbit erythrocyte lysis by approximately 40 % at a concentration of 30 μg/ml [57]. Similarly, human CD46 was shown to inhibit complement-mediated CHO cell lysis by approximately 40 % at a concentration of 25 μg/ml [58]. Also, vaccinia virus complement control protein (VCP) and factor H achieved the same levels of inhibition at 0.1 μM and 0.3 μM, respectively [59]. Lastly, Kaposi sarcoma-associated herpesvirus-complement control protein (KCP) and Rhesus rhadinovirus complement control protein (RCP) achieved 60 and 40 % inhibition, respectively, at 0.5 μM [60]. Therefore, the efficiency of E2 as a complement regulatory protein is highly comparable to that of other viral and cellular Cregs.

E2 as a self-associated molecular pattern (SAMP)

CD46, or MCP (membrane cofactor protein), is a glycoprotein that is widely expressed in the majority of cells, including lymphocytes, macrophages and epithelial cells. It is a complement regulatory protein that protects cells from autoimmune destruction, binding C3b and C4b at the cell surface and acting as a cofactor for factor-I-mediated proteolysis [56, 61, 62]. In recent years, it has been recognized that CD46's functions go far beyond these first findings and that it must have a fundamental role in the organism [63, 64]. In fact, some authors have pointed out that CD46 could be a true innate marker of self/non-self discrimination, constituting a self-associated molecular pattern (SAMP), in clear opposition to the widely studied pathogen-associated molecular patterns (PAMPs) [38, 65]. Moreover, T cells activated via CD3 and CD46 are induced to produce IL-10 and differentiate to a regulatory phenotype (Tr1) [66]. Furthermore, there is evidence suggesting that CD46's functions exceed those of complement regulation and that it might have a direct role in the fertilization and reproduction processes, because it is abundantly expressed in placental tissue and in the inner acrosomal membrane of spermatozoa [63, 64].

In our case, E2 glycoprotein, as a membrane Creg, would be expected to inhibit the destruction of BVDV-infected cells by complement. This would also prevent the production of

inflammatory mediators and the recruitment of inflammatory cells, which together would initiate an immune response. In other words, E2 would be acting as an actual SAMP, allowing BVDV-infected cells to be recognized as self by the host's immune system. This novel strategy of viral immune evasion in BVDV, which could be regarded as innate immunotolerance, would be integrated into its other known evasion strategies, such as fetus infection and elimination of the BVDV-specific T-cell repertoire, which are mechanisms of evasion of the adaptive immune system (adaptive immunotolerance). In short, the growing knowledge of BVDV immunobiology leads to an interesting scenario where BVDV represents a virus that is specialized in integration within its host as a self-component of the organism.

Conclusion

This study demonstrates for the first time the participation of the glycoprotein E2 from BVDV in complement regulation. E2 was capable of inhibiting complement-mediated cell lysis and complement-mediated DNA fragmentation. This mechanism would allow BVDV to evade the innate immune system and persist in its host in conjunction with its ability to generate adaptive immune tolerance.

Acknowledgments I would like to thank the anonymous reviewers of this manuscript for their thoughtful and constructive suggestions, which led to a substantial improvement of this article. This work was supported by a fellowship from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

Compliance with ethical standards

Conflict of interest The author declares that he has no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants.

References

1. Buckwold VE, Beer BE, Donis RO (2003) Bovine viral diarrhea virus as a surrogate model of hepatitis C virus for the evaluation of antiviral agents. Anti Res 60:1–15
2. Peterhans E, Bachofen C, Stalder H, Schweizer M (2010) Cytopathic bovine viral diarrhea viruses (BVDV): emerging pestiviruses doomed to extinction. Veterin Res 41:44
3. Houe H (1999) Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Veterin Microbiol 64:89–107
4. Houe H (2003) Economic impact of BVDV infection in dairies. Biologicals 31:137–143
5. Alcami A, Koszinowski UH (2000) Viral mechanisms of immune evasion. Immunol Today 21:447–455
6. Ploegh HL (1998) Viral strategies of immune evasion. Science 280:248–253
7. Finlay BB, McFadden G (2006) Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. Cell 124:767–782
8. Tortorella D, Gewurz BE, Furman MH, Schust DJ, Ploegh HL (2000) Viral subversion of the immune system. Ann Rev Immunol 18:861–926
9. Ostachuk A, Chiavenna SM, Gómez C, Pecora A, Pérez-Filgueira MD, Escribano JA, Ardila F, Dus Santos MJ, Wigdorovitz A (2009) Expression of a ScFv-E2T fusion protein in CHO-K1 cells and alfalfa transgenic plants for the selective directioning to antigen presenting cells. Veterin Immunol Immunopathol 128:315
10. Chiavenna SM, Ostachuk A, Pecora A, Levy S, Dus Santos MJ, Wigdorovitz A (2009) Truncated E2 glicoprotein expression in CHO-K1 cells to produce a subunit vaccine against BVDV. Veterin Immunol Immunopathol 128:231
11. Escribano JM, Wigdorovitz A, Gil F, Ostachuk A, Pérez-Filgueira M, Domínguez J, Serrano C, Dus Santos MJ, Alonso C (2009) Fusion protein that directs vaccine antigens to antigen presenting cells and applications thereof. World Intellectual Property Organization (WIPO) WO/2009/112603
12. Ostachuk A, Gil F, Alonso C, Escribano JA, Pérez-Filgueira MD, Wigdorovitz A, Domínguez J, Nuñez C, Dus Santos MJ (2009) Proteína de fusión con direccionamiento de antígenos vacunales a células presentadoras de antígeno y sus aplicaciones. Instituto Nacional de la Propiedad Industrial (INPI) P080101073
13. Pecora A, Aguirreburualde MSP, Aguirreburualde A, Leunda MR, Odeon A, Chiavenna S, Bochoeyer D, Spitteler M, Filippi J, Dus Santos MJ, Wigdorovitz A (2012) Safety and efficacy of an E2 glycoprotein subunit vaccine produced in mammalian cells to prevent experimental infection with bovine viral diarrhoea virus in cattle. Veterin Res Commun 36:157–164
14. Aguirreburualde MSP, Gómez MC, Ostachuk A, Wolman F, Albanesi G, Pecora A, Odeon A, Ardila F, Escribano JA, Dus Santos MJ, Wigdorovitz A (2013) Efficacy of a BVDV subunit vaccine produced in alfalfa transgenic plants. Veterin Immunol Immunopathol 151:315–324
15. Maurer K, Krey T, Moennig V, Thiel HJ, Rumenapf T (2004) CD46 is a cellular receptor for bovine viral diarrhea virus. J Virol 78:1792–1799
16. Krey T, Moussay E, Thiel HJ, Rümenapf T (2006) Role of the low-density lipoprotein receptor in entry of bovine viral diarrhea virus. J Virol 80:10862–10867
17. Agnello V, Ábel G, Elfahal M, Knight GB, Zhang QX (1999) Hepatitis C virus and other Flaviviridae viruses enter cells via low density lipoprotein receptor. Proceed Nat Acad Sci 96:12766–12771
18. Gladue DP, Baker-Bransetter R, Holinka LG, Fernandez-Sainz IJ, O'Donnell V, Fletcher P, Lu Z, Borca MV (2014) Interaction of CSFV E2 protein with swine host factors as detected by yeast two-hybrid system. PLoS One 9:e85324
19. Kemper C, Atkinson JP, Hourcade DE (2010) Properdin: emerging roles of a pattern-recognition molecule. Ann Rev Immunol 28:131–155
20. Favoreel HW, Van de Walle GR, Nauwynck HJ, Pensaert MB (2003) Virus complement evasion strategies. J General Virol 84:1–15
21. Stoermer KA, Morrison TE (2011) Complement and viral pathogenesis. Virology 411:362–373
22. Bernet J, Mullick J, Singh AK, Sahu A (2003) Viral mimicry of the complement system. J Biosci 28:249–264
23. Vossen MT, Westerhout EM, Söderberg-Nauclér C, Wiertz EJ (2002) Viral immune evasion: a masterpiece of evolution. Immunogenetics 54:527–542

24. Meri S, Jördens M, Jarva H (2008) Microbial complement inhibitors as vaccines. Vaccine 26S:I113–I117
25. Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. J Mole Biol 340:783–795
26. Hirokawa T, Boon-Chieng S, Mitaku S (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics 14:378–379
27. Gupta R, Jung E, Brunak S (2004) Prediction of N-glycosylation sites in human proteins **(In preparation)**
28. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31:3497–3500
29. Berg T, King B, Meikle PJ, Nilssen O, Tollersrud OK, Hopwood JJ (2001) Purification and characterization of recombinant human lysosomal [alpha]-mannosidase. Mole Genet Metab 73:18–29
30. Goulart HR, dos Santos Arthuso F, Capone MVN, de Oliveira TL, Bartolini P, Soares CRJ (2010) Enhancement of human prolactin synthesis by sodium butyrate addition to serum-free CHO cell culture. J Biomed Biotechnoly 2010:405872
31. Carvalhal AV, Santos SS, Calado J, Haury M, Carrondo MJT (2003) Cell growth arrest by nucleotides, nucleosides and bases as a tool for improved production of recombinant proteins. Biotechnol Prog 19:69–83
32. Kumar N, Gammell P, Clynes M (2007) Proliferation control strategies to improve productivity and survival during CHO based production culture. Cytotechnology 53:33–46
33. Liu CH, Chen LH (2007) Promotion of recombinant macrophage colony stimulating factor production by dimethyl sulfoxide addition in Chinese hamster ovary cells. J Biosci Bioeng 103:45–49
34. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9:671–675
35. Liszewski MK, Fang CJ, Atkinson JP (2008) Inhibiting complement activation on cells at the step of C3 cleavage. Vaccine 26S:I22–I27
36. Nauta AJ, Daha MR, Tijsma O, van de Water B, Tedesco F, Roos A (2002) The membrane attack complex of complement induces caspase activation and apoptosis. Euro J Immunol 32:783–792
37. Cragg M, Howatt W, Bloodworth L, Anderson V, Morgan B, Glennie M (2000) Complement mediated cell death is associated with DNA fragmentation. Cell Death Different 7:48–58
38. Elward K, Griffiths M, Mizuno M, Harris CL, Neal JW, Morgan BP, Gasque P (2005) CD46 plays a key role in tailoring innate immune recognition of apoptotic and necrotic cells. J Biol Chem 280:36342–36354
39. Huang X, Halicka HD, Traganos F, Tanaka T, Kurose A, Darzynkiewicz Z (2005) Cytometric assessment of DNA damage in relation to cell cycle phase and apoptosis. Cell Proliferation 38:223–243
40. Robinson JP, Rajwa B, Patsekin V, Davisson VJ (2012) Computational analysis of high-throughput flow cytometry data. Expert Opin Drug Dis 7:679–693
41. Sekiguchi DR, Sutter JA, Rickels MR, Naji A, Liu C, Propert KJ, Rogers WT, Luning Prak ET (2011) Analysis of B cell subsets following pancreatic islet cell transplantation in a patient with type 1 diabetes by cytometric fingerprinting. J Immunol Methods 363:233–244
42. Rogers WT, Holyst HA (2009) FlowFP: A Bioconductor package for fingerprinting flow cytometric data. Advances in Bioinformatics 2009:193947
43. Rogers WT, Moser AR, Holyst HA, Bantly A, Mohler ER III, Scangas G, Moore JS (2008) Cytometric fingerprinting: quantitative characterization of multivariate distributions. Cytometry Part A 73:430–441
44. Ihaka R, Gentleman R (1996) R: a language for data analysis and graphics. J Comput Graph Stat 5:299–314
45. Venables WN, Smith DM, R Development Core Team (2002) An introduction to R
46. Development Core Team R (2008) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria
47. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J et al (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5:R80
48. Hahne F, LeMeur N, Brinkman R, Ellis B, Haaland P, Sarkar D, Spidlen J, Strain E, Gentleman R (2009) flowCore: a Bioconductor package for high throughput flow cytometry. BMC Bioinform 10:106
49. Sarkar D, Le Meur N, Gentleman R (2008) Using flowViz to visualize flow cytometry data. Bioinformatics 24:878–879
50. Sarkar D (2008) Lattice: multivariate data visualization with R. Springer
51. Kolde R (2010) Package pheatmap
52. Tegla C, Cudrici C, Patel S, Trippe R 3rd, Rus V, Niculescu F, Rus H (2011) Membrane attack by complement: the assembly and biology of terminal complement complexes. Immunol Res 51:45–60
53. Avirutnan P, Fuchs A, Hauhart RE, Somnuke P, Youn S, Diamond MS, Atkinson JP (2010) Antagonism of the complement component C4 by flavivirus nonstructural protein NS1. J Exp Med 207:793–806
54. Chung KM, Liszewski MK, Nybakken G, Davis AE, Townsend RR, Fremont DH, Atkinson JP, Diamond MS (2006) West Nile virus nonstructural protein NS1 inhibits complement activation by binding the regulatory protein factor H. Proceed Nat Acad Sci 103:19111–19116
55. Dunkelberger JR, Song WC (2010) Complement and its role in innate and adaptive immune responses. Cell Res 20:34–50
56. Liszewski MK, Post TW, Atkinson JP (1991) Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. Ann Rev Immunol 9:431–455
57. Van den Berg CW, de la Lastra JMP, Llanes D, Morgan BP (1997) Purification and characterization of the pig analogue of human membrane cofactor protein (CD46/MCP). J Immunol 158:1703–1709
58. Christiansen D, Milland J, Thorley B, McKenzie I, Mottram P, Purcell L, Loveland B (1996) Engineering of recombinant soluble CD46: an inhibitor of complement activation. Immunology 87:348
59. Sahu A, Isaacs SN, Soulika AM, Lambris JD (1998) Interaction of vaccinia virus complement control protein with human complement proteins: factor I-mediated degradation of C3b to iC3b1 inactivates the alternative complement pathway. J Immunol 160:5596–5604
60. Okroj M, Mark L, Stokowska A, Wong SW, Rose N, Blackbourn DJ, Villoutreix BO, Spiller OB, Blom AM (2009) Characterization of the complement inhibitory function of rhesus rhadinovirus complement control protein (RCP). J Biol Chem 284:505–514
61. Oglesby T, Allen C, Liszewski M, White D, Atkinson J (1992) Membrane cofactor protein (CD46) protects cells from complement-mediated attack by an intrinsic mechanism. J Exp Med 175:1547–1551
62. Kojima A, Iwata K, Seya T, Matsumoto M, Ariga H, Atkinson JP, Nagasawa S (1993) Membrane cofactor protein (CD46) protects cells predominantly from alternative complement pathway-mediated C3-fragment deposition and cytolysis. J Immunol 151:1519–1527

63. Riley-Vargas RC, Gill DB, Kemper C, Liszewski MK, Atkinson JP (2004) CD46: expanding beyond complement regulation. Trends Immunol 25:496–503
64. Liszewski MK, Kemper C, Price JD, Atkinson JP (2005) Emerging roles and new functions of CD46. Springer Seminars in Immunopathology. pp 345–358
65. Elward K, Gasque P (2003) "Eat me" and "don't eat me" signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system. Mole Immunol 40:85–94
66. Kemper C, Chan AC, Green JM, Brett KA, Murphy KM, Atkinson JP (2003) Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. Nature 421:388–392