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VHSV G glycoprotein major determinants implicated in triggering the host type I IFN antiviral response as DNA vaccine molecular adjuvants

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ABSTRACT

We have recently identified the two major determinants of the glycoprotein G of the viral hemorrhagic septicaemia rhabdovirus (gpG_{VHSV}), peptides p31 and p33 implicated in triggering the host type I IFN antiviral response associated to these rhabdoviral antigens. With the aim to investigate the properties of these viral glycoprotein regions as DNA molecular adjuvants, their corresponding cDNA sequences were cloned into a plasmid (pMCV1.4) flanked by the signal peptide and transmembrane sequences of gpG_{VHSV}. In addition, a plasmid construct encoding both sequences p31 and p33 (pMCV1.4-p31 + p33) was also designed. *In vitro* transitory cell transfection assays showed that these VHSV gpG regions were able to induce the expression of type I IFN stimulated genes as well as to confer resistance to the infection with a different fish rhabdovirus, the spring viremia of carp virus (SVCV). *In vivo*, zebrafish intramuscular injection of only 1 μg of the construct pMCV1.4-p31 + p33 conferred fish protection against SVCV lethal challenge up to 45 days post-immunization. Moreover, pMCV1.4-p31 + p33 construct was assayed for molecular adjuvantcity's for a DNA vaccine against SVCV based in the surface antigen of this virus (pAE6-G_{SVCV}). The results showed that the co-injection of the SVCV DNA vaccine and the molecular adjuvant allowed (i) a ten-fold reduction in the dose of pAE6-Gsvcv without compromising its efficacy (ii) an increase in the duration of protection, and (iii) an increase in the survival rate. To our knowledge, this is the first report in which specific IFN-inducing regions from a viral gpG are used to design more-efficient and cost–effective viral vaccines, as well as to improve our knowledge on how to stimulate the innate immune system.

1. Introduction

Two decades ago deoxyribonucleic acid-based immunization (DNA vaccination) initiated a new era of vaccine research and since then it has become an extremely powerful tool to develop new antiviral vaccines [1]. Four DNA vaccine products have already been licensed for animal health applications [2,3]. Among them, the vaccine against the fish novirhabdovirus infectious hematopoietic necrosis (IHNV) is one of the most successful DNA vaccines developed so far. This vaccine is protective at small doses (as little as 10 ng in trout fry) and efficacious for up to two years after vaccination [3–5].

DNA vaccines possess several advantages such as their unique ability to readily induce both humoral and cellular immune responses [6], their rapid and cost-effective production and the high stability of DNA preparations compare to other types of vaccines. Furthermore, DNA vaccines are not affected by anti-vector immunity [7] commonly associated to virus-based vectors used for vaccination. However it is also true that in some cases the development of DNA vaccines present limitations mainly associated to their intrinsic low immunogenicity. Consequently, research has been conducted to improve the immunogenicity of the vaccines by combining them with adjuvants, either co-administered with the vaccine or encoded in the same plasmid [7]. Up until today, different molecules of the innate immune system [8–10], β-glucans [11], poly I:C [12–14] and other strategies [15–18] have been used to improve DNA vaccination protection rates. However, these strategies have worked only to a very limited extent. Therefore, other approaches are needed to find novel adjuvants.

Recent studies have showed that virus-related products such as the defective interfering (DI) RNA produced by the Sendai virus

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strain Cantell [19] or influenza virosomes [20] can efficiently work as viral vaccine adjuvants. We recently identified two linear determinants of gpG_{VHSV} (peptides p31 and p33) implicated in triggering a cell antiviral response mediated by type I IFN [21]. Moreover, we demonstrated that these short viral glycoprotein segments were able to protect cells against VHSV infection. Taking into account that type I IFN-related responses are the first line of the host defense controlling viral infections [22], we hypothesized that these gpG_{VHSV}-derived peptides might have the potential to act as DNA molecular adjuvants. To that end, the DNA sequences encoding the p31, p33 or p31 + p33 amino acid sequences were cloned into a plasmid. Subsequently, these plasmids were used to transfect zebrafish cells *in vitro* and *in vivo*. The results showed that the plasmid construct encoding the sequences of both peptides (pMCV1.4-p31 + p33) conferred fish partial protection against a lethal challenge with the spring viremia carp virus (SVCV) at 45 days post-immunization. Furthermore, the protection rates recorded after SVCV lethal challenge in zebrafish immunized with a DNA vaccine encoding the gpG sequence of SVCV (pAE6-G_{SVCV}) in combination with pMCV1.4-p31 + 33 were higher than those conferred by the immunization with the vaccine alone. These results indicate that the gpG_{VHSV}-derived peptides implicated in activating the type I IFN system are potent viral DNA vaccine molecular adjuvants. On the other hand, to our knowledge, this is the first report in which specific IFN-inducing peptides from a viral glycoprotein are used as potential adjuvant candidates for DNA vaccines.

2. Materials and methods

2.1. Plasmid constructs

For the expression of the VHSV glycoprotein G (gpG_{VHSV}, Genbank accession A10182)-derived peptides, p31 (from amino acid 280 to 310) and p33 (from amino acid 340 to 370) (Fig. S1), three different plasmid constructs were designed. The constructs were made by cloning the DNA sequences of p31, p33 or a combination of p31 + p33 linked by six glycines (Fig. S1, gray box) into the vector pMCV1.4 [23] (Fig. S2) obtaining pMCV1.4-p31, pMCV1.4-p33 and pMCV1.4-p31 + p33. Furthermore, to generate cell membrane anchored adjuvants, in all of the cases the peptide DNA sequences were flanked by that of the signal peptide (SP) and the transmembrane domain (TM) of the gpG_{VHSV} (Fig. S1). DNA sequences were synthesized by Genscript (CA, USA).

In addition, the plasmids pAE6-Gsvcv [24] and pMCV1.4-G_{VHSV} [1] encoding the glycoprotein G of SVCV and VHSV, respectively, were also used in this work.

2.2. Cell culture and virus

ZF4 (zebrafish embryonic fibroblast, ATCC numbers CRL-2050) [25] and RTG-2 (fibroblastic cell line derived from rainbow trout gonad, ATCC CCL-55) cell lines were used in this work. ZF4 cells were maintained in a 5% CO_2 atmosphere with RPMI-1640 Dutch modified (Gibco, Invitrogen corporation, UK) at 28 °C. Likewise, RTG-2 cells were maintained with minimal essential medium (MEM) at 20 °C. In both cases, the cell culture medium was supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, USA), 1 mM pyruvate (Gibco, Invitrogen Corporation, UK), 2 mM Glutamine (Gibco), 50 μg/mL gentamicin (Gibco) and 2 μg/ml fungizone.

The isolate 56/70 of SVCV isolated from carp [26] was propagated in ZF4 cells at 22 °C [24]. On the other hand, VHSV-07.71, isolated in France from rainbow trout, *Oncorhynchus mykiss* [27] was propagated in EPC cells at 14 °C [28]. In both cases, supernatants from infected EPC cell monolayers were clarified by centrifugation at 4000 × *g* for 30 min and kept in aliquots at −80 °C. Clarified supernatants were used for both *in vitro* and *in vivo* the experiments.

2.3. Fish

Rainbow trout (*Oncorhynchus mykiss*) from 5 cm to 6 cm, obtained from a VHSV-free commercial farm (Piszolla group, Zaragoza, Spain), were maintained in 50 L tanks at the animal experimentation facilities at the University Miguel Hernandez (UMH) at temperatures from 12 to 14 °C. Adult zebrafish (*Danio reriro*) of 2–3 g (~4 cm in length) were obtained from a local fish pet shop and maintained at 28 °C in 30 L tanks. Tanks were equipped with a re-circulating dechlorinated water system. Prior to experiments all fish were acclimatized to the laboratory conditions for 2 weeks. Fish were fed daily with a commercial feed diet.

2.4. Cell transfection assays

ZF4 and RTG-2 cells were transfected with pMCV1.4-p31, pMCV1.4-p33, pMCV1.4-p31 + p33 or the empty plasmid, pMCV1.4 (henceforth pMCV1.4-coiled). Cell transfections were carried out as previously described [29] using Fugene as transfection reagent. Cells treated with poly (I:C) (40 μg/mL) were used as a positive control of *mx* induction. After 27 h, total RNA was extracted and the transcriptional expression of each peptide as well as the zebrafish *isoform C* (*zfmx*) or trout *mx3* gene was evaluated by RT-qPCR using specific primers (Table S1).

2.5. RNA isolation, cDNA synthesis and qPCR assays (RT-qPCR)

The E.Z.N.A HP and Tissue RNA kit (Omega bio-tek, R6812 and R6688, respectively) were used for total RNA extraction in the *in vitro* and the *in vivo* experiments according to the manufacturer instructions. The isolated RNAs were stored at −80 °C until used

The cDNA was obtained as previously described in [30]. Likewise, previously published protocols were followed for quantitative PCR (qPCR) [21]. Primers and probes are listed in Table S1. Non-template controls (NTCs) and controls without RT were included for each gene in all RT-qPCR assays (data not shown). Gene expression results were analyzed by means of the $2^{-\Delta Ct}$ method to evaluate the expression of transcripts for each plasmid relative to the elongation factor 1 alpha (EF1-α). For the zebrafish *zfmx* and trout *mx3* gene expression, the results were analyzed with the $2^{-\Delta\Delta Ct}$ method [31], using the elongation factor 1 alpha (EF1-α) gene as an endogenous control for quantification. Non-treated ZF4 cells served as calibrator cells (control), and fold increases were calculated relative to transcriptional expression in these cells.

2.6. In vitro cell infection assays

ZF4 cells were transfected as above indicated. Transfected cells were then infected with SVCV (multiplicity of infection (MOI) of 2.10^{-3}) in a final volume of 100 μL/well of cell culture medium supplemented with 2% FBS. At 24 h post-infection the supernatants from infected cell cultures were harvested and viral yields assessed by an immunostaining focus assay [32]. Briefly, different dilutions of the supernatants from SVCV infected ZF4 cells (from 0.1 to 0.0001) were added to EPC cell monolayers, grown in 96-well plates, at 22 °C for 90 min. Then, cells were washed with PBS, fresh medium added and plates further incubated for 24 h. After incubation period, cells were fixed with a solution of 4% formaldehyde (Sigma, F1635) for 15 min, washed with PBS and further fixed with cold methanol (−20 °C) for 15 min. Fixed cells were stained with the monoclonal antibody BIO 331 (Bio-X Diagnostics, Jemelle, Belgium) against SVCV as previously described [24]. Stained infected cell foci

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were counted with an inverted microscope (Nikon Eclipse TE2000-U, Nikon Instruments Inc., NY, USA). The results were expressed as percentage of infectivity by the formula, (no. of SVCV foci forming units in transfected cells/no. of SVCV foci forming units in non-transfected cells) * 100.

2.7. Detection of the trout Mx proteins by IF

RTG-2 cells were transfected with the different plasmid constructs as above described. Three days after transfection, cells were fixed with cold methanol and incubated with the rabbit anti-trout Mx3 polyclonal serum [21] diluted 200-fold in PBS at room temperature for 2 h. The indirect staining was carried out using a goat anti-rabbit antibody conjugated with rhodamine (Sigma). Stained cells were viewed and photographed with an inverted fluorescence microscope provided with a digital camera (Nikon DS-1QM).

2.8. Specific IgMs against p31 and p33 in sera from 30-days-immunized/infected trout

Rainbow trout were anaesthetized by immersion in 50 μg/mL buffered MS-222 prior to plasmid injections and then divided into the experimental groups (6 fish each). Groups were intramuscularly immunized with one of the following: (i) 50 μL of PBS (control trout), (ii) 50 μL of PBS containing 1 μg of pMCV1.4-p31, (iii) 1 μg pMCV1.4-p33 or iv) 1 μg pMCV1.4-p31 + p33. In addition, a group of 20 trout were infected by bath immersion with VHSV-07.71 (10^6 $TCID_{50}$/mL). At day 30 post-immunization/infection, six trout from each group were sacrificed and blood extracted from the caudal vein let to clot overnight at 4 °C, then centrifuged for 10 min at 1600 × *g*. The sera from each experimental group was pooled and stored at −70 °C until analyzed. The presence of specific antibodies (IgMs) to p31 and p33 peptides in trout sera was determined through enzyme-linked immunosorbant assay (ELISA) using a p31 and p33 synthetic peptides as solid-phase antigen. ELISA was carried out using the monoclonal antibody to trout IgM, 1G7 [1]. The 1-Step ultra TMB-ELISA (Thermo scientific) was used to develop the peroxidase reaction (450 nm). As negative and positive controls, sera from non-infected or VHSV infection surviving trout were included in the assays.

2.9. Zebrafish DNA immunization protocol

Two DNA immunization assays were performed. In both cases, zebrafish were anaesthetized by immersion in 50 μg/mL buffered MS-222 prior to immunization and then divided into four experimental groups (30 fish each). Groups were intramuscularly immunized with one of the following, First trial. (i) 10 μL of PBS (control fish), (ii) 10 μL of PBS containing 1 μg of pMCV1.4-p31, (iii) 1 μg pMCV1.4-p33 or (iv) 1 μg pMCV1.4-p31 + p33. At five days post-injection muscle samples from 3 fish were excised and the transcriptional expression of all the antigens as well as of the *zfmx* gene was analyzed by RT-qPCR.

Second trial. (i) 10 μL of PBS (control fish), (ii) 10 μL of PBS containing 0.5 μg of pMCV1.4-p31 + p33, (iii) 10 μL of PBS containing 1 μg of pAE6-Gsvcv or (iv) 10 μL of PBS containing 0.5 μg of pMCV1.4-p31 + p33 and 1 μg of pAE6-Gsvcv.

2.10. In vivo SVCV challenge assays

Zebrafish ($n = 27$) were challenged by bath immersion with lethal dose of SVCV by bath immersion for 90 min at 22 °C 45 (first trial) or 60 days after immunization (second trial), as previously described [24]. Mortality was recorded daily for 22 days following the challenge. Data has been represented as Relative Percentage of Survival (RPS) calculated by the formula: RPS = (1 − no dead fish/no dead control fish) * 100.

2.11. Viral recovery from the second trial of immunization 4 days after infection

Four days after SVCV challenge, virus was recovered from three fish per group, chosen randomly, and tissue samples from fins, spleen, gills and head kidney removed and disaggregated. Virus titers present in tissue homogenates were determined by plaque assays and expressed as plaque forming units (PFU) per mL as previously described [24]. Briefly, different dilutions of the tissue homogenates were added to ZF4 cell monolayers at 22 °C for 90 min. Then, cells were washed and infected cell monolayers covered with a solution of cell culture medium containing a 2% aqueous solution of methyl cellulose (Sigma). Cell plates were incubated at 22 °C for 5 days and then the media with methyl cellulose was removed. Finally, wells were stained with crystal violet-formalin to count plaques.

2.12. Statistics

To compare the effects of each construct in relation to its appropriate control, all data was analyzed using Student's *t*-test. Differences were considered statistically significant at $P < 0.05$ (*) or $P < 0.01$ (**).

3. Results

3.1. Expression of mx3 in RTG-2 cells transfected with peptide-plasmids

A cell line from rainbow trout (RTG-2), a common host of VHSV, was transfected with the DNA constructs to test whether or not these plasmids could drive the expression of the gpG_{VHSV}-derived antigens in fish cells. Antigen expression was evaluated at the transcriptional level and compared to that of the whole glycoprotein, encoded in pMCV1.4-G_{VHSV} [1]. The results showed that expression of the whole antigen and the gpG_{VHSV}-derived peptides after transfection with the DNA plasmids were similar in trout cells (Fig. 1A).

Next, the magnitude of the type I IFN response in these trout cells expressing the gpG_{VHSV}-derived peptides was measured, as transcripts of trout *mx3*, at 72 h post-transfection. *The mx3* expression was significantly up-regulated in transfected cells, compared to control cells (Fig. 1B). However, the *mx3* expression levels differed among cells transfected with the different plasmids. The gpG_{VHSV}-derived peptides, p31 and p31-33 up-regulated the *mx3* gene expression by 220- and >6.000-fold, respectively. In contrast, *mx3* induction levels of 20-, and 14,3-fold were detected in the cells expressing p33 or gpG_{VHSV}, respectively. In response to pMCV1.4-coiled cell transfection, the *mx3* gene expression was also up-regulated 5,43-fold (Fig. 3B).

The expression of trout *mx3* was also evaluated at the protein level, by Western Blot (WB), using an antiserum against trout Mx proteins (Fig. 1C). A densitometric analysis of the Mx protein bands revealed that cells expressing p31 + p33 showed the highest Mx levels (>10-fold) compared to non-transfected cells (bar graph, Fig. 1C). To a lesser extent (<5-fold), gpG_{VHSV}- and p33-expressing cells as well as cells treated with poly (I:C) or transfected with pMCV1.4-coiled also induced increased levels of Mx. These results were corroborated by immunofluorescence (IF) (Figs. 1D and S3). However, in the case of pMCV1.4-coiled-transfected cells no Mx expression was detected by IF (Fig. S3)

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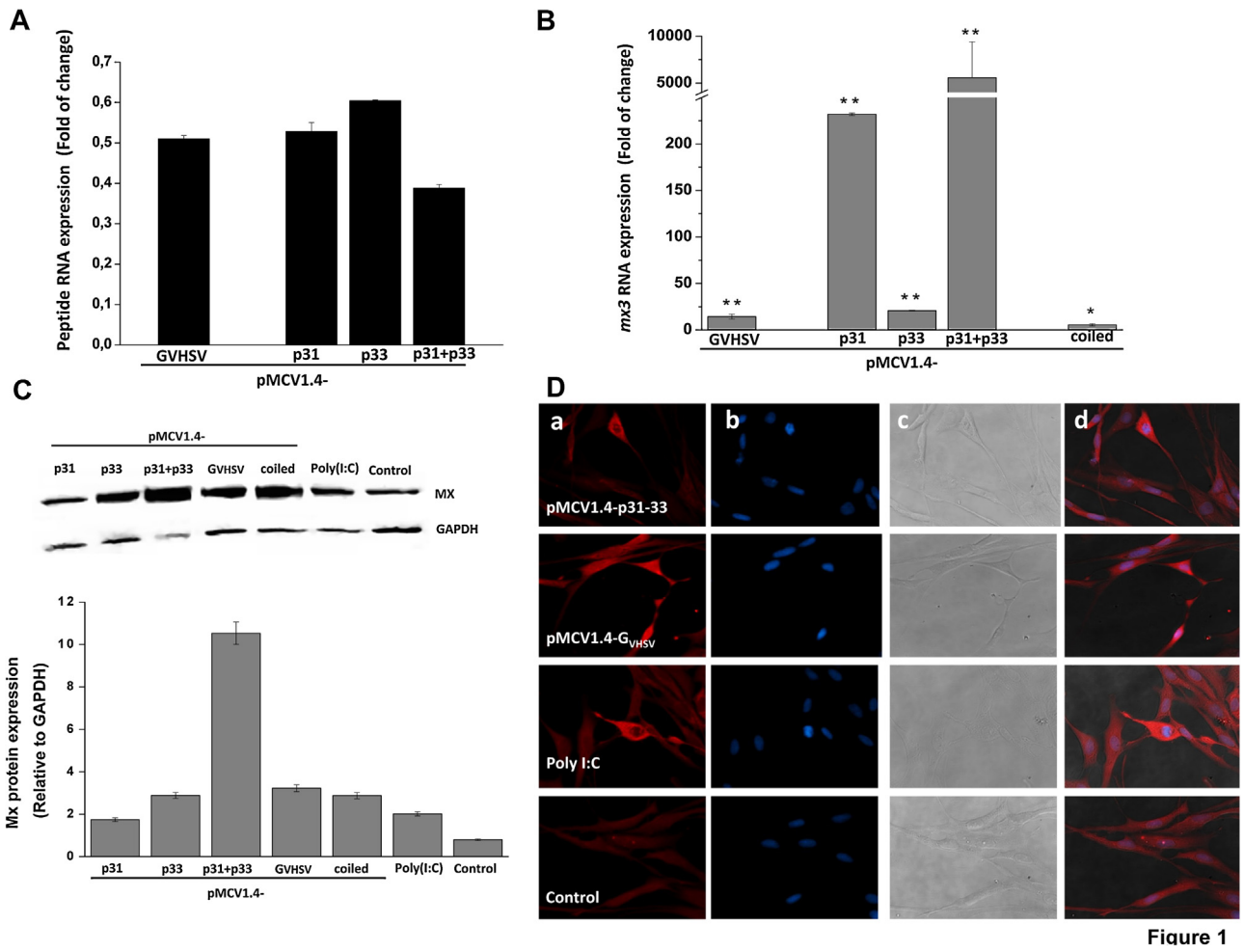

Fig. 1. Expression of gpG_{VHSV} – derived peptides and trout *mx3* gene and protein in RTG-2 transfected cells. RTG-2 cells were transfected with each of the gpG_{VHSV} – derived peptides as well as with the pMCV1.4-coiled. Seventy two hour post-transfection, cells were harvested and processed. (A) Expression of gpG_{VHSV} – derived peptides transcripts estimated by RT-qPCR. Asterisks indicate significant differences in peptide expression relative to EF1α. (B) Expression of trout *mx3* gene transcripts estimated by RT-qPCR. Asterisks indicate significant differences in *mx3* gene expression relative to control cells. In both cases, data are mean ± S.D. from two different experiments, each performed in triplicate. * $P < 0.05$; ** $P < 0.01$. (C) Analysis of Mx3 protein expression by western blot. (D) Analysis of Mx3 protein expression by IF. (a) Polyclonal anti-Mx antibody (red), (b) DAPI DNA stain (blue), (c) phase contrast and (d) merged fields. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2. Presence of specific IgMs in the sera of trout immunized with the plasmids encoding p31 and p33

Higher levels of Mx in trout cells suggested that the gpG-derived peptides were being expressed in the cells. However, since specific antibodies against these small peptides were not available we alternatively checked for the presence of antibodies raised against these gpG regions in trout immunized with the plasmids encoding p31 and p33. To that end trout were injected intramuscularly with the DNA plasmids and the presence of specific IgMs against p31 and p33 in the sera was evaluated at 30 days post-injection (Fig. 2). Specific IgMs against all of the gpG_{VHSV}-derived peptides were detected in immunized fish (Fig. 2), corroborating the successful *in vivo* expression of the peptides. Likewise, specific antibodies to p31 and p33 could be detected in the sera of VHSV infection-surviving trout.

3.3. In vitro expression of gpG_{VHSV}-derived peptides, induction of *zfmx* and anti-viral properties

Before using the gpG_{VHSV}-derived peptides as DNA vaccine adjuvants against SVCV using the zebrafish as a host model species [24,33,34], we tested whether p31, p33 and p31 + p33 were expressed in a cell line derived from zebrafish (ZF4) following transfection with our DNA constructs.

All gpG_{VHSV}-derived peptides were transcriptionally expressed in ZF4 cells at 72 h post-transfection (Fig. 3A). Furthermore, all of the gpG_{VHSV}-derived peptides induced an increment of the zebrafish *mx* (*zfmx*) gene expression in transfected cells with no differences in the induction among the gpG_{VHSV}-derived peptides (Fig. 3B). Overall, a 4 fold of *zfmx* gene expression increment compared to control cells was detected. Poly (I:C), a positive control for IFN induction, up-regulated the *zfmx* induction by 3,5-fold.

In vitro transfection/infection assay showed that all the gpG_{VHSV}-derived peptides were able to reduce the infectivity of SVCV in zebrafish cells (Fig. 3C). Virus infectivity was reduced by 45, 50 and 35% in ZF4 cells transfected with pMCV1.4-p31, pMCV1.4-p33 or pMCV1.4-p31 + p33, respectively, compared to pMCV1.4-coiled-transfected cells. Only SVCV residual infectivity (≤15%) was observed in cells treated with Poly (I:C) (Fig. 3C).

3.4. In vivo antiviral properties of the gpG_{VHSV}-derived peptides

The expression and IFN induction of the gpG_{VHSV}-derived peptides was tested *in vivo* at the injection site (skeletal muscle) in adult zebrafish (black and gray bars, Fig. 4A). Interestingly, although all

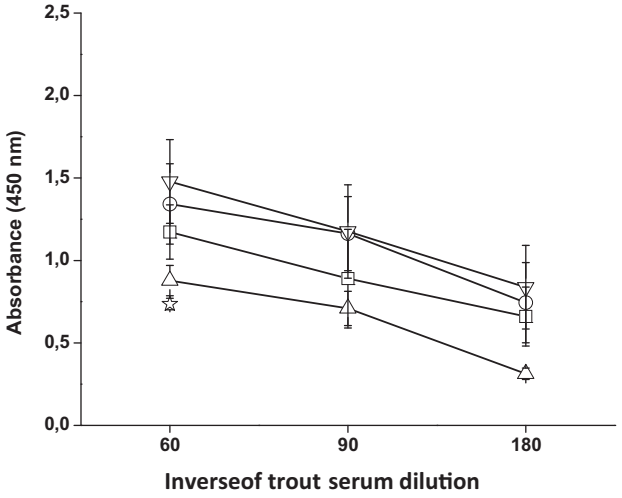

Fig. 2. Presence of anti-p31 and -p33 specific antibodies (IgMs) in sera from trout immunized with each of the plasmid encoding the gpG_{VHSV}-derived peptides or surviving to VHSV infection. The presence of specific IgMs in trout sera was analyzed by ELISA. Data are mean ± S.D. of sera from six fish per group, each assay in triplicate. Sera obtained from VHSV infection surviving trout was used as a positive control (inverted open triangle). Non-immunized trout (open star), trout injected with pMCV1.4-31 (open square), pMCV1.4-33 (open circle) or pMCV1.4-p31 + p33 (open triangle).

of the plasmids drove the expression of the peptides, the expression levels of both p33 and p31 + p33 were > 10-fold higher than those obtained with p31. Regarding IFN induction, only a significant increment of *zfmx* expression levels (from 2 to 4-fold) was observed in fish injected with the plasmid encoding p31 + p33 (gray bars, Fig. 4A).

For the immunization assays, fish were injected with the different plasmid constructs and exposed to a lethal dose of SVCV 45 days after the immunization. More than 65% of the control zebrafish died in the 20 days that followed the SVCV infection presenting clear signs of SVCV infection (Fig. 4 C1) compared to healthy fish (Fig. 4C2). Expression of p31 and p33 moderately protected against the infection, showing RPS values of 28.6 and 36.3%, respectively (Fig. 4B). Maximal RPS occurred in fish expressing p31 + p33 (49.7% Fig. 4B). This result suggests that, in this case, there is correlation between the *zfmx* gene induction levels at the immunization site and fish protection rates after virus challenge. Consequently, pMCV1.4-p31 + p33 plasmid was selected as the best adjuvant candidate for the following immunization experiments.

3.5. Adjuvant effects of pMCV1.4-p31 + p33 in combination with pAE6-G_{SVCV}

Finally, to prove that pMCV-p31 + p33 was a good molecular adjuvant, we combined it with the DNA vaccine against SVCV, pAE6-G_{SVCV}. Additionally, four days after infection virus yields were

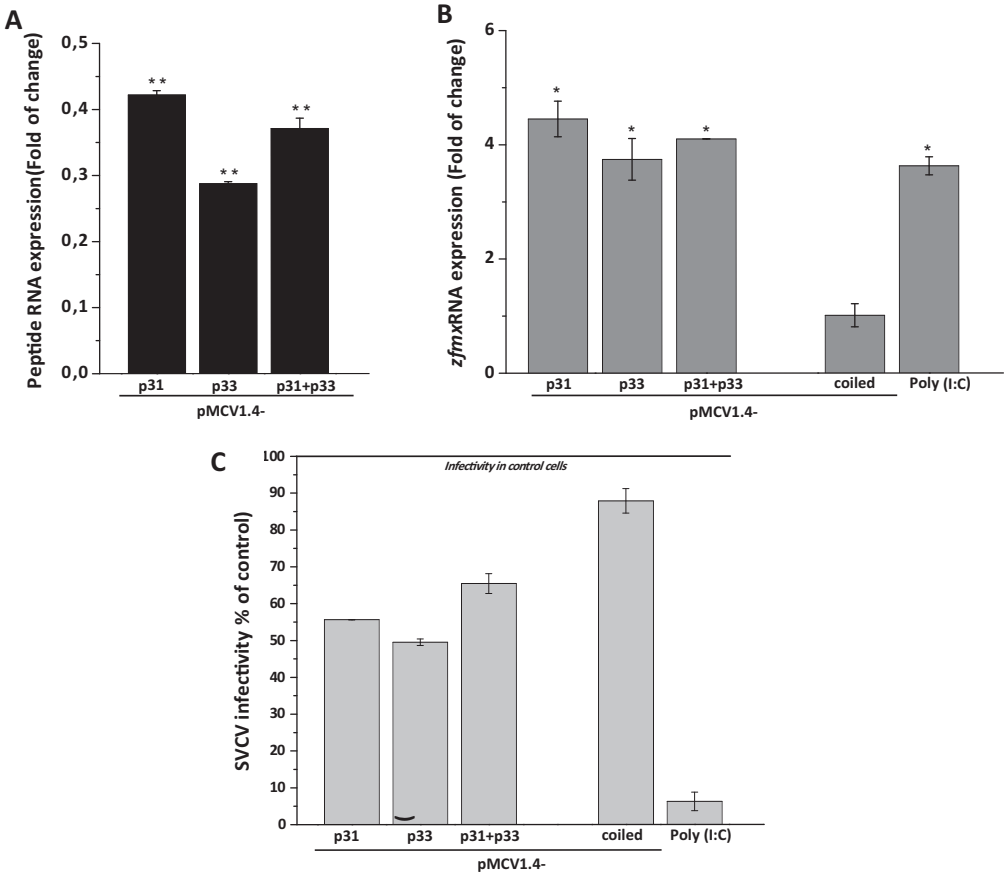

Fig. 3. Expression of gpG_{VHSV} – derived peptides (A), *zfmx* gene (B) and SVCV infectivity in ZF4 transfected cells (C). ZF4 cells were transfected with each of the gpG_{VHSV} – derived peptides as well as with the pMCV1.4-coiled. Seventy two hour post-transfection, cells were harvested and processed or infected with SVCV. (A) Expression of gpG_{VHSV} – derived peptides transcripts estimated by RT-qPCR. Asterisks indicate significant differences in peptide expression relative to EF1α. (B) Expression *zfmx* gene transcripts estimated by RT-qPCR. Asterisks indicate significant differences in *zfmx* genes expression relative to control cells. In both cases, data are mean ± S.D. from two different experiments, each performed in triplicate. * $P < 0.05$; ** $P < 0.01$. (C) Percentage of SVCV infectivity in transfected ZF4 cells. Data are mean values ± S.D. from two different experiments, each performed in triplicate.

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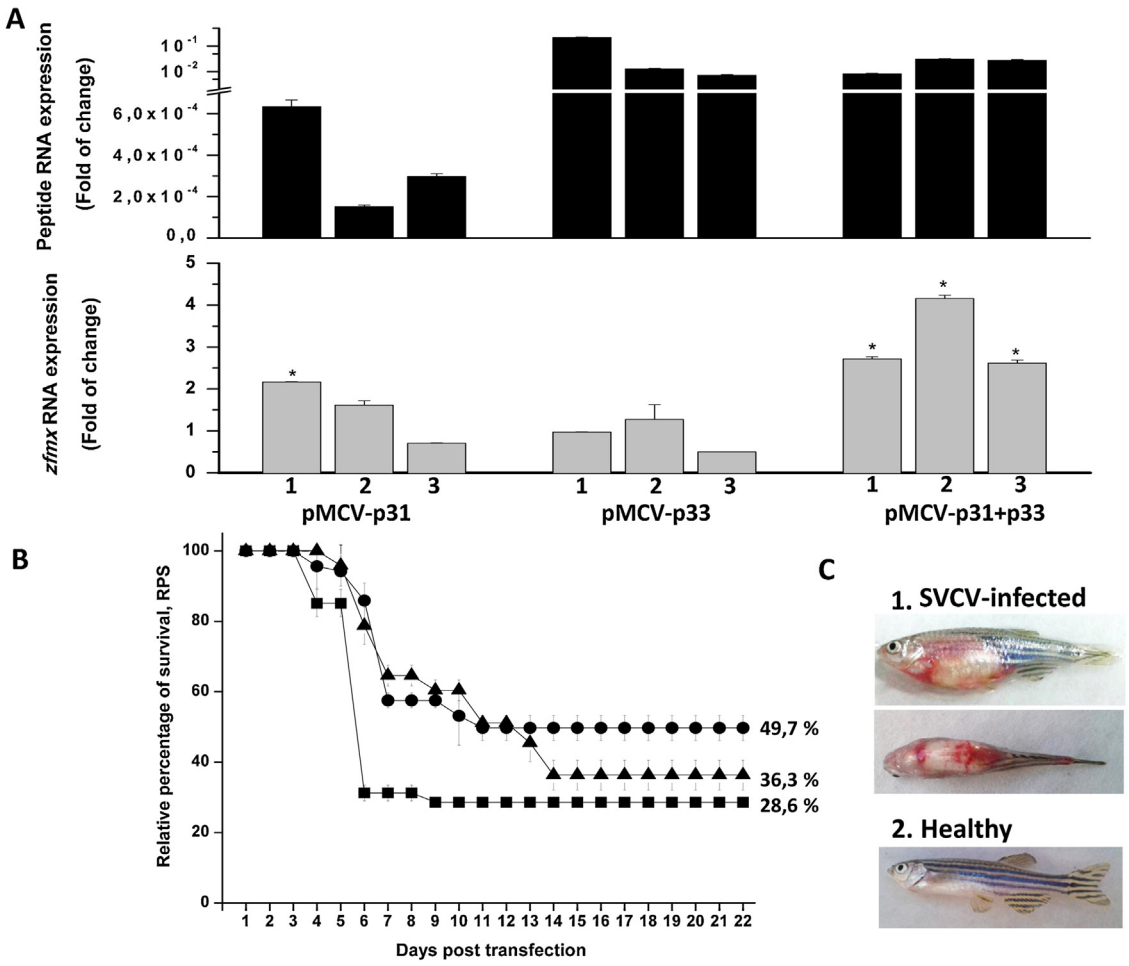

Fig. 4. Expression of gpG_{VHSV} – derived peptides in skeletal muscle of zebrafish and protection conferred against SVCV challenge. Adult zebrafish were injected intramuscularly with PBS, pMCV1.4-31, pMCV1.4-33 or pMCV1.4-p31 + p33. (A) After 5 days, muscle from three individual zebrafish from each group were sampled to evaluated the transcript expression of both transfected peptides and *zfmx* gene by RT-qPCR. Asterisks in the lower panel indicate statistically significant differences in *zfmx* gene expression levels between the fish injected with the plasmids and the fish injected with PBS (control fish) according *t*-test at *P* values of: * $P < 0.01$. (B), after 45 days, the remaining zebrafish were exposed to a lethal dose of SVCV by bath immersion. Mortality was monitored daily. Results were expressed as relative percentage of survival (RPS). Data are mean ± S.D. from two different experiments. pMCV1.4-p31 + p33 immunized fish (black circle), pMCV1.4-p31 immunized fish (black square), pMCV1.4-p33 immunized fish (black triangle). In contrast to healthy fish (C2), all dead fish exhibited severe skin hemorrhages (C1).

estimated from internal organs from pools of three fish from each group.

Viral yields from control fish (non-immunized) were the highest among all four groups (Table 1) with differences ranging between 10- and 10,000-fold (PFU/mL) with the other groups. This is consistent with the 100% mortality of this group in the first 12 days following infection (Fig. 5). Lower viral titers were recovered from fish immunized either with pAE6-G_{SVCV} or pMCV-p31 + p33 (3.8×10^3 and 1.3×10^4 PFU/mL, respectively). These viral yield reductions also translated into significantly higher RPS values in these groups, 42.3% (pMCV-p31 + p33) and 56.5% (pAE6-G_{SVCV}) (Fig. 5). Zebrafish injected with pAE6-G_{SVCV} + pMCV-p31 + p33 were the most resistant to virus infection, with RPS values of 66.8% and virus yields of 15 PFU/mL (Table 1).

Table 1
Recovered SVCV from 4 days infected zebrafish.

Group	Recovered SVCV (PFU/mL)
Control	$7.8 * 10^5$
pAE6-Gsvcv	$3.8 * 10^3$
pMCV1.4-p31 + p33	$1.3 * 10^4$
pAE6-Gsvcv + pMCV1.4-p31 + p33	$1.5 * 10^1$

4. Discussion

The most important finding reported in this work is the demonstration that eukaryotic expression plasmids coding fragments of a viral glycoprotein (gpG_{VHSV}-derived peptides) are an unexpectedly powerful vaccine molecular adjuvant. This study opens the door to search for a new generation of vaccine adjuvants based on pathogens components rather than on host immune system molecules. Similar conclusions could also be extracted from studies using sendai [19] and influenza [20] virus-related products.

The gpG_{VHSV}-derived peptides used in this work were those previously identified to be implicated in triggering the cell antiviral response mediated by type I IFN [21]. Firstly, after the expression plasmid's construction, we evaluated if the endogenous expression of each of these regions (pMCV1.4-p31 and pMCV1.4-p33) as well as a combination of both (pMCV1.4-p31 + p33) were able to activate *in vitro* the type I IFN system. The interferon stimulated gene (ISG) *mx* was chosen since Mx proteins have been proven to be very specific and sensitive markers for type I IFN induction [35–37]. A direct relationship between the gpG_{VHSV} expression and type I IFN production has been described in the rainbow trout cell line RTG-P1 [38] and RTG-2 [39]. Similarly, we have observed that

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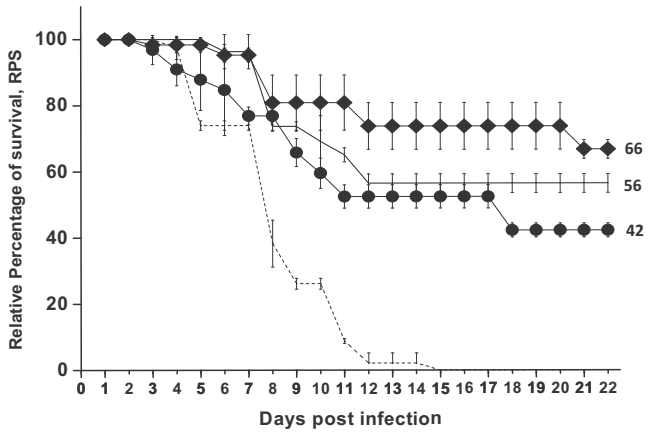

Fig. 5. Challenge of zebrafish with SVCV after co-immunization with pCMV-p31 + p33 and pAE6-Gsvcv plasmids. Adult zebrafish were injected intramuscularly with a PBS solution containing 1 μg of pAE6-G_{SVCV}, 0.5 μg of pMCV1.4-p31 + p33 or a combination of both. After 60 days, the remaining zebrafish were infected with SVCV by bath immersion. Mortality was monitored daily. Results were expressed as Relative Percentage of Survival (RPS). Data are mean ± S.D. from two different experiments. pAE6-G_{SVCV} immunized fish (black line), pMCV1.4-p31 + p33 immunized fish (black circle), pAE6-G_{SVCV} together with pMCV1.4-p31 + p33 immunized fish (black diamond), control fish (scratched line).

each of the plasmids encoding the gpG_{VHSV}-derived peptides could successfully activate the *in vitro mx* gene induction after RTG-2 cell transfection. Moreover, the *mx* gene expression levels induced by the gpG_{VHSV}-derived peptides were even higher than those observed in the cell transfected with a plasmid encoding the whole sequence of the gpG_{VHSV}. On the other hand, pMCV1.4-p31 + p33 induced the highest transcript and protein expression levels of Mx compared to pMCV1.4-p31, pMCV1.4-p33 or the gpG_{VHSV} itself, which suggested that there was a synergistic effect when these two peptide regions were combined. Altogether, the results indicated that activation of type I IFN system by these gpG_{VHSV}-derived peptides is preserved, or even improved, when they are endogenously synthesized by the cells and expressed on their membranes. Similarly, other molecular adjuvants/immunostimulants specifically engineered to be expressed as membrane-anchored molecules [40], attached to liposomes [41] or anchored to the envelope of virus-like particles [42] have also shown improved immunogenicity.

Having characterized the system in RTG2, a common host the rhabdovirus VHSV, our purpose was to use the gpG_{VHSV}-derived peptides as DNA vaccine adjuvants for the DNA vaccine against the heterologous rhabdovirus SVCV (pAE6-G_{SVCV}) using the model zebrafish/SVCV. All gpG_{VHSV}-derived peptide constructs induced an increment of the *zfmx in vitro* (ZF4 cells) that in turn resulted in a reduction of the SVCV infectivity. However, *in vivo*, only the fish injected with pMCV1.4-p31 + p33 showed a notable significant induction of *zfmx*, at the injection site, skeletal muscle. Besides, the survival in zebrafish injected with pMCV1.4-p31 + p33 construct at 45 days post-injection was around 49.7% RPS, which suggested that the combined expression of p31 and p33 induced in fish a strong and long lasting antiviral immune response. This may be explained by the fact that fish survival to rhabdoviruses [43] as well as to other RNA and DNA viruses [44–51] is highly dependent on the innate antiviral responses mediated by IFNs [52]. Thus, ideally, the way to maximize the effect of a viral vaccine should be to combine it with an adjuvant that activates similar early events of the host innate immune response to that of the viruses, thus mimicking their natural infection. Therefore, we hypothesized that these gpG_{VHSV}-derived peptides, as they are strictly speaking virus-derived components, may be the best way to strengthen the immunogenicity of viral vaccines and so, pMCV1.4-p31 + p33 was selected as the best potential molecular adjuvant for the SVCV DNA vaccine.

The combination of pAE6-G_{SVCV} and pMCV1.4-p31 + p33 increased survival rates (≈67% RPS) and reduced viral yields (>100-fold lower) compared to pMCV1.4-p31 + p33 or pAE6-G_{SVCV} alone. Curiously, these results are different to those found when the whole gpG_{VHSV} was used as a molecular DNA adjuvant for an inactivated salmonid alphavirus (SAV) vaccine [53], where no adjuvant effect was found. Other studies have used cell host molecules as DNA adjuvants codified in plasmid constructs administered together with the corresponding vaccine. The cell response to these adjuvants has been measured, though, in early points after injection. For example, the effects of the interleukin 8 (IL8) were measured at 3, 7 or 12 days post-injection, observing the decrease of the inflammatory response over time both in spleen and head kidney [10], suggesting that the effect of this adjuvant does not last for a long period of time. Concurrently, in a previous work, a host molecule (zebrafish beta-defensin 2, zfBD2) was used as DNA adjuvant for gpG_{SVCV}, achieving similar protection rates against SVCV (≈64% RPS) than those obtained using pMCV1.4-p31 + p33. However, the gpG_{VHSV}-derived peptide has several advantages in comparison with zfBD2 which are the long lasting immunity (up to 2 months) and the reduced size of the expressed DNA adjuvant. Besides, our results prove that the combination of pAE6-G_{SVCV} with a viral product used as molecular DNA adjuvant (pMCV1.4-p31 + p33) allowed a reduction of the vaccine dose (~10-fold) without affecting its efficacy, reaching a survival rate near 70% sixty days after immunization.

Probably these gpG-derived peptides might have specific determinants that could be recognized by the viral specific pattern recognition receptors PRR, simulating a natural infection. Altogether, our results show that viral molecular associated patterns can efficiently work as broad range vaccine adjuvants. Work is in progress to evaluate this adjuvant effects with unrelated virus as the pancreatic necrosis virus (IPNV) a fish virus belonging to the Birnaviridae family.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.07.111.

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