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An ELISA for detection of trout antibodies to viral haemorrhagic septicemia virus using recombinant fragments of their viral G protein

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An enzyme linked immunosorbent assay (ELISA) method to study serum antibodies to viral haemorrhagic septicemia virus (VHSV) was designed by using recombinant fragments of their G protein. By using this fragment-ELISA, we describe the binding of antibodies against recombinant G fragments of 45–445 amino acids present in VHSV-hyperimmunized trout sera. Fragments were designed by taking into account their tridimensional pH-dependent structure and functional domains. Sera were obtained from hyper-immunized trout following 4–5 intraperitoneal injections of VHSV antigens by using Freund's or saponin adjuvants. Sera from different hyperimmunized trout differed quantitatively rather than qualitatively in their recognition of solid-phase frg11 (56–110), frg12 (65–109), frg13 (97–167), frg14 (141–214), frg15 (65–250), frg16 (252–450) and G (G21–465) by Western blot and ELISA. However, titres were higher when using frg11, frg15 or frg16, rather than G21–465, suggesting higher accessibility to G epitopes. Further knowledge of the antigenicity of the G protein of rhabdoviruses by using fragments might be used to improve current vaccines. On the other hand, they might be used to dissect the trout antibody response to VHSV infections, to complement *in vitro* neutralizing assays, and/or to quantitate anti-VHSV antibodies in VHSV-infected/vaccinated trout, other fish and/or other body fluids such as mucus.

1. Introduction

In trout infected naturally with viral haemorrhagic septicemia virus (VHSV), it is known that: (i) *in vitro* neutralizing antibodies can only be detected in ~50% of the survivors (Olesen et al., 1991; Olesen and Jorgensen, 1991), (ii) the majority of the survivors, resist a second VHSV infection but with no detectable increase in their N-antibody (Ab) levels (Olesen et al., 1991; Olesen and Jorgensen, 1991) and (iii) antibodies could not be detected by immunoblotting in most sera from trout surviving VHSV (Lorenzen et al., 1993a). On the other hand, the injection with recombinant G proteins made in *Escherichia coli* (Estepa et al., 1994; Lorenzen et al., 1993b), yeast (Estepa et al., 1994) and/or baculovirus (Koener and Leong, 1990), induced low and irreproducible protection levels despite the production of antibodies. In contrast, high protection levels have been obtained by intramuscular injection of the G gene, generally with low N-Ab levels (Anderson et al., 1996a,b; Fernandez-Alonso et al., 2001; LaPatra et al., 2001; Lorenzen et al., 1998, 2000, 2001; Lorenzen and LaPatra, 2005; McLauchlan et al., 2003). Therefore, the role of anti-protein G antibodies in trout sera and/or their target epitopes in protection of fish surviving rhabdoviral infections after infection and/or vaccination, is still a matter of study (Kurath et al., 2007).

For instance, while antibodies could not be detected by immunoblotting in most sera from protected trout surviving VHS, antibodies were detected by immunoblotting in trout injected with G, but the trout were, usually, not protected (Lorenzen et al., 1993a). Since most of the negative immunoblotting sera were positive by enzyme linked immunosorbent assay (ELISA), immunofluorescence and/or neutralization, the most likely reason for negative or weak immunoblotting results, is that trout produce N-antibodies mainly to disulphide-dependent conformational epitopes (which are lost after immunoblotting in reducing conditions), rather than to other epitopes (linear or disulphide-independent conformational epitopes). Confirming that hypothesis, the reduction of the 6 intramolecular disulphide bonds of native G (Einer-Jensen et al., 1998), further decreased the small immunoblotting reactivity of the few positive anti-VHSV trout sera (Lorenzen et al., 1990; Olesen et al., 1993).

Because at least one *in vitro* non-neutralizing monoclonal Ab (MAb) induced *in vivo* protection after serum passive transfer (Lorenzen et al., 1990), it is still not clear what could be the relative importance in protection of anti-G antibodies directed against non-neutralizable B-cell epitopes. Previous attempts to identify short linear B-cell epitopes on the VHSV G by the use of overlapping 15-mer synthetic peptides, showed that N-monoclonal antibodies

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Table 1
Design and properties of fragments from the glycoprotein G of VHSV07.71.

G general location	G fragment number	Position on the G protein	Number of aminoacids	Molecular weight (kDa)	Number of Cys
Domain of frg15	frg11	56–110	55	10.3	2
frg11 without Cys	frg12	65–109	45	9.2	0
Domain of frg15	frg13	97–167	71	12.6	2
Domain of frg15: fusion loops	frg14	141–214	74	13.0	3
Amino terminal G	frg15	65–250	186	25.2	5
Carboxy terminal G	frg16	252–450	199	26.3	4
G without transmembrane and carboxy terminal regions	G	21–465	445	49.1	12

Fragments 11–16 were made in *E. coli* while the G was made in insect larvae. The amino acid position on the protein G is shown by including their signal peptide in the numbers. Number of amino acids, corresponding number of amino acids of the protein G. In addition to their protein G sequence, the fragments contained amino terminal polyHis tails with 33 amino acids (~4 kDa) sequences. Removal of the polyHis tail by the enterokinase target sequence provided in the pRSET was not possible because the change of conformation at the physiological pH required for enterokinase activity precipitated the fragments and yields were very low.

could not be mapped and highly neutralizing trout sera only significantly recognized some peptides in the 99–113, 199–213 and 329–393 regions (Fernandez-Alonso et al., 1998). In contrast, by sequencing MAb resistant VHSV mutants, the N-MAb C10 epitope could be mapped to positions 140 and 433 defining a conformational epitope (Bearzotti et al., 1995) while the 3F1A12 epitope was mapped to position 253 located between two close cysteines forming one disulphide bond (Lorenzen, personal communication). Disulphide-dependent conformational neutralizing B-cell epitopes may be more immunogenic in trout than other epitopes. Alternatively, the antigenicity of B-cell epitopes might be lost more easily in immunoblotting assays and/or larger G regions might be required to detect their disulphide-independent yet conformational epitopes. Therefore, to explore the possibility of detecting the presence of those kinds of epitopes on the G of VHSV, ELISA assays were performed with affinity-purified recombinant G larger fragments (of 45–445 amino acids of length).

The G21–465 devoid of their signal peptide and transmembrane domain was expressed in insects while the rest of the fragments were designed by taking into account their mapping on the pH-dependent tridimensional structures of protein G modelled after that of vesicular stomatitis virus, a well known mammalian rhabdovirus (Roche et al., 2006, 2007). The results obtained by using the fragments as solid phase for ELISA, showed that some disulphide-independent epitopes could be detected among sera antibodies from hyperimmunized trout. Absorbance values were highest when using fragments rather than by using G21–465, most probably because of the higher accessibility of their epitopes. While these assays may even have some diagnostic potential, they might be used for the improvement and follow-up of vaccination attempts, such as reported before for frg11 (Fernandez-Alonso et al., 2001). Future work by using fragments to screen large numbers of sera from trout infected with VHSV, might help to understand further the complex trout Ab response against VHSV infection. In addition, antigenic G fragments could be used to detect anti-VHSV antibodies in other fish species, and/or to detect mucus immunoglobulins. The downsizing antigen method could be tested also in other fish viruses for similar purposes.

2. Materials and methods

2.1. Construction of recombinant baculoviruses expressing the viral haemorrhagic septicemia viral glycoprotein G

The glycoprotein G was cloned in the appropriated plasmids by substituting their hydrophobic signal peptide by that of honey bee melitin (see below) and in the presence or absence of their transmembrane region (G21–507 and G21–465). The corresponding DNA sequences were PCR amplified from the pMCV1.4-G (G1–507) of VHSV-0771 (Gene Bank accession number X59148) (Rocha et al., 2005) by using the following primers: 5′GCGGATCCTCAGATCACTCAACGACC (forward) and 5′GCTCTAGAGACCGTCTGACTTCTGGAG (G21–507) or 5′GCTCTAGATGATGGCCAAAGACTCCAG (G21–465) (reverse), respectively (Table 1 and Fig. 1). All primers were flanked by *BamHI*/*XbaI* restriction sites, for subcloning into the carboxyterminal polyhistidine tail-containing pFastMelB2 vector containing the melitin signal peptide (Invitrogen) (Gomez-Sebastian et al., 2008). The PCR amplification was carried out by denaturation at 95 °C 5 min, 30 cycles of amplification (94 °C for 20 s, 52 °C for 30 s, 72 °C for 1 min) and a final extension of 72 °C for 10 min. Constructs were transformed in DH10 Bac™ competent *E. coli* cells following the Bac-to-Bac® baculovirus expression system (Invitrogen). *Spodoptera frugiperda* (Sf21) cells were transfected with recombinant bacmid DNAs and 72 h after transfection, supernatants containing the recombinant baculoviruses, were collected and amplified by re-infecting fresh Sf21 cells. Wild type baculovirus with no insert was used (BacNi) as negative control (Perez-Filgueira et al., 2007).

2.2. Cloning of viral haemorrhagic septicemia viral glycoprotein G fragments in E. coli

The DNA sequence (Thiry et al., 1991) corresponding to the glycoprotein G of VHSV 07.71, isolated in France (LeBerre et al., 1977) from rainbow trout *Oncorhynchus mykiss* (Walbaum), was used through the experiments (Gene Bank accession number X59148). The corresponding DNA sequences of frg11 (amino acid 56–110), frg12 (65–109), frg13 (97–167), frg14 (141–214), frg15 (65–250) and frg16 (252–450) (Table 1 and Fig. 1) flanked by *BamHI* and *XhoI* restriction enzyme sequences were synthesized (BioS & T Inc., Montreal, Canada) and subcloned into the pRSETa plasmid (Invitrogen, San Diego, CA). The constructs confirmed by sequencing were used to transform *E. coli* DH5α (Invitrogen, Barcelona, Spain) by electroporation. Large amounts of plasmid were prepared from *E. coli* pellets by using a modification of a commercial DNA purification system (Promega, Madison, USA). Their DNA concentration was then estimated by SYBR binding (Sigma Chemical Co., St. Louis, MO), by diluting the initial solution of SYBR 500-fold in 5 M NaCl, 100 mM $NaPO_4H_2$ (pH 7) and measuring their fluorescence at 485/535 nm (linearity from 5 to 200 μg DNA/ml) and confirmed by nanodrop ND1000 spectrophotometry measurements (Nanodrop Technologies Inc., Wilmington, DE, USA). Plasmid solutions were adjusted to 0.5–1 mg/ml and kept frozen until used.

2.3. Recombinant G proteins and their fragments expression in insect and E. coli, respectively

Trichoplusia ni (cabbage looper) larvae were injected with 10 μl of $\sim 30 \times 10^6$ pfu/ml of the corresponding recombinant

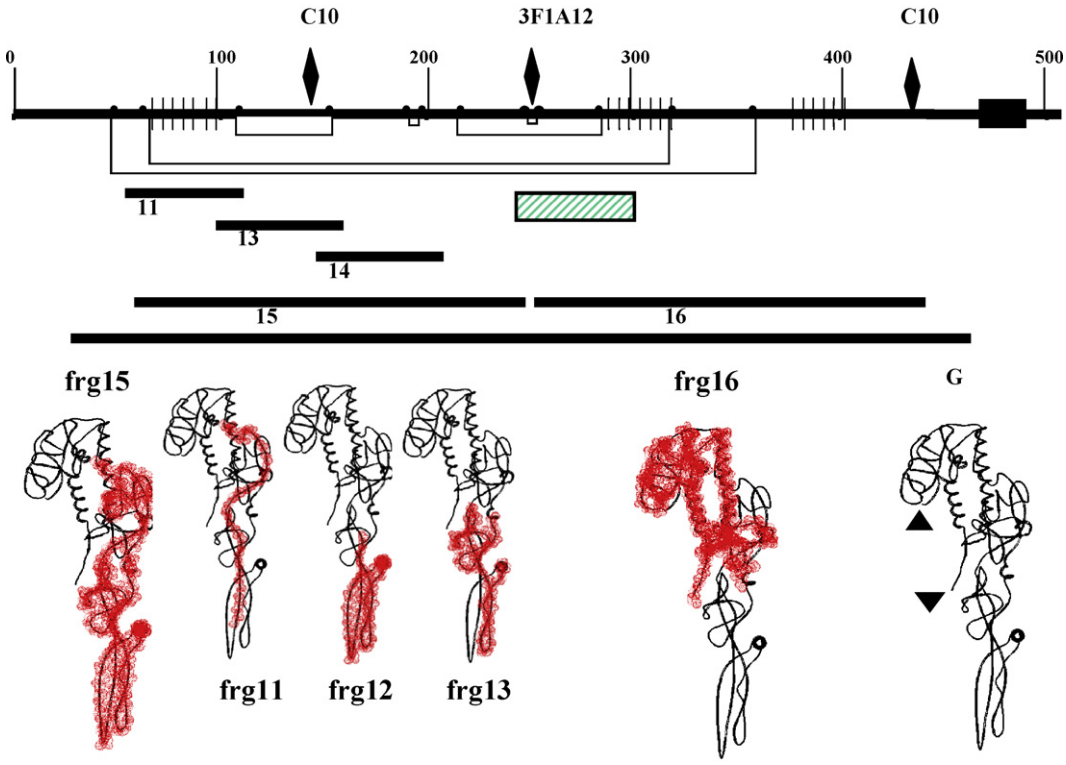

Fig. 1. Approximated location of fragments in the protein G amino acid linear sequence (up) and in their post-fusion low pH tridimensional monomeric structure (down). Numbering goes from the amino terminal (0) to the carboxyterminal (517) amino acids of the protein G of VHSV07.71 by including the signal peptide. □ (Black), Sequence of VHSV protein G and their cloned fragments, frg11 (amino acid 56–110), frg12 (65–109), frg13 (97–167), frg14 (141–214), frg15 (65–250), frg16 (252–450) and G21–465. The frg12 (65–109), very similar to frg11, has been omitted for clarity.▲, Terminal NH2 of the G21–465. ▼, Terminal COOH of the G21–465. Horizontal hatched rectangle, high variability region (245–300) (Benmansour et al., 1997; Einer-Jensen et al., 2004). ●, Cysteines connected by horizontal lines meaning its pairing by disulphide bridges (Einer-Jensen et al., 1998). |||||||, Non-cannonical hydrophobic heptad repeat regions (Coll, 1995). ♦, C10 MAb resistant mutants mapping at positions 140 and 433 and 3F1A12 MAb resistant mutants mapping at position 253. ■, Highly hydrophobic transmembrane region. The tridimensional structure of the glycoprotein G of VHSV at the post fusion state at low pH (down) was modelled after the X-ray structure of vesicular stomatitis virus G protein (2CMZ) from the RCSB protein data bank (http://www.rcsb.org/pdb) with the Swiss pdbv viewer (http://www.expasy.org/spdbv). Due to the existence of small gaps and 2 different disulphide bridges, the resulting approximation has an estimated precision of at least ±3 amino acids.

baculoviruses and maintained at 28 °C (Medin et al., 1995; Perez-Filgueira et al., 2006, 2007). About 72 h later, when they become swollen, pale and lethargic, larvae were frozen at −70 °C. Infected larvae (∼15 g) were homogenised in 6 M guanidinimun chloride, 1 M sodium chloride, 25 mM imidazole and protease inhibitor cocktail (Complete, Roche) and 40 mM phosphate buffer (pH 7.8). After removing the pellet by centrifugation at 10,000 × *g* 10 min and filtering through Miracloth paper (Calbiochem), the extracts were sonicated at 10 W with a needle Virtis sonicator until a clear lysate was obtained. They were kept frozen until processed.

For expression of the cloned fragments in bacteria, *E. coli* BL21 DE3 cells were transformed with the pRSETa-frg plasmids. The *E. coli* colonies were grown in 100 ml of TB medium supplemented with 100 μg/ml ampicilin at 32 °C and agitated continuously. Next day, the temperature was down shifted to 28 °C and 100 mM IPTG was added to induce expression. Six hours later 100 mM IPTG was added again and the culture was maintained overnight at 28 °C. Finally, the cells were pelleted by centrifugation and treated subsequently with 30 ml of lysis buffer (20 mM Na_2HPO_4, 0.5 M NaCl, 6 M guanidine HCl, pH 7.8). The lysate was submitted to sonication bursts as above until cleared and then centrifuged 20,000 × *g* for 30 min and kept frozen until processed.

2.4. Purification of polyhistidine containing protein G and their fragments

The extracts were applied to a Ni affinity column (ProBond™, Invitrogen) equilibrated previously with binding buffer (6 M guanidinium chloride, 1 M NaCl, 25 mM imidazole, 40 mM NaH_2PO_4 pH 7.8). The same buffer was passed through the column to wash and elution buffer with 150 mM imidazole was used to obtain the polyHis containing fragments. Peak fractions by absorbance at 280 nm were pooled, adjusted to pH 4 to avoid their precipitation, chromatographed through Sephadex G-100 in 20 mM sodium acetate pH 4 and dialyzed extensively against 20 mM sodium acetate pH 4. In addition to their protein G sequence, the fragments contained amino terminal polyHis tails with a 33 amino acids (4 kDa) sequence (MRGSHHHHHHGMASMTGGQQMGRDLY-DDDKDRW). Protein concentrations were determined by using the BCA method and purity confirmed by densitometry of Coomassie-blue stained protein bands separated on 10–20% polyacrylamide gradient gels (PAGE, BioRad).

2.5. Characterization of G fragments by Western blotting

Confirmation of fragment recognition by trout antibodies was performed by Western blot on SDS–15% polyacrylamide gels in buffer containing 2-β-mercaptoethanol. The proteins in the gel were transferred to nitro-cellulose membranes (Bio-Rad, Richmond, VA), blocked with 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). Three millimeters wide membrane strips were incubated with the corresponding trout Ab and with the peroxidase-conjugated rabbit anti-mouse (RAM-PO) Ab and developed with diaminobenzidine (DAB) staining

Table 2
Sera from hyperimmunized trout used in this work.

Trout serum	Antigen	Adjuvant	Number injections	Protein (mg/ml)
Tc1	–	–	0	35.8
T30	VHSVp + G4 + Gs	Freund's	5	42.5
T31	VHSVp + Gs	Freund's	2	22.5
T32	N3 + G4 + Gs	Freund's	4	57.9
T34	VHSVp	Saponin	5	73.2
T35	VHSVp	Saponin	5	90.3
Pooled	VHSV infected	–	–	62.3

Protein concentrations were estimated by nanodrop absorbances at 280 nm after a 10-fold dilution of the sera. Their value ranges are within the expected values reported before (Olesen, 1986). The Coomassie blue-staining profiles of the PAGE of all hyperimmune sera were similar (not shown). Gs, soluble protein G obtained from supernatants of VHSV-infected EPC cell monolayers purified by affinity chromatography on concanavalin A (Perez et al., 1998). G4, recombinant glycoprotein G of VHSV made on yeast (Estepa et al., 1994). VHSVp, supernatants from infected EPC cells concentrated by polyethylene glycol (PEG). Pooled, the pooled trout sera comes from 3 VHSV-infected trout (gift of Dr. J. Castric).

Western blotting analysis was performed also for quantitation and detection of the specific proteins contained in the protein extracts from insect larvae. A mixture of protein-A purified anti-G monoclonal antibodies C10 (Bearzotti et al., 1995; Gaudin et al., 1999), I16 (INRA, unpublished) and 3F1A12 (Aarhus, unpublished) obtained from INRA (Dr. M. Bremont) and Denmark Centre of Aarhus (Dr. N. Lorenzen), respectively were used. Thus, 30 μg of protein extract per lane was loaded in 12% SDS–polyacrylamide gels (SDS–PAGE). After electrophoresis, gels were transferred to nitrocellulose membranes (Schleicher & Schuell). Briefly, membranes were treated overnight with a blocking solution of phosphate buffer saline–0.05%Tween 20 (PBS–T) with 4% skim milk. Membranes were then incubated for 1 h at room temperature with the specific anti-G antibodies mixture 50-fold diluted. After several washes with PBS–T, membranes were incubated with an anti-mouse horseradish peroxidase-conjugated IgGs (Sigma). Finally, the bands were visualized with ECL (Amersham).

2.6. VHSV virus and Gs protein

VHSV 07.71, the VHSV was grown using *epithelial papullosum cyprini* (EPC) cells in cell culture medium as described previously (Basurco and Coll, 1992; Winton et al., 2010). VHSV was concentrated-purified from infected EPC supernatants using 7% polyethylene glycol (PEG) 6000 in 2.3% NaCl, pH 7.8 as previously described (Basurco and Coll, 1992) or by ultracentrifugation at 25,000 × *g* during 3 h. Purification of VHSV soluble G (Gs) was made by using supernatants from infected EPC cell cultures and concanavalin A affinity chromatography as described previously (Perez et al., 1998).

2.7. Production of anti-VHSV polyclonal antibodies in trout

Groups of trout (1000 g of body weight) were held in 100 l aquaria at 14–20 °C. Hyperimmunized trout serum was obtained by 4–5 intraperitoneal injections during 3 months with 400 μl of the antigen preparations. To trout T30, T31 and T32 several combinations of 30 μg of heat inactivated (37 °C 30 min) PEG-concentrated VHSV, 30 μg of recombinant G4 or N3 (Estepa et al., 1994) and 30 μg of lectin affinity purified Gs (Perez et al., 1998) (Table 2) were injected diluted 1:1 with complete Freund's adjuvant during the first injection and the rest of injections diluted 1:1 with incomplete Freund's. T34 and T35 were injected with concentrated VHSV in saponin. Concentrated VHSV (30 μg) was sonicated in the presence of 30 μg of saponin (Superfos, Quil A), 620 μg of cholesterol and 6 μg of phosphatidylcholine in 1 ml of distilled water (Table 2). The use of heat (de Kinkelin et al., 1980; DeKinkelin and Scherrer, 1970) and membrane-dissolving oil (Freund's) or saponin adjuvants contributed to inactivate completely purified VHSV as shown by lack of detectable infection by cell culture. Non-injected trout serum was obtained from 15 healthy non-infected trout (500 g of body weight) from a farm with no history of VHSV infections in more than 30 years (Escuela de Montes, Madrid, Spain) and used either single or pooled according to the experiments.

2.8. Anti-VHSV G and -fragments ELISA assays of trout sera

To assay for trout anti-VHSV G antibodies in trout sera, polystyrene plates of Maxisorb® 96-wells (Nunc Immunoplates) were used. They were coated with 1–5 μg/well of the purified recombinant G protein or their fragments in 100 μl of distilled water, and kept overnight at 37 °C (Rocha et al., 2002) or at 4 °C in diluted carbonate buffer. To reduce non-specific binding, the coated wells were blocked with 100 μl/well 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 20% of rabbit serum and washed with 0.05% Tween 20 before performing the ELISA. The wells were incubated for 60 min at room temperature with 100 μl/well of trout sera serially diluted in dilution buffer. After washing, they were incubated for 30 min with anti-trout immunoglobulin MAb 1G7 diluted 5000-fold (Sanchez et al., 1991). After washing with 0.05% Tween 20, 10,000-fold diluted high specific activity horseradish peroxidase-conjugated rabbit anti-mouse antibody (Sigma Chemical Co., St. Louis, MO, catalog number A9044) was added. The colour reaction was developed by adding 1 mg/ml o-phenylenediamine in citrate buffer containing 3 mM H_2O_2. Optical density was measured by using dual wavelength measurements at 492–620 nm in a Labsystems ELISA reader. The optical density at 620 nm was used to correct for individual differences between wells as recommended by the manufacturer. Backgrounds estimated by performing the ELISA in the absence of any added trout serum ranged from 0.15 to 0.2 absorbance units at 492–620 nm.

ELISA performed by coating the fragments in the absence or in the presence of 0.1 M β-mercaptoethanol was used to estimate the possible importance of disulphide bonding in the subsequent binding reaction to hyperimmunized T30 trout sera. After coating the plates were thoroughly washed with 0.05% Tween 20 and then used for the rest of the ELISA. The results were expressed in percentages as calculated by the formula (absorbance in the presence of β-mercaptoethanol/absorbance in the absence of β-mercaptoethanol) × 100. Averages and standard deviations from 2 experiments were calculated.

2.9. VHSV microneutralization: focus neutralization test (FNT)

About 2×10^3 $TCID_{50}$ per ml of VHSV 07.71 were incubated overnight at 4 °C with serial dilutions of trout serum containing anti-VHSV antibodies in cell culture medium with 2% fetal calf serum, 2% of fresh non-immunized trout serum (as a source of complement) and 100 mM Tris pH 7.8. Next day cultures of EPC cells in 96-well plates coated with poly-Lys were infected with 100 μl of the different virus/trout serum mixtures, adsorbed for 2 h at 14 °C with continuous agitation, washed with cell culture medium, filled with 100 μl/well with culture medium and incubated overnight at 14 °C. The VHSV infected EPC monolayers were then fixed for 10 min in cold methanol and dried. To detect the VHSV N antigen, the MAb 2C9 (Sanz and Coll, 1992a,b) 1000-fold diluted in dilution buffer (130 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.24 mM merthiolate, 5 g of Tween 20/l, 50 mg of phenol red/l, pH 6.8) was added to the wells (100 μl/well) and incubated for 1 h. After washing with distilled water, 100 μl of peroxidase-labelled

rabbit anti-mouse IgG (Sigma, St. Louis, MO, USA) was added per well and incubation was continued for 30 min. After 3 washings with distilled water, 50 μl of 1 mg/ml/well of DAB (Sigma Chemical Co.) in citrate/phosphate buffer was added, and the reaction was allowed to proceed until brown foci were detected by inspection with an inverted microscope in the controls containing no antibodies. FNT titre was defined as the reciprocal of the maximal dilution that reduced DAB positive foci to 50% of the number obtained with non-immunized trout serum (Lorenzo et al., 1996). FNT titres were rounded to the nearest value of the means of 4 independent determinations made in two different laboratories (INIA and Univ. Miguel Hernandez).

3. Results

3.1. Design, purification and properties of VHSV G fragments

Fig. 1 shows the location of the VHSV fragments cloned in the amino acid sequence and in the modelled tridimensional structure on their protein G. Table 1 shows the frg locations, number of amino acids, molecular weights and number of cysteines.

The fragments were designed by avoiding the presence of cysteines as much as possible, to reduce possible disulphide-dependent conformations. The whole G protein sequence was divided into 2 parts: an amino terminal part of 186 amino acids called frg15 (65–250) and a carboxy terminal part of 199 amino acids called frg16 (252–450) (Fig. 1). Frg15 contains the projected 2 loop sequences implicated in fusion (Estepa et al., 2001; Nuñez et al., 1998; Rocha et al., 2004) and frg16 (252–450) contains the head of the molecule and the rest of epitopes including the Mx-inducing peptides (Chico et al., 2010). The amino terminal frg15 was also divided on frg11 (55 amino acids, from 56 to 110), frg12 (45 amino acids, 65–109), frg13 (71 amino acids, 97–167) and frg14 (74 amino acids, 141–214). Frg11 contains most of the linear epitopes of the G protein mapped by Pepscan by using hyperimmunized trout sera (Fernandez-Alonso et al., 1998; Rocha et al., 2002). Frg12 is about the same as frg11 but differs in the absence of an amino terminal cysteine-containing short peptide (^{56}MPIRPAQNRC) and a carboxy terminal cysteine ^{110}C. Frg13 contained the two loops ended by the hydrophobic tips which are though to penetrate the lipid membrane, while frg14 only contained one of the loops and additional sequences at their carboxy end. The fragments mentioned above were expressed in *E. coli* and purified by affinity and Sephadex chromatography (Fig. 1).

Complete protein G of VHSV, except either the signal peptide with 487 amino acids (amino acid 21–507) or the signal peptide plus transmembrane with 445 amino acids (21–465) were cloned and expressed in *T. ni* insect larvae. The specificity of the recombinant proteins was confirmed by Western blotting by using both an anti-G monoclonal antibody mixture (C10, I16 and 3F1A12) and commercial anti-polyHis monoclonal antibodies (not shown). Both of the expressed affinity purified recombinant proteins migrated as 3–5 bands in polyacrylanide gel electrophoresis (PAGE) under reducing conditions with apparent molecular weights of ~55–60 kDa, most probably because of different glycosylation patterns. They were absent in the BacNi purified proteins (not shown). The amount of purified G21–465 was ~10-fold higher than the amount of G21–507. Therefore only the G21–465 was used for the rest of the experiments. Preparative experiments with 50 insect larvae yielded ~15 mg of G (~0.3 mg per larvae) as estimated after affinity and Sephadex chromatography purification.

During purification procedures, all the recombinant forms of the protein G of VHSV commented above, showed a strong tendency to precipitate at physiological pH when the guanidinium chloride used for affinity chromatography was eliminated by Sephadex chromatography or by dialysis. The precipitates were irreversible unless guanidinium chloride was again added. To avoid precipitation of the fragments in the complete absence of guanidinium chloride, their pH had to be lowered to 4 and Sephadex chromatography or dialysis carried out at that pH. Therefore, to avoid precipitates to form, purified fragments were maintained frozen at pH 4 at high concentrations (1–2 mg/ml) and diluted to 10–100 μg/ml just before used for coating the ELISA plates.

Table 3
Properties of the hyperimmunized trout sera used in this work.

Trout serum	Western blots on frg11	50% FNT	ELISA absorbance 492–620 nm on frg11
Tc1	−−	200	0.15 ± 0.02
T30	+++	2000	2.32 ± 0.20
T31	−−−	200	0.04 ± 0.01
T32	+	200	0.99 ± 0.05
T34	++	1500	0.75 ± 0.03
T35	++	500	0.95 ± 0.10
Pooled	++	>12,500	0.36 ± 0.10

Western blots, were carried out by immunodetection of PAGEs with frg11, frg15, frg16 and G21–465. The results were similar in all cases (not shown). −−−, Negative. +, ++, +++, Increasingly positive bands. Focus neutralization test (FNT) titres, were defined as the last dilution which caused 50% reduction in the number of ffu. Neutralization titres were rounded to the nearest value of the means of 4 independent determinations made in 2 different laboratories. ELISA titres (means and standard deviations of duplicated experiments) were defined as the absorbance values above background at 492–620 nm of 200-fold diluted trout sera. Because of their higher titre, T30 was diluted 10-fold before the Western and ELISA tests. Pooled, the pooled trout sera comes from 3 VHSV-infected trout (gift of Dr. J. Castric).

3.2. Properties of VHSV-hyperimmunized trout sera

Table 2 shows the antigens used to obtain the VHSV-hyperimmunized trout sera for this study and some of their properties. Trout sera were obtained by 4–5 repeated intraperitoneal injections of different antigens with Freund's (T30, T31 and T32) or saponin (T34 and T35) adjuvants. Trout T31 could only be injected 2 times, because it became sick and it was bled to death 1 month after last injection (there were no clinical signs of VHS, nor VHSV could be isolated from T31). Tc1 was obtained from a non-immunized trout. The protein content of all their sera ranged between 35.8 and 90.3 mg/ml, except for T31 which was 2–3 times lower than the rest.

3.3. Reduction of non-specific absorbance of non-immunized trout sera

Because both sensitivity and specificity are best estimated when non-specific absorbance measured with non-immunized trout serum in the assay are lower, experiments were carried out to explore the possibilities to lower those absorbance values, without affecting the maximal absorbance obtained with VHSV-hyperimmunized trout. After several preliminary experiments, T30 was selected for optimization of the ELISA since it showed a 10-fold higher titre than T32, T34 and T35 hyperimmunized trout sera (Table 3). To make comparisons under the same range of absorbance, T30 had to be 10-fold diluted. Tc1 a non-immunized healthy trout sera was used as negative control (Table 2). To optimize the ELISA, T30/Tc1 absorbance ratios were optimized.

Because as mentioned above, the G protein and frg11 dramatically changed their conformations with pH, the T30/Tc1 ratios were first assayed in ELISA at different pHs. Thus T30/Tc1 were diluted (1000–5000-fold diluted) in buffers at physiological, medium or fusion pHs (7.6. 6.7 or 5.6, respectively) and their binding to the solid phase coated with different G fragments was estimated. The results showed that maximal T30/Tc1 signal-to-noise ratios were

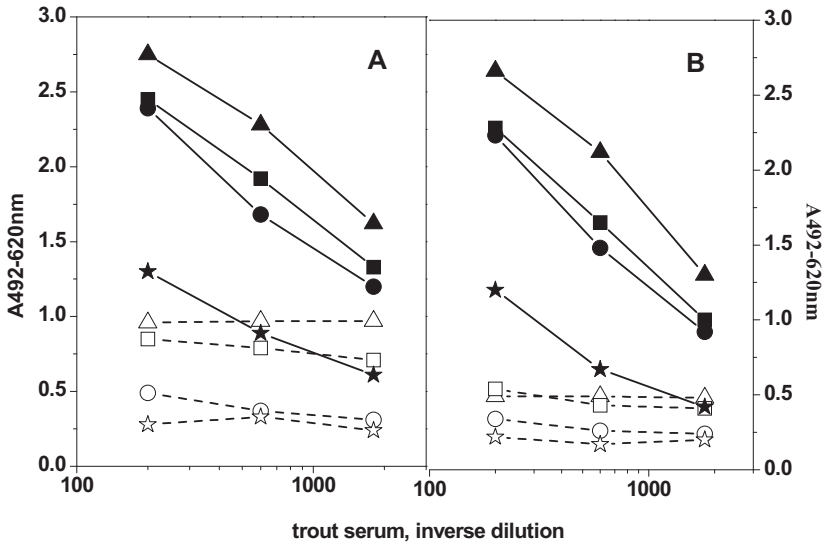

Fig. 2. Absorbance values in VHSV-infected (black symbols) and non-infected (open symbols) trout sera by blocking frg-coated plates with 20% rabbit serum. Plates were blocked with dilution buffer without (A) or with 20% rabbit sera (B) during 1 h at room temperature. Black symbols, VHSV-infected trout sera. Open symbols, non-infected trout sera. The results showed one experiment out of two. Standard deviations were lower than 10%. They have been omitted for clarity. Open symbols, non-infected trout sera. ● ○, Wells coated with frg11. ▲ △, Wells coated with frg15. ■ □, Wells coated with frg16. **, Wells coated with G21–456.

found at pH 6.7 (3.6–5.1 ratios) compared to 1.9–2.2 or 0.6–1.3 ratios found at pH 5.6 and 7,6, respectively ($n = 3$) (not shown).

Nunc immunoplates of different coating affinities for hydrophilic/hydrophobic molecules (Maxi, Medi and Poly Sorb®) surfaces were coated with frg11, frg15, frg16 and G21–4645 and then used for ELISA to estimate T30/Tc1 ratios by using trout sera diluted 200-, 600- and 1800-fold. The highest T30/Tc1 ratios were obtained with Maxi and Poly Sorb® plates while 26–30% lower ratios were obtained with MediSorb® plates depending on the frg (data not shown). MaxiSorb® solid plates were then used for the rest of the experiments.

Then, a checker board titration of dilutions of both anti-trout IgM 1G7 and RAM-PO from 100- to 10,000-fold was used to select those with the highest T30/Tc1 ratios in the ELISA. A dilution of 1000-fold for 1G7 and of 5000-fold for the batch of RAM-PO used for these experiments, were chosen as optimal (data not shown).

The existence of a high non-specific binding of non-immunized trout sera, anti-trout IgM MAb 1G7 and/or RAM-PO to fragments-coated solid-phases, suggested the search for a better blocking buffer. Blocking further the frg-coated surface to reduce the non-specific binding to empty sites on the frg-coated surfaces, with different blocking buffers was then tried. Thus, the addition of 1–5% milk to the dilution buffer, as suggested previously (Kim et al., 2007), completely eliminated backgrounds, but eliminated also T30/Tc1 ratios >1. On the other hand, the use of solid-phases coated with bovine serum albumin (similar to the ELISA in the absence of VHSV in the VHSV-capture ELISA to detect false positives) (Olesen et al., 1991), resulted in low backgrounds (<0.1), suggesting that trout sera could cause the non-specific binding, however concerns arise as to their significance in an indirect ELISA as the one described here. Other possibilities were then explored. Thus, T30/Tc1 ratios were still the highest when blocking was made with dilution buffer, compared to 10-fold dilution buffer, Superblock (Thermo), or dilution buffer without Tween. Incubation after 1 h at room temperature or overnight at 4 °C made no differences (data not shown). Finally, Fig. 2 shows a ~2–3-fold reduction of absorbance values of Tc1 which resulted in a similar improvement of T30/Tc1 ratios for most fragments, when 20% rabbit serum was added to the blocking buffer (dilution buffer +20% rabbit serum). These results suggested that some non-specific binding of trout IgM, anti-trout IgM MAb 1G7 and/or RAM-PO to frg-coated solid phases could be partially competed by the rabbit serum. RAM-PO did not seem to be involved, because incubation of RAM-PO with an excess of rabbit serum did not have any effects on the lowering of absorbance values, while blocking buffer with trout or salmon serum (dilution buffer + trout or salmon serum), did not lower the absorbance either (data not shown). After using several rounds of interdependent variable assays, conditions using 20% rabbit serum were then considered to be optimal to screen the sera from VHSV-hyperimmunized trout.

3.4. Recognition of G fragments by VHSV-hyperimmunized trout sera

To rule out possible reactions of hyperimmunized trout sera with the 36 amino acid polyHis tail contained in all the fragments, the polyHis tail and the frg11 were chemically synthesized and assayed as solid phases by ELISA. Plates coated with 1 μg/ml of synthetic polyHis tails did not showed any difference between T30 and Tc1 sera while plates coated with 1 μg/ml of synthetic frg11 behave similarly to recombinant frg11 (not shown).

To characterize further the G fragments and confirm that the absorbance obtained by ELISA corresponded to true reactivity with the fragments, Western blotting was carried out with each of the fragments and hyperimmunized trout sera. Correlating with the results obtained by ELISA at different pH values, only at pH lower or equal to 6.7, stained bands corresponding to the molecular weight of the fragments were strongly visible by immunoblotting (not shown). Fig. 3C shows the recognition of purified G21–465 by T30. The recombinant G21–465 showed several bands in the 55 kDa region, most probably due to the presence of different glycosylated molecular species (Fig. 3A). Fig. 3D shows the recognition of purified *E. coli* recombinant fragments with hyperimmunized T30 (Fig. 3B). Most of the fragments, were strongly recognized by T30, except frg14. Similar results were obtained with the rest of the trout hyperimmunized sera, except with T31 (Table 3). Pooled sera from 3 VHSV-infected survivor trout (gift of Dr. J. Castric) with 1/10,400 neutralization titres also reacted with all the fragments (not shown).

Binding of T30 to wells coated with β-mercaptoethanol reduced fragments, produced 85.69 ± 1.69, 94.28 ± 1.83, 97.70 ± 2.19, 65.59 ± 5.79, 88.44 ± 0.18, 70.70 ± 8.44 and 88.66 ± 4.99% of the

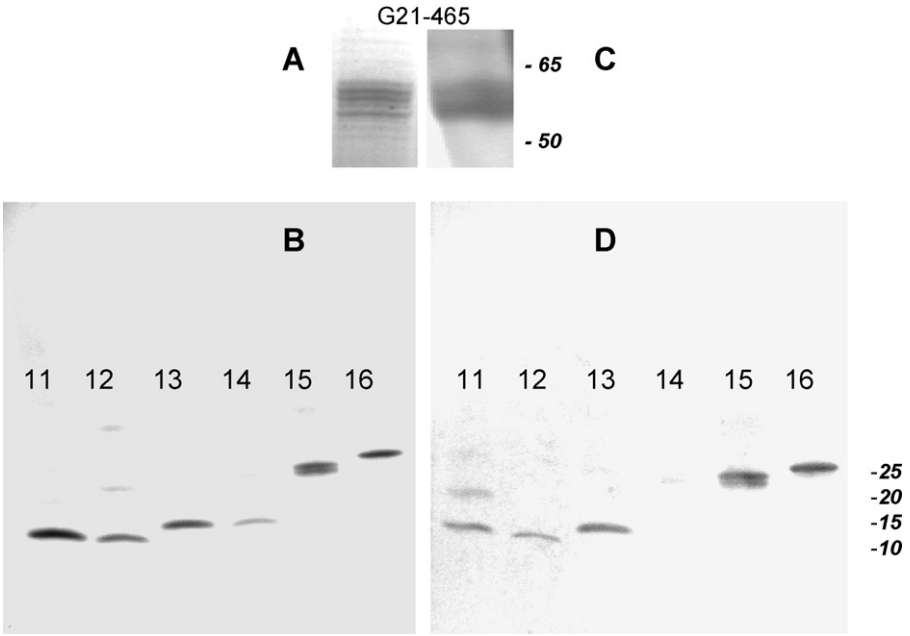

Fig. 3. Polyacrylamide gel electrophoresis (PAGE) of purified recombinant fragments (A and B) and their recognition by T30 trout sera by Western blotting (C and D). The insect (A) or *E. coli* (B) recombinant protein and fragments, respectively extracts were purified by Ni^{2+} affinity column (ProBond™, Invitrogen) in guanidinium chloride buffer and eluted with imidazole. Peak fractions adjusted to pH 4 were chromatographed through Sephadex G-100 and extensively dialyzed against sodium acetate pH 4. Protein concentrations were determined by BCA and fragments electrophoresed in 4–20% polyacrylamide gradient gels (PAGE, BioRad) and stained with Coomassie (A and B). Western blotting was carried out by using T30 trout sera using nitrocellulose membranes. T32, T34 and T35 showed similar binding patterns (not shown). Numbers above the bands correspond to the frg numbers. Numbers in italics to the right of the gels are the molecular weight markers in kDa.

ELISA signal when non-reduced frg11, frg12, frg13, frg14, frg15, frg16 and G21–465, respectively, were used to coat the plates. Frg14 ELISA values were 65.59% lower in the presence of β-mercaptoethanol, thus confirming their negative Western blot signal obtained under reducing conditions (Fig. 3D).

Only the neutralization of T30, T34 or T35 could be detected by FNT but their titres were >10-fold lower than the titres of a pooled sera from 3 VHSV-07.71-infected trout (gift of Dr. J. Castric) (Table 3).

Preliminary ELISAs made to compare T30/Tc1 binding to G fragments on solid-phase ranging from 0.1 to 10 μg/well showed that for most of the frg preparations, absorbance levels increased when the frg coating amounts were increased in the solid-phase until saturation values were obtained at ~5–10 μg/well (not shown).

Fig. 4 shows the different absorbance profiles obtained with different fragments coating the solid phase at 1 μg/ml, when reacting with T30 and Tc1 at dilutions ranging from 600- to 200,000-fold. Under the conditions used for these ELISAs, the absorbance decreased to background levels when using >100,000-fold T30 dilutions. The use of frg11, frg13, frg15 and frg16 as solid-phases resulted in higher relative absorbance values than those obtained with frg12, frg14, G21–465 or purified VHSV. The absorbance obtained by purified VHSV were the lowest, most probably due to the lower relative amounts of G protein. The absorbance obtained with frg12 were ~50% of those of frg11.

Fig. 5 shows the comparison of the binding of 200-fold diluted Tc1, T30, T31, T32, T34 and T35 (Table 2) to solid-phase coated with fragments 11–16 and G21–465 (Table 1) and purified VHSV. The experiment was carried out at several dilutions with similar qualitative results (not shown). The maximal absorbance values were obtained with 10-fold diluted T30, T32, T34 or T35 by using frg11, frg15 and frg16 as solid-phases by any of the 2 different coating methods (to dryness or with carbonate buffer). However with frg15 and frg16, maximal absorbance values for T32, T34 and T35 were obtained only by using the carbonate coating method. The increased binding of T31, T34 and T35 sera to frg15 and frg16

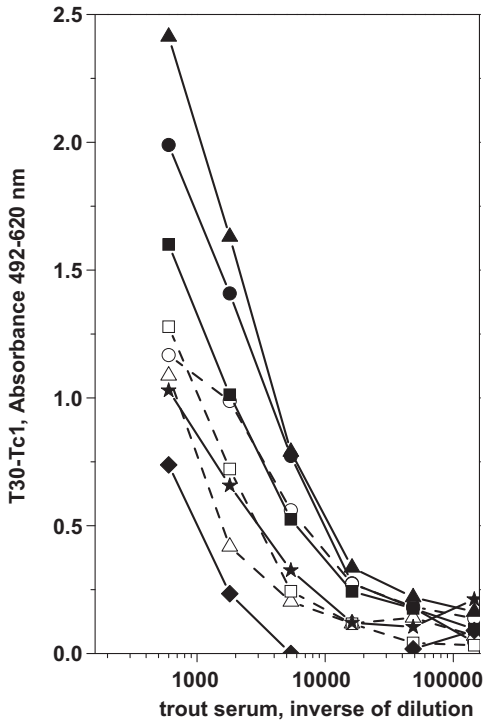

Fig. 4. Recognition of solid-phase fragments by hyperimmunized T30 trout serum. Plates were coated with 1 μg of protein per each frg per well. Trout sera T30 and Tc1 were serially diluted and recognized with anti-trout IgM MAb 1G7 and RAM-PO. Differences between T30 and Tc1 from 3 different experiments were averaged and their means represented. Standard deviations were omitted for clarity. ●, Wells coated with frg11. ○, Wells coated with frg12. △, Wells coated with frg13. □, Wells coated with frg14. ▲, Wells coated with frg15. ■, Wells coated with frg16. *, Wells coated with. G21–456. ♦, Wells coated with purified VHSV.

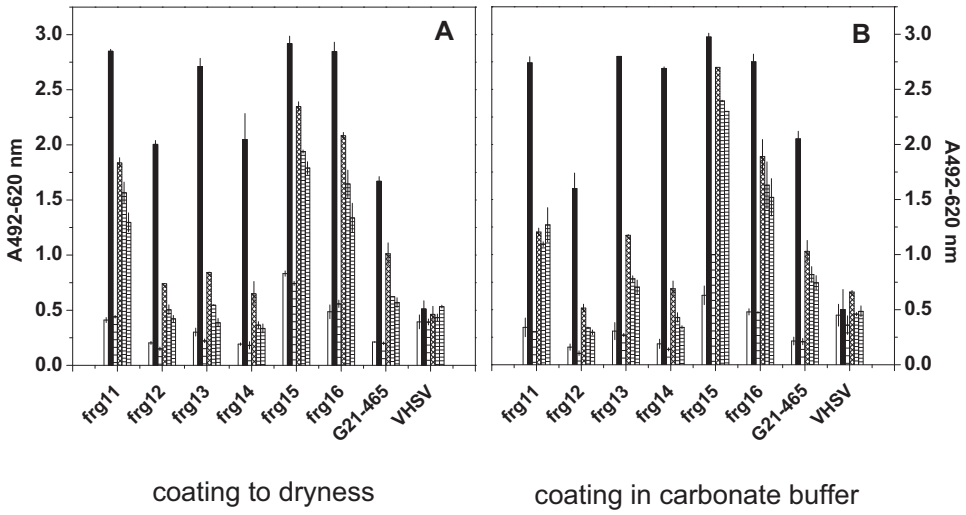

Fig. 5. Recognition of solid-phase fragments by different hyperimmunized trout sera (T30, T31, T32, T34, and T35). Plates were coated with 1 μg per each frg per well. Hyperimmunized trout sera from Table 2 were 200-fold diluted and ELISA performed as indicated in methods by using 5000-diluted anti-trout IgM MAb 1G7 and 10,000 diluted RAM-PO. White bars, non-immunized Tc1 trout serum. Black bars, T30 prediluted 10-fold. Horizontally space hatched bars, T31. Crossed bars, T32. Horizontally dense hatched bars, T34 and T35.

by using the carbonate coating method, were confirmed by using trout sera dilutions between 60- and 14,580-fold (not shown). Binding of trout sera to solid phases coated with both frg15 and frg16 showed an intermediate compared to that obtained when using separately (not shown). The absorbance values obtained with Tc1 (non-immunized) and T31 (low immunization) were about 4–5-fold lower for any of the fragments.

4. Discussion

An ELISA method to study the immunogenicity of serum antibodies to VHSV was designed by using fragments of their G protein as solid phases.

After the elucidation of the pH-dependent tridimensional structures of the protein G of a mammalian rhabdovirus (Roche et al., 2006, 2007), fragments for the VHSV G were designed to contain possible functional domains of the protein G of VHSV. The fragments were cloned and expressed to search for optimal ELISA conditions to detect possible anti-VHSV antibodies reacting with their epitopes. By optimization of the purification method for the G fragments (by using guanidinium chloride and imidazole elution and Sephadex chromatography), ELISA pH, plates, coating methods, blocking buffers, anti-trout immunoglobulin monoclonal Ab 1G7 and RAM-PO concentrations, reproducibility and sensibility of the ELISA were improved to allow comparison of antigenicity among the different G fragments.

Despite the presence of a few cysteines per most fragments except for frg12, binding to disulphide-dependent conformational epitopes on the fragments was only <20% reduced by the addition of β-mercaptoethanol, except that of frg14 (65% reduced). In addition to intramolecular disulphide-dependent conformations, the N-Ab targeted G glycoprotein of rhabdoviruses forms trimers responsible for the low-pH dependent reversible fusion of the viral and host membranes (Coll, 1999). The G protein of mammalian rhabdoviruses can adopt 3 pH-dependent conformational states that are in equilibrium (Gaudin et al., 1993). These are the physiological (83 Å of length), the hydrophobic (fusion activated state) and the low-pH (post-fusion of 113 Å of length, shown in Fig. 1) states. These three conformations are antigenically distinct in mammalians (Roche and Gaudin, 2002), because low pH causes some of the hydrophobic regions of G to be exposed to its surface (Gaudin et al., 1993). Similarly, fish hosts during infection should be exposed to all 3 conformational states and therefore make antibodies to differently exposed epitopes. Accordingly, we first study the pH-dependence of both immunoblotting and ELISA to binding of fragments by hyperimmunized trout sera. The pH at which both immunoblotting and ELISA were optimal was 6.7, an intermediate pH between those causing physiological and post-fusion conformations. Therefore, the pH of 6.7 was chosen to continue the experiments. Reduction to pH 4 during purification to avoid precipitation of the recombinant fragments, might have affected the ELISA results, however, fragment preparations were diluted ~100-fold in either water or bicarbonate buffers to be immobilized to solid phases to minimize those possible effects. Furthermore, the entire ELISA was then performed at the optimal pH of 6.7. On the other hand, the results obtained by using fragments dried in water were similar to those obtained when using bicarbonate buffer for coating (Fig. 5).

Because trout serum anti-VHSV antibodies are tetrameric IgM-like immunoglobulins, and are usually of low affinity, their levels have been difficult to estimate since they bind non-specifically to almost any surface producing high backgrounds and false positives. The use of purified G protein as solid-phase, has also experienced many difficulties due to the problems in obtaining large amounts of lectin-affinity chromatography purified protein G (Perez et al., 1998) and/or recombinant yeast G4 (Estepa et al., 1994). Therefore, we first expressed G21–465 in *T. ni* insects to use the largest possible form of the G (G21–507 was not well expressed in the insects). However, absorbance values obtained by ELISA with G21–465 were lower than those of most *E. coli* made fragments. For instance, the amino terminal (frg15) and the carboxy terminal (frg16) fragments of the G gave 2–3-fold higher absorbance values than the G21–465, thus suggesting that the recombinant G21–465 contains masked epitopes. The higher absorbance values obtained with the fragments, could be explained also because of their lower sizes (55–199 compared to 445 amino acids) would allow a greater number of molecules per surface unit, thus increasing their epitopic density and exposure. Possible disulphide-dependent conformations due to the higher number of cysteines in G21–465 than in the fragments, did not seem to cause a difference in ELISA values and therefore disulphide-independent conformations due to their larger size compared to fragments could be the cause. Alternatively, glycosylaton of the insect-made G21–465 (demonstrated by their

multiple PAGE profile), also could explain a higher masking level of their epitopes and their lower ELISA reactivity.

Optimal relation between exposure of epitopes and length of the fragments was found in frg11 (56–110). Frg11 (56–110) was selected in previous studies because ~40% of sera from trout immunized with VHSV, recognized G protein Pepscan peptides around the fusion-related region (Nuñez et al., 1998; Rocha et al., 2002). The absorbance values obtained with frg12 were ~ 50% of those of frg11. Because frg12 (65–109) only differs from frg11 (56–110), by the absence of a short amino terminal peptide and 2 flanking cysteines (^{56}MPIRPAQNRC and ^{110}C, respectively) and disulphide bonding did not significantly interfere (Table 3), the short amino terminal peptide might contain linear or disulphide-independent epitopes.

Different hyperimmunized trout sera (T30, T32, T34 and T35) recognized frg11, frg15 and frg16 with the highest intensity compared to frg12, frg13, frg14, G21–465 or purified VHSV. The absorbance values depended on the frg used rather than on the different hyperimmunized sera (Fig. 5), despite those being obtained in different trout and/or with different adjuvants and resulting in slightly different neutralization titres (Table 3). Interestingly, the trout sera which could only be injected twice with purified VHSV before it was killed (the rest of trout sera were injected 4–5 times) (Table 2), behave as if it were non-immunized (Fig. 5), although that could be due also to their sick state. This data suggest that to detect the low levels of anti-VHSV antibodies present (if present) in survivor fish (asymptomatic carriers), still higher sensibility ELISAs might be required. The use of chemiluminiscent substrates and/or biotin/streptavidin reagents could be used to boost the sensibility of these ELISAs.

One concern about the possible use of frg-based ELISAs to detect trout antibodies in field samples is the sequence variability of different VHSV genotypes and isolates found in natural outbreaks. The highest sequence variability on the protein G of VHSV was localized in the 245–300 region (Benmansour et al., 1997; Einer-Jensen et al., 2004) and will thus affect the use of frg15 and frg16 for such purposes. Therefore, new frg15- or frg16-like fragments which will avoid the 245–300 hypervariable region could, perhaps, be designed and used to avoid variability and thus to increase the scope of VHSV strains and isolates which could be detected by these fragments.

The existence of natural anti-VHSV antibodies to linear or disulphide-independent conformational G epitopes could be one of the possible explanations of why N-Ab levels do not always correlate with protection (LeFrancois, 1984; Lorenzen and LaPatra, 1999; Lorenzen et al., 1990) nor with the levels of antibodies to whole VHSV by ELISA (Fregeneda-Grandes and Olesen, 2007; Jorgensen et al., 1991; Olesen et al., 1991). Alternatively, such N-antibodies might be present in mucus IgT rather than in sera IgM (Hansen et al., 2005).

All the above mentioned results indicate that further studies of the antigenicity of protein G by the use of their fragments might be useful to clarify their role in the trout protective responses to VHSV and perhaps to improve current DNA vaccines and complement the actual N-antibody assays. The panel of G fragments described in this work, should allow the study of antibody responses to epitopes in different trout sera samples from vaccinated trout as well as for trout surviving VHSV infections.

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