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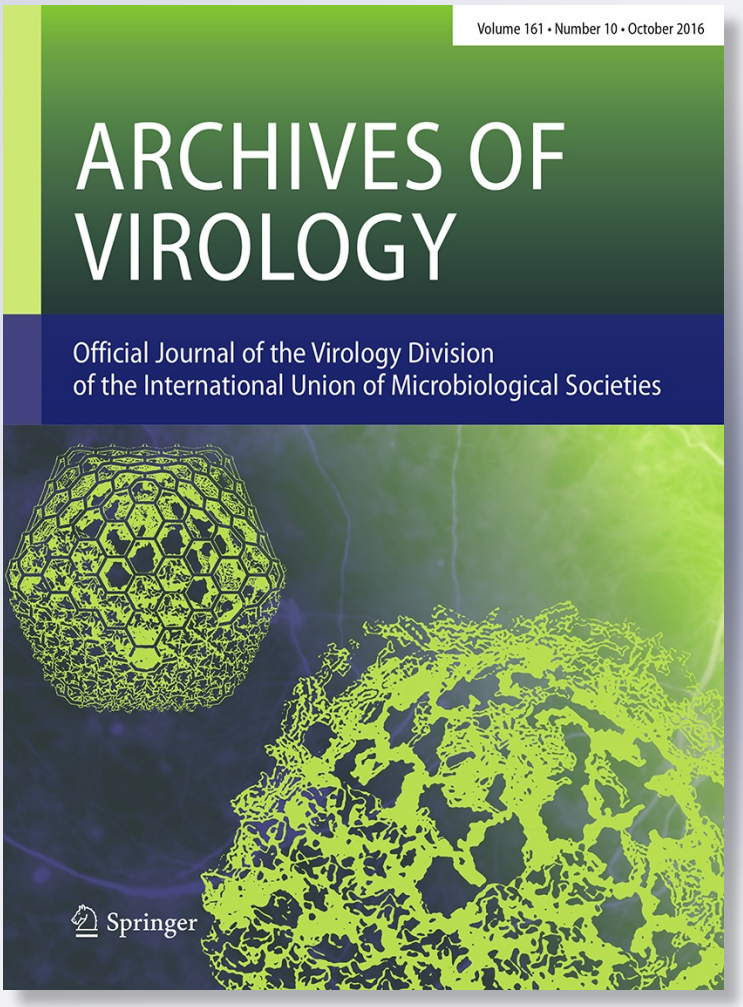
Archives of Virology
Official Journal of the Virology Division of the International Union of Microbiological Societies

ISSN 0304-8608
Volume 161
Number 10

Arch Virol (2016) 161:2653-2665
DOI 10.1007/s00705-016-2934-4



Springer

Arch Virol (2016) 161:2653–2665
DOI 10.1007/s00705-016-2934-4

ORIGINAL ARTICLE

The amino-terminal domain of ORF149 of koi herpesvirus is preferentially targeted by IgM from carp populations surviving infection

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Received: 30 December 2015 / Accepted: 13 June 2016 / Published online: 6 July 2016

Abstract Recombinantly expressed fragments of the protein encoded by ORF149 (pORF149), a structural protein from the common- and koi-carp-infecting cyprinid herpesvirus-3 (CyHV-3) that was previously shown to be antigenic, were used to obtain evidence that its amino-terminal part contains immunodominant epitopes in fish populations that survived the infection. To obtain such evidence, nonspecific binding of carp serum tetrameric IgM had to be overcome by a novel ELISA protocol (rec2-ELISA). Rec2-ELISA involved pre-adsorption of carp sera with a heterologous recombinant fragment before incubation with pORF149 fragments and detection with anti-carp IgM monoclonal antibodies. Only in this way was it possible to distinguish between sera from uninfected and survivor carp populations. Although IgM from survivors recognised pORF149 fragments to a lesser degree than whole virus, specificity was confirmed by correlation of rec2- and CyHV-3-ELISAs, inhibition of rec2-ELISA by an excess of $frgII_{ORF149}$, ELISA using IgM-capture, Western blotting, and reduction of reactivity in CyHV-3-ELISA by pre-adsorption of sera with $frgII_{ORF149}$. The similarity of IgM-binding profiles between $frgI_{ORF149}$ (amino acid residues 42-629) and $frgII_{ORF149}$ (42-159) and their reactivities with previously described anti-CyHV-3 monoclonal antibodies confirmed that most pORF149 epitopes were localised in its amino-terminal part.

Introduction

The recent spread of a new fish herpesvirus called koi herpesvirus (KHV) or cyprinid herpesvirus 3 (CyHV-3), a member of the family *Alloherpesviridae*, affects common

Electronic supplementary material The online version of this article (doi:10.1007/s00705-016-2934-4) contains supplementary material, which is available to authorized users.

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and koi carp (*Cyprinus carpio*) [9, 15, 18]. Since the late 1990s, KHV disease (KHVD) has generated a large number of surviving asymptomatic carrier carp populations with latent and recurrent herpes infections that remain to be studied from an immunological point of view [5, 41].

Fish are lower vertebrates with a primitive immunological system compared to that of mammals (no IgG class switch, tetrameric IgM, no IgM affinity maturation, mucosal IgT/IgZ, phagocytic B-cells, etc.) [48, 50], which generates high levels of nonspecific IgM responses to infection [28]. As a consequence, the CyHV-3 viral proteins targeted by the host [14], host-viral protein interactions [16, 17], and therefore the mechanisms that provide protective immunity, latency, persistence or chronic infections are far from being completely understood [1, 2, 37, 39, 40]. Nevertheless, CyHV-3-ELISA has been used to estimate anti-CyHV-3 IgM levels to follow carp infection [3, 47] or vaccination [36, 43] with high sensitivity. However, because of the technical problems involved in estimating specific carp antiviral tetrameric IgM antibodies (Abs) by virus-free ELISAs, there are few studies on the dissection of IgM responses to CyHV-3 protein targets [3, 14, 35, 43, 47], and no attempts to study possible correlations of IgM levels in survivors with protection, persistence, recurrent infections and/or carrier detection have been reported. Because koi are traded globally, the possibility to spread KHVD by asymptomatic carrier populations remains very high [7]. More knowledge of the carp IgM response to CyHV-3 is expected to contribute not only to improvements in diagnosis and vaccination but perhaps also to reveal novel ideas to fight against mammalian herpesviruses in general.

CyHV-3 possesses a double-stranded DNA genome of ~295 kb, containing 156 open reading frames (ORFs), which are highly conserved among three type-specific isolates from Israel, Japan, and the United States [4]. Mass spectrometric analysis of purified CyHV-3 virions detected the presence of 13 ORFs predicted to encode glycosylated type I integral membrane proteins, at least some of which correspond to hypothetically immunologically relevant antigens [30]. However, a well-defined function has not been elucidated yet for any of them. Most recently, the products of nine of those ORFs (ORF25, 65, 92, 99, 136, 138, 146, 148, and 149) were expressed in bacteria or insect cells to raise polyclonal/monoclonal antibodies (PAbs/MAbs) to study their possible immune dominance [14]. Cells transfected with plasmids coding for pORF25, 65, 148 and 149 (all members of a gene family) predominantly reacted with sera from carp surviving KHVD. Furthermore, four neutralizing MAbs raised against CyHV-3 virions recognized pORF149 in transfected cells and CyHV-3 particles and neutralized CyHV-3 *in vitro*, demonstrating that ORF149 might be one of its major immunodominant proteins [14]. These results prompted us to focus on pORF149 to further explore the IgM response of carp populations surviving KHVD.

Among the fish IgM responses to viral infections, those targeted to disulfide-dependent (conformation-dependent) neutralizing Ab responses of viral membrane glycoproteins, which are detected only in 50 % of viral survivors, are the best known [11, 25–27], while alternative cysteine-free IgM-binding responses are largely unexplored. Thus, the immunological relevance of cysteine-free epitopes only had been studied in trout using pepscan or recombinant fragments (frgs) of the glycoprotein G of viral haemorrhagic septicemia virus (VHSV; G_{VHSV}) [10, 11, 13]. Those studies led to the identification of the immunodominant $frg11_G$, which could substitute for G_{VHSV} in anti-VHSV IgM diagnosis. Furthermore, specific anti-VHSV IgM levels estimated by ELISA (rec-ELISA) using recombinant $frg11_G$ (amino acid residues 56-110 containing only one cysteine) made in *E. coli* were higher when compared to the use of G_{VHSV} made in insects (without its signal peptide and transmembrane region but having intact disulphide-dependent epitopes) [10, 12]. Nevertheless, nonspecific IgM binding was a problem in rec-ELISA as well as in virus-ELISA, not only in VHSV-infected trout sera [10, 11] but also in fish infected with infectious haematopoietic necrosis virus, IHNV (unpublished) and other rhabdoviruses [20–22]. Although inclusion of milk [21], fetal calf serum [20, 22] or rabbit serum [10, 11] in the ELISA buffers reduced non-specific fish IgM binding, their levels remained uncontrolled. In this respect, a protocol involving two parallel ELISAs using heterologous VHSV to correct for nonspecific IgM binding to homologous IHNV was recently proposed to discriminate IHNV-infected from non-infected trout sera [20, 22].

Taking into account all of the data mentioned above, the present study focused on cysteine-free frgs of pORF149 (amino acid residues 1-686) because of the simplicity with which they can be expressed in recombinant *E. coli* with high yields. Both small naturally occurring cysteine-free pORF149 frgs and pORF149 with all of its cysteines mutated to serines were expressed in *E. coli,* and purified. To reduce nonspecific IgM-binding levels, a new ELISA protocol using consecutive heterologous/homologous viral recombinant protein fragments (rec2-ELISA) was developed. To deal with the usually high individual fish variations on IgM responses, we focused the analysis on comparing populations rather than individual fish. Uninfected and KHVD-survivor carp populations could be discriminated by rec2-ELISA with high correlations between the results obtained with $frgI_{ORF149}$ (42-629) or $frgII_{ORF149}$ (42-159) and CyHV-3. Although IgM from survivor carp populations recognised the recombinant frgs to a lesser degree than whole virus, the specificity of rec2-

ELISA was confirmed by its high correlations with CyHV-3-ELISA, its inhibition by excess frg, the binding of $frgII_{ORF149}$ by carp IgM captured by anti-IgM monoclonal antibody, Western blotting and reduction of binding to CyHV-3 by pre-adsorption of the sera with $frgII_{ORF149}$. In addition, reactivities of $frgs_{ORF149}$ with several anti-CyHV-3 monoclonal antibodies described previously [14] suggested that most of the pORF149 cysteine-free epitopes detected by these studies were localised in its amino-terminal part.

In addition, to further study the CyHV-3 mechanisms of infection and persistence through anti-CyHV-3 IgM responses, the rec2-ELISA protocol could also be adapted for screenning of asymptomatic CyHV-3 carriers in KHVD-survivor populations to control koi export/imports and/or to develop new CyHV-3 vaccines.

Materials and methods

CyHV-3 virus and cells employed. CyHV-3 affecting both common and koi carp (*Cyprinus carpio*) was replicated in cells from common carp brain (CCB). To facilitate production and purification of the virus, the KHV-T or CyHV-3-T (Taiwan) isolate was used. The CyHV-3-T isolate is a large-plaque-forming strain with a ~100-fold higher titre in CCB cell culture than wild type, selected at the Graduate Institute of Biotechnology, Central Taiwan University of Science and Technology (Taichung, Taiwan). CCB cells, kindly provided by the Collection of Cell Lines in Veterinary Medicine and the German Reference Laboratory for KHVD (Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany), were grown at 25 °C with 5 % CO_2 in Dutch modified RPMI cell culture medium with 20 mM HEPES supplemented with 10 % fetal calf serum (FCS), 1 mM pyruvate, 2 mM glutamine, and 50 μg of gentamicin and 2.5 μg of fungizone (Sigma, St. Louis, Missouri, USA) per ml. After the CyHV-3-T-infected CCB cell monolayers showed a cytopathic effect, the supernatants were cleared by centrifugation at 10,000*g* for 10 min and kept at -70 °C until use. To obtain concentrated CyHV-3-T, supernatants from infected CCB cell monolayers were centrifuged at 60,000 *g* for 120 min at 4 °C, and pellets were frozen at -70 °C until use. CYHV-3-T titrated by the $TCID_{50}$ assay [6] yielded concentrations of ~10^7 $TCID_{50}$ /ml.

Carp sera used for screening. Carp (*Cyprinus carpio*) were obtained from different sources and locations as summarized in Table S1 and briefly described here.

Survivor carp sera were obtained from FLI (Insel Riems, Germany). Sera from carp with 50-250 g of body weight (n = 91, four independent carp groups) were obtained from fish surviving infection-by-immersion with 10^3 $TCID_{50}$ of CyHV-3-T per ml kept at 22-24 °C for 2 h in a re-circulating system The concentration of CyHV-3-T was calculated to cause 50 % mortality. The infected fish were then released to their original aquaria. All of the infected fish showed clinical symptoms from 1 to 4 weeks after infection (100 % morbidity), deaths were observed between 18 and 26 days after infection, and total mortality was ~40 % after 4 weeks. At this time, CyHV-3-T could be detected in all of the fish by gill swabs [32]. After other additional 4 weeks, the surviving fish had recovered and were bled from the tail vein. Sera from carp surviving a KHVD outbreak (n = 8) with high neutralizing titres were kindly provided by Dra. D. Chee (Animal & Plant Health Laboratories, Aquatic Animal Health Section Laboratories Group, Singapore).

Sera from uninfected koi carp (n = 32) with 100 to 500 g of body weight were obtained from an outdoor closed-circuit farm with no previous history of KHVD for more than 3 years at "Escuela de Ingenieros de Montes" of UPM (Madrid, Spain). Sera from carp that were not infected with CyHV-3 were obtained in 2005 from carp surviving a laboratory infection with spring viremia of carp virus, SVCV (n = 17), at the Cefas Laboratory at Lowestoft, England (Dr. P. Dixon). Sera from carp maintained for 10 years free from any infections were obtained from FLI, Germany (n = 10). Koi sera imported to Spain from Israel (n = 9), Thailand (n = 9), the Czech Republic (n = 9), Germany (n = 6), or commercial sources from Spain (n = 9) and sera from wild carp captured from Vegas del Guadiana (Extremadura, Spain) (n = 16) and Bornos (Cadiz, Spain) (n = 29) were obtained from J.L. Gonzalez (Consultores Biología Conservación, CBC, S.L.).

The experimental CyHV-3-T challenge was approved by the Animal Protection Committee of the Friedrich-Loeffler Institut, Greifswald, and the Federal State of Mecklenburg-Vorpommern, Germany (LALLF M-V/TSD/7221.3-2.1-006/10, FLI TV 04/10). Carp and koi were handled in accordance with the National and European Guidelines and Regulations on Laboratory Animal Care. Fish work was approved by the INIA Animal Ethics Committee (authorization PROEX Oct 2014, 219/14). CyHV-3-T-infected carp were monitored 2-4 times daily and those with altered behavior were euthanized by an overdose of methanesulfonate 3-aminobenzoic acid ethyl ester (MSS2) to minimize their suffering. When appropriate, carp were anaesthetized using MSS2 prior to bleeding. Blood was individually collected from the caudal vein, allowed to clot overnight at 4 °C, and centrifuged, and the resulting serum was kept frozen at -20 °C. After recovery, carp were reintegrated into their former environment.

Construction of recombinant fragments (frgs) of pORF149 of CyHV-3. pGST-K0149 (a fusion protein of pORF149 and glutathione S-transferase, here called

frg0$_{ORF149}$) was described before [14]. Briefly, the complete ORF149 amplified from the CyHV-3-I DNA by PCR using primers flanked by *EcoRI* and *XhoI* sites was subcloned into the pGEX-4T-1 prokaryotic expression plasmid (GE Healthcare). The frg0$_{ORF149}$ (pORF149 sequence in Fig.S1A), was expressed in the *E. coli* strain XL1-Blue MRF' (Agilent), electroeluted from SDS polyacrylamide gels, washed with PBS, and lyophilized. For the rest of the fragments (frgs), recombinant expression and purification were performed as previously described for frg11 (amino acid residues 56-110) of the protein G of viral haemorrhagic septicemia virus, VHSV (frg11$_G$) [10, 42]. Briefly, the protein sequence of the CyHV-3-U reference strain (GenBank accession number NC009127) was used to identify the ORF149 sequence and design its derived frgs$_{ORF149}$ (frgI$_{ORF149}$ amino acid residues 42-629, frgII$_{ORF149}$ 42-159, and frgIII$_{ORF149}$ 441-629) according to the sequences described in Fig. S1A and B and the scheme shown in Fig. S2A. The cysteine TGT or TGC codons were changed to serines (AGT or AGC) by substituting the first T to A in the frgI$_{ORF149}$ design (Fig. S1B). Fgs$_{ORF149}$ and frg11$_G$ nucleic acid coding sequences were then chemically synthesized and cloned into pRSETa expression vector between the *BamHI* and *XhoI* restriction sites. pRSETa added amino-terminal sequences containing poly-histidine (polyH) tails to the frg sequences (GeneArt, Regensburg, Germany). In addition to its amino-terminal polyH tail, an additional carboxy-terminal tail sequence containing two more polyH stretches was added to frgII2xpolyH$_{ORF149}$ [19] (^{NH2}GSGSGSGHHHHHHGSG GSGGSGGSGHHHHHHGSGS^{COOH}). *E. coli* BL21 was transfected with the pRSETa plasmid constructs containing the nucleic acid sequences coding for the frgs, and protein expression was induced by adding 0.1 M IPTG. *E. coli* extracts were then purified by Ni^{2+} affinity chromatography as described previously [10, 12, 42]. Purified frgs$_{ORF149}$ were kept frozen in 20 mM sodium acetate buffer, pH 4.5, to increase their solubility. Gradient SDS polyacrylamide gel electrophoresis showed >95 % purity, as estimated by Coomassie blue staining of bands with the expected molecular weight (Fig. S2B).

Assays of anti-frgs$_{ORF149}$ in carp sera by homologous ELISA protocols (virus- and rec-ELISA). Maxisorb 96-wells polystyrene plates (Nunc) were coated with per well 2 µg of purified CyHV-3-T (virus-ELISA) or pORF149 recombinant frgs (frg0$_{ORF149}$, frgI$_{ORF149}$, frgII$_{ORF149}$, homologous rec-ELISA) in 50 µl of water and dried overnight at 37 °C. The remainder of the steps were performed at 20-25 °C.

Because preliminary ELISA experiments showed a high level of nonspecific IgM binding, which prevented discrimination between uninfected and KHVD survivor sera, optimal concentrations of skimmed milk for blocking the solid phase and of *E. coli* extracts (EE) for pre-incubating sera were first determined using an small population of carp (n = 8). After optimization, frgs$_{ORF149}$- coated plates were first blocked with 100 µl of dilution buffer (0.5 % bovine serum albumin, 0.1 % Tween-20, 0.01 % Merthiolate, 0.005 % phenol red in phosphate buffered saline, pH 6.7) containing 10 µg of skimmed milk (Sigma) per well for 60 min. The carp sera were then diluted 100-fold in dilution buffer containing 20 µg of *E. coli* extract (EE) (supernatant from sonicated *E. coli* BL21 centrifuged at 10,000 *g* for 20 min) and incubated with frg$_{ORF149}$-containing solid phases in 50 µl per well for 60 min.

To quantify IgM binding, the wells were washed with 0.5 % Tween 20 in phosphate-buffered saline (PBS) and incubated for 30 min with anti-carp IgM MAbs (Aquatic Diagnostics Ltd) in 50 µl of dilution buffer per well. After washing, 50 µl of 2500-fold diluted horseradish-peroxidase-conjugated rabbit anti-mouse antibody RAM-PO (Sigma Chem. Co, St. Louis, MO) was added to each well and incubated for 30 min (Fig. S3, rec-ELISA). After washing three times, the colour reaction was developed by adding 50 µl of *o*-phenylenediamine (1 mg/ml) in citrate buffer, pH 5.5, containing 3 mM H_2O_2, and the reaction was stopped by adding 50 µl of 0.1 M sulfuric acid 20 min later. The optical density was measured at 492-620 nm in a Labsystems ELISA reader to correct for individual differences between wells [45].

Assays of anti-frgs$_{ORF149}$ in carp sera by consecutive heterologous-homologous ELISA protocol (rec2-ELISA). Plates were coated and blocked as for the rec-ELISA protocol. Fifty µl of 100-fold-diluted carp sera was first pre-incubated for 60 min with frg11G (heterologous solid-phase) (Fig. S3, rec2-ELISA). Fifty µl of the frg11$_G$-pre-incubated supernatant containing non-adsorbed IgM was then incubated for 60 min on frg$_{ORF149}$ (homologous solid-phase) (Fig. S3). To quantify IgM binding to the homologous solid phase (rec2-ELISA), the sample was incubated with anti-carp IgM MAbs and RAM-PO, the colour was developed, and absorbance was measured as indicated above for the rec-ELISA.

Estimation of IgM binding to frgs$_{ORF149}$ by Western blotting. Confirmation of frgs recognition by Western blot on frgs$_{ORF149}$ separated in SDS-4-20 % polyacrylamide gradient gels (Bio-Rad, Richmond, VI, USA) in buffer containing β-mercaptoethanol was estimated using 100-fold-diluted sera pooled from uninfected (n = 7) or survivor (n = 7) carp. The proteins were transferred to nitro-cellulose membranes (Bio-Rad) and blocked with the same dilution buffer used for the ELISA protocols. Three-mm-wide membrane strips were incubated with anti-carp IgM monoclonal antibody and with peroxidase-conjugated rabbit anti-mouse (RAM-PO) and developed with diaminobenzidine (DAB) staining as described before [10].

Western blotting analysis was performed by photography, densitometry and quantification in arbitrary units using a Gel Doc™ Xr apparatus provided with the Image Lab™ 5.2 software (Bio-Rad). To best compare with the ELISA absorbance units, the mean optical densities (n = 3) of the bands in Western blots were corrected using the following formula: Optical density of the bands in arbitrary units × 0.2 / Mean optical density in arbitrary units of the bands corresponding to the uninfected pool.

Results

Selection of CyHV-3 pORF149 and cysteine-free derived fragments

The CyHV-3 protein encoded by ORF149 (pORF149) was selected because it was previously identified as one of the most immunologically relevant targets of carp IgM and the best inducer of mouse MAbs [14]. Focusing our studies on the cysteine-free epitopes of pORF149 avoided the production of inclusion bodies, instability and low yields obtained when the cysteine-complete GST-KO149 ($frg0_{CyHV-3}$) described before [14] (Fig. S1A and Fig. S2A) was expressed in *E. coli* (results not shown). In addition, *E. coli*-expressed GST-KO149 was recognized in Western blots in only 16 % of survivor carp sera, despite using different *E. coli* strains for optimal expression (not shown). On the other hand, alternative GST-KO149 expression in insect cells [14] also resulted in conformation-related problems arising during purification (unpublished).

pORF149 fragments (frgs) containing cysteine-free epitopes were designed either by mutating its 15 cysteines (Fig. S1B) to serines to produce $frgI_{ORF149}$ (amino acid residues 42-629) (Fig. S1A-B and S2A) or by selecting naturally occurring cysteine-free frgs or frgs with only one cysteine residue to obtain amino-terminal $frgII_{ORF149}$ (42-159) or carboxy-terminal $frgIII_{ORF149}$ (441-629), respectively (Fig. S1A and S2A). In addition, the highly hydrophobic signal peptide and carboxy-terminal transmembrane regions were eliminated to increase expression of $frgI_{ORF149}$ in *E. coli* (see Fig. S1B for the resulting amino acid sequence).

To predict possible interference by cross-reaction with other carp herpesviruses, the frg_{ORF149} amino acid sequences were compared to other existing protein sequences. A BLAST search using the pORF149 sequence showed 42 and 37 % identity to the ORF65 of CyHV-1 (YP_007003728) and CyHV-2 (AKC01976), respectively (Fig. S1C). However, because most of the identical amino acids were distributed randomly throughout the pORF149 molecule, its derived frgs had a low probability of inducing cross-reactions. Thus, $frgII_{ORF149}$ contained only 36 % amino acid identity to the most similar ORF65 frg of CyHV-1 (Fig. S1D, 58-149). $FrgIII_{ORF149}$ did not have any detectable sequence similarity to other herpesvirus proteins (not shown). On the other hand, the sequence of $frgII_{ORF149}$ was identical in the three CyHV-3 reference isolates [4], while the $frgIII_{ORF149}$-J strain contained an insert of 13 amino acid residues in the PTT-rich domain (not shown).

Because purified $frgIII_{ORF149}$ lost 100 % of its IgM-binding capacity after being frozen (results not shown) and $frgI_{ORF149}$/$frgII_{ORF149}$ yielded ~10 times more recombinant protein than $frg0_{ORF149}$ or $frgIII_{ORF149}$, $frgI_{ORF149}$ and $frgII_{ORF149}$ were chosen for the rest of the experiments.

Uninfected carp populations from different locations exhibited variable levels of IgM binding

Preliminary experiments showed that IgM binding to $frgII_{ORF149}$ by homologous rec-ELISA (Fig. S3, rec-ELISA) of sera from uninfected carp generated a high level of nonspecific IgM-binding, which interfered with the analysis of KHVD survivor sera (not shown). Therefore, blocking with milk and pre-incubation of the sera with *E. coli* extracts (EE) were optimized using a small number of carp serum samples (eight uninfected and eight survivors). Nevertheless, non-specific IgM-binding levels still remained unpredictable when using a larger number of carp serum samples from different locations. To further investigate the nonspecific IgM-binding in larger carp

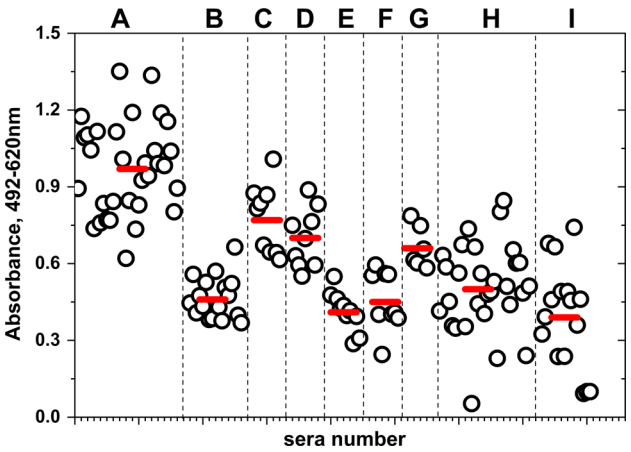

Fig. 1 IgM-binding values by rec-ELISA of carp populations from different locations with no previous history of KHVD. Rec-ELISA was performed using $frgII_{ORF149}$ as solid phase. Carp populations were obtained as follows: A, Spanish koi maintained for 3-4 years without any KHVD; B, carp surviving laboratory-induced spring viremia carp virus infection, SVCV; C-G, koi populations imported from different countries to Spain; H-I, carp populations from different locations in Spain. Open circles, mean of individual carp serum calculated from 2-3 independent assays per serum; red horizontal bars, mean absorbance value per location calculated from 2-3 independent assays per location

populations, sera from healthy carp from different international locations without any previous history of KHVD (here called uninfected) were collected. When all of these sera were assayed by rec-ELISA using $frgII_{ORF149}$ as the solid phase, highly variable levels of nonspecific IgM-binding were found both at the individual and population level. Thus, the means of ELISA absorbance values for locations A-I were 0.97 ± 0.03 (n = 32 individual carp), 0.46 ± 0.02 (n = 17), 1.77 ± 0.04 (n = 9), 0.70 ± 0.03 (n = 9), 0.41 ± 0.02 (n = 10), 0.45 ± 0.03 (n = 9), 0.66 ± 0.03 (n = 6), 0.50 ± 0.03 (n = 29), 0.39 ± 0.05 (n = 18) (Fig. 1A-I).

That the nonspecific IgM binding was dependent on IgM in the carp serum rather than on the anti-IgM MAb was confirmed using three alternative anti-carp IgM MAbs (AquaTic Diagnostics), which yielded similar results. Furthermore, absorbance values less than 0.01 were obtained when the carp serum was omitted from the rec-ELISA (not shown). Removal of polyH tails from purified frgs was not possible despite numerous attempts (unpublished), and further purification of the frgs by affinity and/or size-exclusion chromatography did not eliminate nonspecific IgM-binding (not shown), suggesting that the binding was due to polyH-containing EE impurities or to EE proteins similar in size to the frgs. In addition, nonspecific IgM binding could not be reduced after increasing $frgII_{ORF149}$ purity by including two additional consecutive polyH stretches to its carboxy terminal part (Fig. S2B, panel B). The nonspecific IgM binding precluded discrimination between uninfected and CyHV-3-T-infected carp sera, regardless of whether a $frg0_{ORF149}$ (Fig. 2A), $frgII_{ORF149}$ (Fig. 2B) or $frgII_{ORF1492xpolyH}$ (not shown) solid phase was used for rec-ELISA. In conclusion, additional reduction of nonspecific IgM binding was still required to study carp IgM responses to pORF149.

Discrimination between nonspecific binding and KHVD-survivor carp by rec2-ELISA

To discriminate nonspecific and specific IgM binding of sera from carp populations, a novel consecutive heterologous-homologous rec-ELISA protocol (rec2-ELISA) was devised (Fig. S3, rec2-ELISA) based on previously described parallel heterologous/homologous salmonid rhabdovirus-ELISAs [22]. The rec2-ELISA protocol

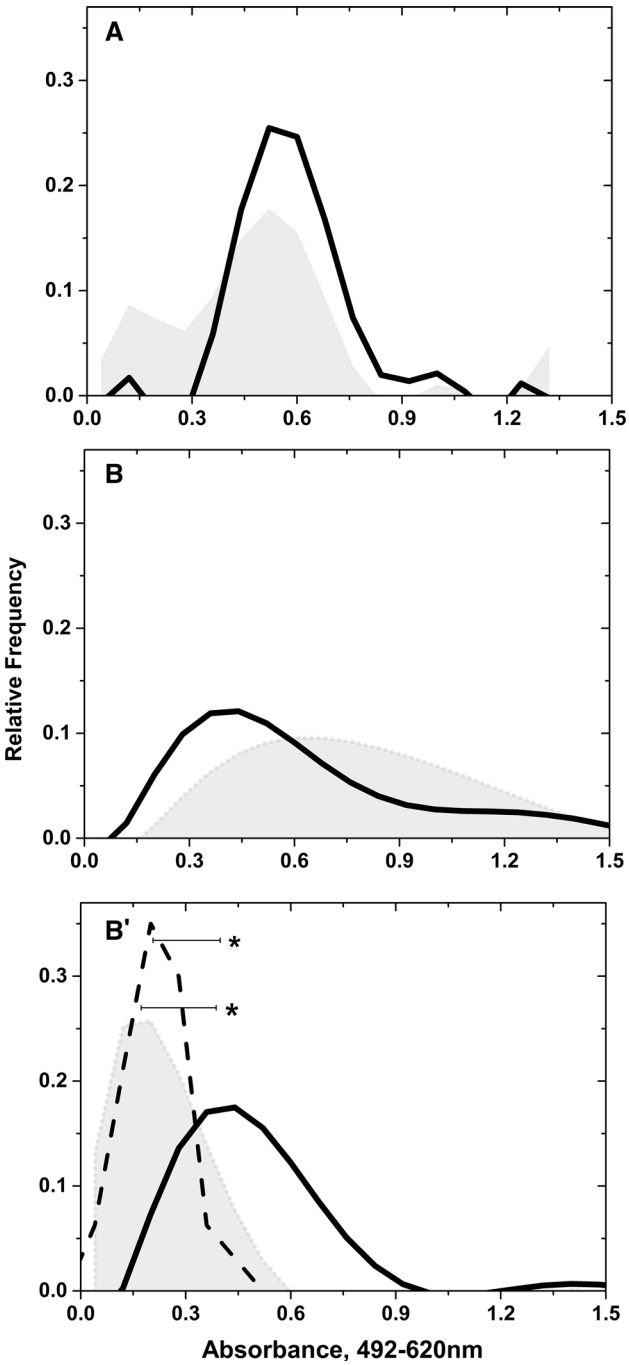

Fig. 2 Comparison of rec- and rec2-ELISAs and specific inhibition of rec2-ELISA by $frgII_{ORF149}$. Rec-ELISA was performed with $frg0_{ORF149}$ (A) or $frgII_{ORF149}$ (B) solid-phases. Rec2-ELISA was performed with a first $frg11_G$ and a final $frgII_{ORF149}$ solid-phase (B'). The mean absorbance values for each serum were calculated from two different experiments (n = 2) and classified in 0.08 absorbance increments. The resulting distribution of frequencies relative to the total number of sera in each population (relative frequency), was adjusted by 9th-order polynomial fitting by fixing a 0-intercept (OriginPro 2016) and represented. Shaded areas, IgM binding of sera from uninfected carp populations (n = 23). Continuous black lines, IgM binding of sera from CyHV-3-T survivor carp populations (n = 48). Dashed black line, IgM binding of sera from CYHV-3-T survivors in the presence of 20.8 µg of $frgII_{ORF149}$ per well by rec2-ELISA. *, significantly higher than uninfected (A, B) or uninfected and $frgII_{ORF149}$-inhibited (B') sera at the $p < 0.05$ level

Table 1 Binding of $frgII_{ORF149}$ to carp IgM captured by monoclonal antibody

Carp serum	Mean ± sd	N
Uninfected	0.23 ± 0.03	48
Survivor	*0.41 ± 0.03	48

Solid phases were coated with purified anti-carp IgM monoclonal antibody (2 µg/well) and incubated for 60 min with 50 µl of 100-fold-diluted sera from uninfected and CyHV-3-T survivor carp. After washing, 3 µg per well of $frgII_{ORF149}$ was added in 50 µl of dilution buffer and incubated for 30 min. After washing, 50 µl of 300-fold-diluted horseradish-peroxidase-labeled anti-polyH monoclonal antibody (Sigma) was added and incubated for 30 min to estimate $frgII_{ORF149}$ binding. Colour development was done as described in Materials and methods. N, number of carp sera. Mean ± sd, means and standard deviations. *, significatively different from uninfected sera at the $p < 0.05$ level

consisted of a pre-incubation step in which each carp serum were pre-incubated with a solid phase coated with heterologous $frg11_G$ from VHSV obtained by the same procedures that were used to obtain $frgs_{ORF149}$ to remove any cross-reactivity (Fig. S2B, panel C). A second incubation step with the resulting supernatant/non-adsorbed sera with homologous $frgs_{ORF149}$ and the rest of the ELISA steps were then performed (Fig. S3, rec2-ELISA).

Uninfected and CyHV-3-T survivor carp serum populations that could not be discriminated by homologous $frg0_{ORF149}$ (Fig. 2A) or $frgII_{ORF149}$ (Fig. 2B) rec-ELISA could be discriminated by $frgII_{ORF149}$ rec2-ELISA (Fig. 2B'). The broad distribution of serum IgM binding from uninfected carp populations from 0.3 to 1.2 absorbance units (Fig. 2B, shadow surface) obtained by $frgII_{ORF149}$ rec-ELISA, was reduced to a sharper distribution ~ 0.2 units when using the $frgII_{ORF149}$ rec2-ELISA protocol (Fig. 2B', shaded area). In contrast, the distribution of sera from survivor carp populations remained ~0.4 in rec- and rec2-ELISA (Fig. 2B and 2B', respectively, black lines).

The specificity of $frgII_{ORF149}$ IgM binding of survivors compared to uninfected populations was confirmed by its inhibition by co-incubation of the sera with an excess of $frgII_{ORF149}$ during the homologous step of the rec2-ELISA protocol (Fig. 2B', dashed line). Similar discrimination between uninfected and survivor carp sera populations was obtained when carp IgM was first captured on a solid phases coated with purified anti-carp IgM monoclonal antibody and $frgII_{ORF149}$ binding was detected with horseradish-labelled anti-polyH monoclonal antibody (Table 1). In addition, a similar level of discrimination was also obtained by Western blotting of pooled uninfected (n = 7) or survivor (n = 7) sera recognizing $frgI_{ORF149}$ or $frgII_{ORF149}$ separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membranes (Table 2).

Table 2 Recognition of $frgI_{ORF149}$ and $frgII_{ORF149}$ by pooled carp sera by Western blot

Pooled carp serum	$frgI_{ORF149}$ Mean ± sd	$frgII_{ORF149}$ Mean ± sd	n
Uninfected	0.20 ± 0.04	0.20 ± 0.04	3
Survivor	*0.42 ± 0.04	*0.36 ± 0.03	3

Selected sera from seven uninfected or seven surviving carp were pooled. Western blotting was performed by incubation of 100-fold-diluted pooled sera with $frgI_{ORF149}$ or $frgII_{ORF149}$ separated by 4-20 % polyacrylamide gradient gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). The bands were developed with anti-carp IgM, horseradish-peroxidase-labeled anti-mouse IgG and diaminobenzidine DAB using the same buffers and incubation times as described for rec-ELISA. Photography, densitometry and quantification in arbitrary units were performed as indicated in Materials and methods. To compare the results with those from rec2-ELISA, each of the optical densities was corrected by the following formula: Optical density in arbitrary units of each of the uninfected or survivor bands × 0.2/Mean optical density in arbitrary units of the corresponding non-infected bands (n = 3). Mean ± sd, means and standard deviations from three independent Western blot experiments (n); *, significantly different from uninfected pooled sera samples at the $p < 0.05$ level

Comparison between survivor carp population responses by $frgs_{ORF149}$ rec2-ELISA and CyHV-3-T-ELISA

To further investigate carp IgM responses, sera from survivor carp populations obtained from four different CyHV-3-T experimental infection groups (Fig. 3, red, black, blue, and green lines) were assayed by frgsORF149 rec2-ELISA and compared with the results obtained with CyHV-3-T-ELISA (Fig. 3). Uninfected (Fig. 3, shaded area) and survivor carp populations (Fig. 3, colored lines) were distinguished with CyHV-3-T by virus-ELISA and with $frgI_{ORF149}$ or $frgII_{ORF149}$ by rec2-ELISA. All survivor serum groups showed 3- to 5-fold lower mean absorbances (Fig. 3B and C) than when using CyHV-3-T (Fig. 3A). The distribution of absorbance values in CyHV-3-T-ELISA was slightly broader than that in rec2-ELISA, confirming the expected IgM binding to a greater variety of epitopes. On the other hand, the distribution of absorbance values in the uninfected carp population obtained with $frgI_{ORF149}$ / $frgII_{ORF149}$ was sharper (Fig. 3B and C, shaded areas) than that obtained with CyHV-3-T (Fig. 3A, shaded areas), suggesting an advantage in the use of frgs to discriminate uninfected from KHVD-survivor carp populations. The differences in mean absorbance obtained in CyHV-3-T and rec2-ELISA varied for the different groups of survivor sera, suggesting that different epitopes contribute to the differentiation of each of the carp population responses.

Using the rec2-ELISA with either $frgI_{ORF149}$ or $frgII_{ORF149}$, the correlation of the individual serum absorbance values of uninfected and survivor carp sera with CyHV-3-T-ELISA

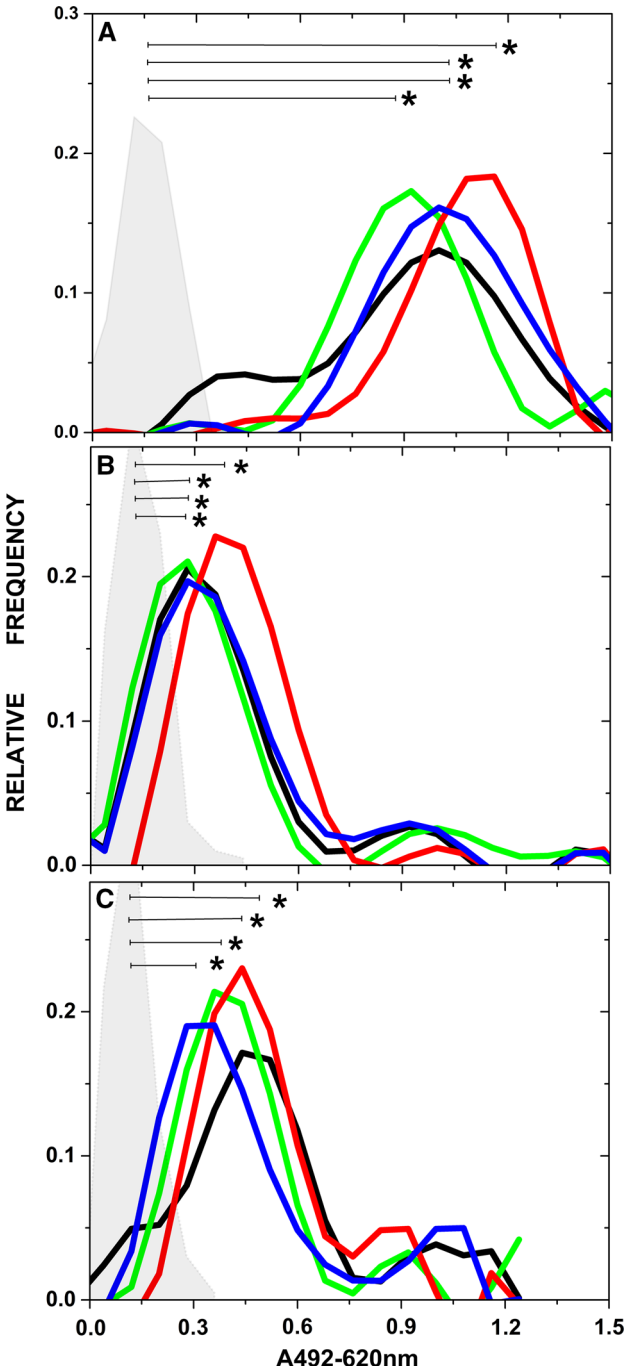

Fig. 3 Comparison of CyHV-3-T- and rec2-ELISAs. Uninfected fish and four groups of survivors of CyHV-3-T experimental infections were tested by CyHV-3-T- (A) or rec2-ELISAs using either $frgI_{ORF149}$ (B) or $frgII_{ORF149}$ (C) final solid phases. Absorbance means were obtained from two different experiments (n = 2), distributed into classes, and representation as indicated in Figure 2. Shaded areas, sera from the uninfected carp population (n = 43). Black lines, survivor carp sera from population 1 (n = 23). Green lines, survivor carp sera from population 2 (n = 23). Red lines, survivor carp sera from population 3 (n = 23). Blue lines, survivor carp sera from population 4 (n = 23). *, significantly higher than uninfected sera at the $p < 0.05$ level

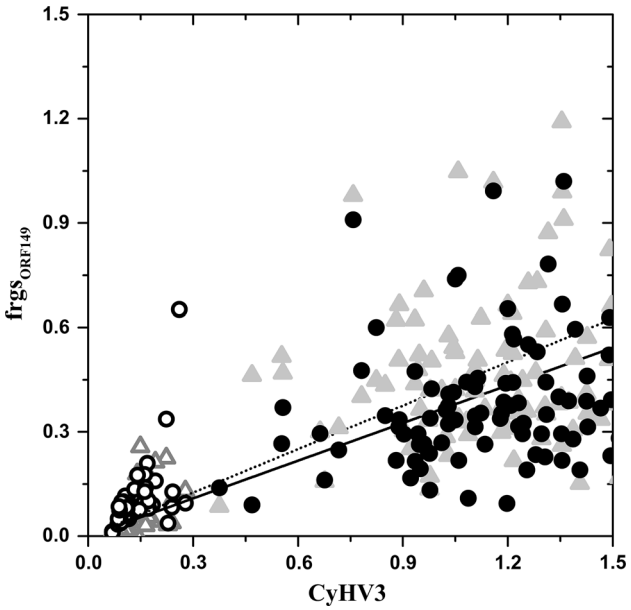

Fig. 4 Correlation between CyHV-3-T- and rec2-ELISA values. The data from Figure 3 were compared. ○, uninfected sera tested in CyHV-3-T- and rec2-ELISA using $frgI_{ORF149}$ (n = 43); Δ, uninfected sera tested in CyHV-3-T- and rec2-ELISA using $frgII_{ORF149}$ (n = 43); ●, CyHV-3-T survivor sera tested in CyHV-3-T- and rec2-ELISA using $frgI_{ORF149}$ (n = 92); ▲, CyHV-3-T survivor sera tested in CyHV-3-T- and rec2-ELISA using $frgII_{ORF149}$ (n = 92). The corresponding Pearson's linear correlation coefficients were 0.87 (black circles/black line, CyHV-3-T/$frgI_{ORF149}$) and 0.90 (grey triangles/dotted line, CyHV-3-T/$frgII_{ORF149}$)

were very high (Pearson's 0.87 and 0.90, respectively). However, as commented above, the absorbance values obtained with survivor sera in $frgI_{ORF149}$ /$frgII_{ORF149}$ were lower than those obtained with CyHV-3-T (Fig. 4).

The similarities between the absorbance profiles obtained with $frgI_{ORF149}$ and $frgII_{ORF149}$ and their differences with CyHV-3-T suggested that 20-33 % of these cysteine-?independent? responses to CyHV-3-T might map to the pORF149 amino-terminal sequence. To further estimate the relative levels of anti-$frgII_{ORF149}$ and anti-CyHV-3-T antibodies, anti-$frgII_{ORF149}$ was removed by incubating the carp sera with a solid phase coated with $frgII_{ORF149}$ before addition of anti-CyHV-3-T antibodies. Table 3 shows that the mean IgM binding of survivor sera to CyHV-3 was reduced from 1.13 to 0.68, showing that ~60 % of the anti-$frgII_{ORF149}$ could be depleted from the whole response of anti-CyHV-3-T antibodies. These data also confirmed the specificity of the rec2-ELISA.

Recognition of CyHV-3 pORF149 fragments by anti-CyHV-3 monoclonal antibodies

To test the immunogenicity of pORF149 in heterologous hosts, several available MAbs obtained after immunization

Table 3 IgM-binding of carp sera by CyHV-3-T-ELISA after incubation with solid-phase $frgII_{ORF149}$

Carp serum	-$frgII_{ORF149}$ Mean ± sd	+$frgII_{ORF149}$ Mean ± sd	N
Uninfected	0.24 ± 0.08	0.22 ± 0.08	48
Survivor	$^{+}$1.13 ± 0.23	$^{+}$*0.68 ± 0.14	48

Sera from uninfected and CyHV-3-T survivor carp were diluted 100-fold and assayed for anti-CyHV-3-T IgM-binding by ELISA without (-) or with (+) previous incubation of sera with solid phases coated with $frgII_{ORF149}$. -frgIIORF149, binding of the sera to CyHV-3-T solid-phase. +frgIIORF149, binding of the sera supernatants obtained after binding to $frgII_{ORF149}$ solid-phase to CyHV-3-T solid-phase. N, number of carp sera assayed. Mean ±sd, means and standard deviations from two independent experiments. +, significantly different from the corresponding mean values of uninfected sera at the $p < 0.05$ level. *, significantly different from the mean values of -$frgII_{ORF149}$ at the $p < 0.05$ level

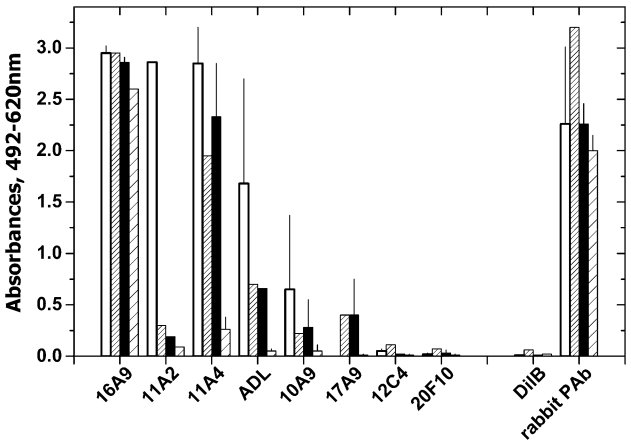

Fig. 5 Binding of anti-CyHV-3 monoclonal antibodies (MAb) to recombinant $frgs_{ORF149}$. Two µg of protein per well of *E. coli*-expressed $frg0_{ORF149}$ (GST-KO149 [14]), $frgI_{ORF149}$, $frgII_{ORF149}$ or $frgIII_{ORF149}$ were used as solid phases. Protein-G-purified MAbs at 0.2-0.4 mg/ml were diluted 20-fold with dilution buffer and incubated with the solid phases. The presence of frgs bound to the solid phases was confirmed by binding of peroxidase-labeled anti-polyH (Sigma) with absorbances >2.2 for all frgs except for $frg0_{ORF149}$, which gave a value of 0.02. Absorbances <0.01 were obtained by each of the MAbs when the frgs were omitted from the solid phase. Means and standard deviations are shown (n = 2 experiments). ADL, anti-KHV P14 purchased from Aquatic Diagnostics Ltd; rabbit PAb, rabbit anti-GST-KO149 antiserum [14]; DilB, dilution buffer; white bars, solid-phase coated with $frg0_{ORF149}$ (GST-KO149) [14], a cysteine-intact and complete pORF149 expressed in *E. coli* [14]; dense hatched bars, solid-phase coated with $frgI_{ORF149}$; black bars, solid phase coated with $frgII_{ORF149}$; sparse hatched bars, solid phase coated with $frgIII_{ORF149}$

of mice with purified CyHV-3 (Table S2) were used to map epitopes in $frg0_{ORF149}$ (cysteine intact pORF149, previously described as GST-KO149) [14], $frgI_{ORF149}$, $frgII_{ORF149}$ or $frgIII_{ORF149}$. After the coating and blocking steps, all of the frgs were present on the solid phase, as demonstrated by the high-binding levels obtained for each solid phase with rabbit anti-CyHV-3 polyclonal Abs (Fig. 5, rabbit PAb). Furthermore, peroxidase-labelled anti-polyH MAb only recognized $frgI_{ORF149}$, $frgII_{ORF149}$ and $frgIII_{ORF149}$, but not $frg0_{ORF149}$, since $frg0_{ORF149}$ was fused to GST [14] rather than to polyH (results not shown). The recognition of CyHV-3 epitopes in each frg_{ORF149} was also confirmed by MAb 16A9, which bound similarly to all frgs. MAb 11A4 differed from 16A9 in that it recognized $frgI_{ORF149}$ and $frgII_{ORF149}$ but not $frgIII_{ORF149}$, confirming the immunogenicity of the amino-terminal part of the pORF149 molecule in heterologous hosts such as mice. MAbs 11A2 and ADL (P14) mostly recognized $frg0_{ORF149}$, suggesting recognition of conformational cysteine-dependent epitopes. The MAbs 10A9 and 17A9 (neutralizing MAbs) [14], 12C4, and 20F10, did not recognize most epitopes on the frgs, possibly due to their specificity for other CyHV-3 proteins or to the specificity to cysteine-dependent conformational epitopes destroyed by the coating process.

Discussion

Using recombinant pORF149 fragments of CyHV-3 and a novel rec2-ELISA protocol, amino-terminal epitopes have been identified as immunodominant in serum IgM responses of carp populations surviving experimental CyHV-3-T infections. Because of the high variability of fish responses to infection, focusing on populations (frequency distributions) rather than pooled samples, was instrumental for detecting low but significant differences between uninfected and survivor carp. The specificity of the discrimination was further supported at the population level by i) the similar mean absorbance levels in the rec- and the rec2-ELISA protocols, ii) inhibition of reactivity by the addition of an excess of $frgII_{ORF149}$ during the homologous incubation step of rec2-ELISA, iii) similar profiles between uninfected and survivor sera when using an alternative IgM-captured ELISA and iv) Western blotting of pooled sera. Furthermore, CyHV-3-ELISA reactivity was reduced when the sera were pre-incubated with $frgII_{ORF149}$. Therefore, when analysing populations, false positive results due to lack of specificity are more rarely to be expected with the ?refined? rec2-ELISA than with the standard rec-ELISA protocol. The ability to discriminate between uninfected and survivor carp serum populations was only 3- to 5-fold lower than when using whole CyHV-3, an unexpected finding given the large number of proteins encoded by the genome of herpesviruses.

Up to now, attempts to map CyHV-3 IgM targets [14] have been limited because of the technical difficulties in estimating tetrameric IgM binding specific for recombinant viral frgs (rec-ELISA) in fish despite the existence of

highly specific neutralization and CyHV-3-ELISA assays [18, 29, 36, 38, 46, 47]. Thus, previous mapping of immunologically relevant viral epitopes in salmonids has met with nonspecific IgM binding attributed to the low affinity of fish IgM [10, 11, 20–22]. Preliminary experiments with CyHV-3 recombinant proteins also showed nonspecific IgM binding, which was attributed to altered conformations caused during the 37 °C *E. coli* biosynthesis or during the downstream purification steps (unpublished). High levels of nonspecific IgM binding was also observed when using recombinant $frgs_{ORF149}$ in this work. To discriminate uninfected from survivor IgM binding, the parallel VHSV/IHNV ELISA protocol recently described for salmonids [20, 22] was tested with $frgs_{ORF149}$ rec-ELISA. However, when parallel $frg11_G$ (heterologous) and $frgII_{ORF149}$ (homologous) solid phases were used with uninfected and CyHV-3-T survivor carp sera, many of the resulting differential absorbance values were negative (not shown). A possible explanation for those results was the existence of high levels of anti-*E. coli* IgM in carp sera recognizing contaminating epitopes that were only accessible when in the solid phase, as suggested by the results obtained when using *E. coli* extracts as the solid phase (not shown). Thus, those high levels of non specific binding precluded any epitope analysis until a pre-binding step with heterologous $frg11_G$ from VHSV was included (rec2-ELISA protocol). Only by the rec2-ELISA protocol could we identify survivor carp sera, although only with 2- to 3-fold higher absorbance than with uninfected sera and 3- to 5-fold lower than when using whole virus. Nevertheless, the significance of the recognition at the population level was confirmed by different methods, as mentioned above.

It was not unexpected to find anti-*E. coli* IgM in carp sera, since to survive in an aquatic environment where bacterial concentrations (including those of *E. coli*) might be high, fish need to maintain appropriate antibody levels. On the other hand, it is known that *E. coli* can colonize and multiply in fish intestine after initial delivery through their feed, and therefore, the presence of anti-*E. coli* IgM binding activity in their sera might not necessary come from water contamination [8]. Variation in feeds and/or bacterial exposure could also explain why different carp populations showed a wide variation in their nonspecific IgM binding levels. Nevertheless, the high values of nonspecific IgM binding in some of the carp populations studied here raised some intriguing questions about the possible presence of other natural Abs and/or infection-dependent co-induction of broadly specific IgM. A complete explanation, therefore, requires further experimentation. For example, among many other possibilities, cross-reaction or lack of cross-reaction with other bacteria and/or proteins should be further studied, as similar cases have been reported for mammalian IgM [23].

The similarity of the IgM binding levels obtained with $frgI_{ORF149}$ (amino acid residues 42-629 with all of its cysteines mutated to serines) and $frgII_{ORF149}$ (42-159), suggested that many of the host-targeted epitopes of pORF149 could be localized in its amino-terminal part. Although destruction of some cysteine-dependent epitopes could also explain that similarity, it is unlikely that single amino acid mutations could destroy all such epitopes in $frgI_{ORF149}$. The similar absorbance profiles of surviving serum populations obtained with $frg0_{ORF149}$ and $frgI_{ORF149}$ (amino acid 1-686, containing all of its cysteines) seem to confirm the first assumption mentioned above. That the amino-terminal part of pORF149 contains immunologically relevant epitopes capable of inducing Abs not only in carp but also in mice was shown by the recognition of $frg0_{ORF149}$, $frgI_{ORF149}$ and $frgII_{ORF149}$, but not of $frgIII_{ORF149}$ (441-629) by MAb 11A4, one among seven randomly generated MAbs to CyHV-3. To our knowledge, no such immunodominance has been reported in other herpesviruses. In addition, cysteine-free epitopes would be best expressed in *E. coli*, and higher yields in downstream purification processes would be obtained. These could be also important advantages for novel vaccine designs based on pORF149 frgs.

From a diagnostics point of view, a practical method to detect anti-CyHV-3 Abs in survivors would be most desirable. For massive screening, any type of ELISA will be preferred to labour-intensive neutralization assays. Among ELISAs, an assay using recombinant viral proteins rather than whole virus would be more practical, since it bypasses time-consuming and unsafe cell culture procedures. Since the use of insect-expressed native G_{VHSV} [12] neither improved ELISA sensitivity nor lowered nonspecific IgM binding [10], *E. coli*-expressed cysteine-free epitopes would be preferred. In this respect, pORF149 frgs could be adapted to practical diagnostic ELISAs to detect asymptomatic CyHV-3 carp carrier populations. It could be argued that restriction to the amino-terminal sequence of pORF149 reduced sensitivity compared to whole CyHV-3, and that for diagnostic purposes, better discrimination between uninfected and survivor populations would be required. To increase sensitivity, alternative recombinant fragments from other immunodominant CyHV-3 ORFs could be added to $frgII_{ORF149}$ to fill the gap of absorbance obtained with CyHV-3. On the other hand, because fish anti-viral Abs of the tetrameric IgM class bind nonspecifically to many surfaces, some false positives might appear [34], and therefore, individual positives would have to be confirmed by neutralization and/or immunofluorescence. However, a few false positives will not have a large effect on the frequency distribution of ELISA values when estimating values from a carp population, and therefore, the rec2-ELISA protocol could allow the identification of carp

populations with a high probability of having survived KHVD. Nevertheless, because of the large number of variables in natural KHVD outbreaks, more fine-tuning will be necessary to reach definitive conclusions in the diagnostics area.

Little is known about the function of pORF149, except for its relationship with pORF25, 65, 148 and 149 (members of the same gene family) and its location in the CyHV-3 virions [14]. Protein sequences with 43 % identity were found in the pORF148 of CyHV-3, but none with significant similarity to any other herpesvirus proteins. Nevertheless, the PTT repeat-rich $frgIII_{ORF149}$ domain showed 65-75 % identity to heterologous proteins from several sources, such as sialidases, chitinases, collagen, C-type lectins and mucins, suggesting that $frgIII_{ORF149}$ might be implicated in interactions with host cellular membrane glycoproteins. It is also unknown how the cysteine-free epitopes identified in this work might be related to *in vitro* neutralizing epitopes, but all pORF149-specific MAbs, including MAb 11A4 (Dr. K.Thompson, personal communication), neutralized CyHV-3 *in vitro* [14]. *In vitro* neutralizing anti-CyHV-3 Abs were detected in most sera from carp surviving the infection for many months [36], as was observed in trout surviving VHSV infection [25–27]. Although most of the epitopes targeted by *in vitro*-neutralizing Abs in trout belong to disulphide-dependent viral surface glycoprotein epitopes, only a maximum of ~50 % of the survivor sera contained detectable *in vitro* neutralizing Abs [25–27], and those were gradually replaced over time by anti-viral IgM targeting cysteine-free viral epitopes [10, 11, 13, 24]. It remains to be investigated how the CyHV-3-T two-month IgM responses investigated in this work would evolve over longer times.

In addition to other CyHV-3 proteins that have been proposed as subunit vaccine candidates such as pORF81 [44, 52], pORF25 [51], or pORF72 [49], the corresponding eukaryotic expression constructs of $frgs_{ORF149}$ might also be suitable for the development of subunit or DNA vaccines.

Acknowledgments Thanks go to Paula Perez Gonzalez, who helped with the experimental work. Thanks are due to Dra. D. Chee (Animal & Plant Health Laboratories, Aquatic Animal health Section Laboratories Group, Singapore) for providing sera from carp surviving CyHV-3, to Dra. K. Thompson (AquaTic Diagnostics, Ltd) for providing some of the anti-CyHV-3 monoclonal antibodies, and Dra. P. Fernandez (MAGRAMA, Algete, Madrid, Spain) for her help with CyHV-3-U. We also thank J.L. Gonzalez and his team (CBC, SL) for obtaining common carp sera samples from all over Spain and from imported koi, and P. Encinas, B. Chinchilla and Dr. E. Gomez-Casado, who helped with Spanish koi sera at UPM (Madrid). This work was supported by RTA2013-00008-00-00 of INIA, and AGL2014-51773-C3 and AGL2014-53190 REDC of the Ministerio de Economía y Competitividad of Spain.

Compliance with ethical standards

This study was funded by RTA2013-00008-00-00 of the Instituto Nacional de Investigaciones y Tecnología Agraria y Alimentaria, INIA, and AGL2014-51773-C3 and AGL2014-53190 REDC of the Ministerio de Economía y Competitividad of Spain. The authors declare that they have no conflict of interest. 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 by any of the authors.

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