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Expression of a foreign epitope on infectious pancreatic necrosis virus VP2 capsid protein subviral particle (SVP) and immunogenicity in rainbow trout

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ABSTRACT

Infectious pancreatic necrosis virus (IPNV) is a major viral pathogen of salmonid fish and causes serious economic losses to salmonid aquaculture. Previously, we demonstrated that the IPNV capsid protein, VP2, expressed in yeast self-assembles into subviral particles (SVPs) and injection of these IPNV rVP2 SVPs into rainbow trout elicits an immune response. Immunized fish had reduced viral loads compared to unimmunized fish when challenged with IPNV. To evaluate the suitability of IPNV rVP2 SVPs for future development of multivalent vaccines, a linear epitope of a human oncogene, *c-myc*, was cloned into the IPNV rVP2 SVP backbone as a model epitope and expressed in yeast. Western blot analyses with anti-*c-myc* and anti-IPNV antibodies provided positive identification of both the *c-myc* and VP2 epitopes on the *c-myc* VP2 SVPs. Transmission electron microscopy of purified chimeric *c-myc* VP2 SVPs revealed the formation of ~20 nm particles. Rainbow trout immunized with *c-myc* VP2 SVPs elicited both anti-*c-myc* and anti-IPNV immune responses. When immunized fish were challenged with IPNV, the viral load in the *c-myc* VP2 SVP immunized fish was significantly lower than the sham-vaccinated controls. The results indicate that IPNV rVP2 SVPs can tolerate the insertion of foreign epitopes without affecting either the antigenic potential of the epitopes of the backbone protein or the inserted foreign epitope. This opens the possibility of using the IPNV rVP2 SVP platform to express epitopes of other viruses, which could pave the way for development of multivalent subunit vaccines or novel marker vaccines.

1. Introduction

The structural protein(s) of many viruses form specific aggregates through self-assembly in a variety of different expression systems (Ludwig and Wagner, 2007; Noad and Roy, 2003). These virally derived particles generally imitate the native viruses in size and morphology and are referred to as virus-like particles (VLPs) (Noad and Roy, 2003). When viral proteins aggregate to form particles that do not mimic the virus capsid in size yet form predictable complex(es) they have been referred to as subviral particles (SVPs). VLPs and the SVPs do not contain the genetic material of the virus and are, therefore, incapable of self-replication in the cell but are generally similar to the native virus in cellular uptake and intracellular trafficking (Grgacic and Anderson, 2006). Due to structural similarities, VLPs elicit effective immune responses, and serve as excellent candidates for the production of vaccines (Chackerian, 2007; Noad and Roy, 2003; Ramqvist et al., 2007). Therefore, VLPs and SVPs provide an attractive platform for the display of foreign epitopes or targeting molecules including antigens that are unable to self-assemble, deliver oligonucleotides or plasmids, and drugs (Garcea and Gissmann, 2004; Georgens et al., 2005; Braun et al., 1999; Kimchi-Sarfaty and Gottesmann, 2004; Patient et al., 2009). VLPs have been produced from a wide range of viruses such as Norwalk virus (Mason et al., 1996), infectious bursal disease virus (Shivappa et al., 2005; Remond et al., 2009), Simian virus 40 (Kimchi-Sarfaty and Gottesmann, 2004), human immunodeficiency virus (Doan et al., 2005); hepatitis C virus (Lorenzo et al., 2001), and infectious hypodermal and hematopoietic necrosis virus (Hou et al., 2009). The genetic constructs made to express the capsid proteins have been used for the expression of the proteins and self-assembly into VLPs and SVPs in hosts ranging from bacteria (Braun et al., 1999), insects (Shivappa et al., 2005), insect cell culture (Shivappa et al., 2005), yeast (Allnutt et al., 2007), plant cell culture (Mason et al., 1996) and whole plants (Santi et al., 2006).

Infectious pancreatic necrosis, caused by the infectious pancreatic necrosis virus (IPNV), is an important viral disease in salmonids, and is particularly damaging to juvenile fish (Wolf et

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al., 1960; Roberts and Peason, 2005). IPNV is a member of the family *Birnaviridae*, a group of viruses that are non-enveloped, ~60 nm in diameter, and contain two segments of dsRNA as genetic components, Segments A and B (Coulibaly et al., 2005; Duncan et al., 1991; Gorbalenya et al., 2002). Segment B encodes a non-structural protein that has helicase, protease, and RNA-dependent-RNA-polymerase (RdRp) domains. Segment A contains structural proteins that are expressed as a large polypeptide that is post-translationally cleaved into three structural proteins: capsid protein precursor pVP2, protease VP4, and capsid protein VP3. Following capsid assembly, the pVP2 protein is cleaved further to produce VP2 (Dobos, 1995; Galloux et al., 2004). Most of the neutralizing epitopes of IPNV were mapped to VP2 (Tarrab et al., 1993; Frost et al., 1995; Fridholm et al., 2007). Consequently, VP3 is believed to be a more internal capsid protein than VP2 (Dobos et al., 1977), although some neutralizing epitopes have been linked to it (Tarrab et al., 1995).

Recently, we reported that the IPNV VP2 gene expressed in a yeast expression system forms SVPs that are antigenic and are capable of providing immunity against the native IPNV (Allnutt et al., 2007). This is a promising method to develop a VLP-based vaccine against IPNV. We were interested in determining whether a foreign epitope could be displayed on IPNV rVP2 SVP without affecting the antigenicity of the native as well as the foreign epitopes. The *c-myc* human oncogene epitope was used as a proof-of-principle since it is a well-characterized, simple, linear epitope for which an antibody is commercially available thereby enabling easy detection of the peptide in chimeric SVPs. In addition, the sequence representing the *c-myc* epitope is unique enough that a homologous sequence is unlikely to be found in the rainbow trout genome. The results from the present study demonstrated that the IPNV rVP2 SVP can tolerate the insertion of a foreign epitope and that the injection of these chimeric *c-myc* VP2 SVPs elicits antibody response against the IPNV rVP2 protein as well as the foreign peptide in immunized rainbow trout. When rainbow trout vaccinated with *c-myc* VP2 SVPs were challenged with native IPNV, there was a significant reduction in viral load in vaccinated fish compared to the sham-injected fish opening the possibility of developing multivalent vaccines against viral diseases in salmonids using the IPNV rVP2 SVP platform.

2. Materials and methods

2.1. Sequence analysis of IPNV VP2 gene and cloning of human oncogene c-myc epitope in VP2 SVP vector

The IPNV VP2 amino acid sequence of West Buxton Strain (GenBank accession number AAC71003) was compared to the homologous sequence of other IPNV strains (GenBank accessions BAA05532, P05844, P22495, AAK32155–AAK32160). The N-terminal amino acid positions 241–288 were found to be highly variable and were taken for subsequent analyses using a computer program (BioMedCache, Hitachi Medical, Japan) to predict possible insertion sites that would have surface exposure and minimal impact on SVP assembly. Four amino acid sequence analysis protocols were used for this analysis: Parker antigenicity, Hoops and Woods antigenicity, Goldman/Engleman/Steitz hydrophilicity, and Karplus and Schulz flexibility. A composite score of one through twelve reflecting relative surface exposure was assigned to each amino acid, and a window of seven residues was used for each protocol. The scores were noted for each residue for each protocol and placed into a spreadsheet. The four scores were added together, and a linear graph was made displaying the scores. The higher the composite score of an amino acid, the greater the chance of the corresponding amino acid to remain on the hydrophilic exterior of the protein. In addition, each residue was noted as favorable (shown as a triangle, Fig. 1), unfavorable (shown as a diamond, Fig. 1), or indifferent (shown as a circle, Fig. 1) to turns using the Levitt protocol. Based on the sequence analysis, the amino acid position 252 was chosen to insert the *c-myc* epitope into the IPNV rVP2 SVP vector.

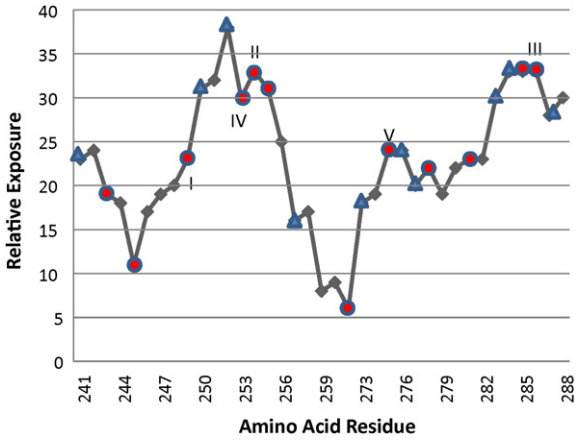

Fig. 1. Comparison of the predicted surface exposure of specific amino acids in the IPNV VP2 capsid protein. Relative hydrophilicity and/or exposure of IPNV VP2 capsid protein was determined using an arbitrary unit derived from a number of different measures of exposure. A combined score of the sequence of the VP2 protein using Parker Antigenicity, Hoops and Woods Antigenicity, Goldman/Engleman/Steitz Hydrophilicity, and Karplus and Schulz Flexibility protocols was used to generate the graph. The amino acid positions are indicated on the *X*-axis and the composite score for each amino acid for the corresponding position is indicated on the *Y*-axis. The residues that are turn favoring, turn disfavoring, and turn indifferent are represented respectively as triangles, diamonds, and circles, respectively. Five candidate sites identified based on our analyses were marked as *I* through *V*.

The cloning of IPNV VP2 gene in a yeast expression vector pESC-ura (Stratagene, San Diego, CA) was previously described (Allnutt et al., 2007). The resulting IPNV rVP2 clone was designated as pIPA1-Z1. A linear epitope of human oncogene *c-myc* was then cloned into IPNV VP2 amino acid position 252 in the pIPA1-Z1 backbone using The GeneEditor™ *in vitro* Mutagenesis System (Promega, Madison, WI). The *c-myc* insertion oligonucleotide (Pannunzio et al., 2004) (5′CTAGCAGCCGAACCCGTCgaacagaagttgatttccgaagaagacctcAACG-AGACGCGGTTCGACTT3′, *c-myc* sequence is in lower case the flanking sequences represent the IPNV VP2 sequence) was custom synthesized (Invitrogen, Carlsbad, CA). The plasmid DNA was isolated from the recombinant chimeric *c-myc* IPNV VP2 clones and sequenced. The recombinant plasmid was then used to transform yeast (*Saccharomyces cerevisiae* strain YPH501, Stratagene, La Jolla, CA) using the EZ Yeast Transformation Kit (Zymed, San Francisco, CA). Mutant yeast colonies were selected for growth on autotrophic SG-ura medium containing galactose and grown at 30 °C for 4 days. Cells were collected by centrifugation and crude protein extracts were prepared by using Y-PER yeast breaking buffer (Pierce Biotechnologies, Rockford, IL). Cell lysates were electrophoresed on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membrane by electroblotting. The blots were probed with sheep-anti-IPNV polyclonal antibody (Microtek International, Inc., Saanichton, B.C., Canada) and anti-*c-myc* monoclonal antibody (Covance Inc., Emeryville, CA). The IPNV VP2 detection was carried out using a rabbit-anti-sheep polyclonal antibody conjugated to HRP (Bethyl Laboratories, Montgomery, TX), and *c-myc* detection was performed using rabbit-anti-mouse polyclonal antibody conjugated to HRP (Bethyl Laboratories, Montgomery, TX). The colorimetric substrate tetramethyl benzidine (TMB) in a one step solution was used as described by the manufacturer (Pierce, Rockford, IL) for detection.

Table 1
Experimental design for vaccination trial of rainbow trout with chimeric IPNV *c-myc* SVPs expressed in yeast cells compared to IPNV rVP2 SVPs from which they were derived.

Treatments	Vaccine delivery (by injection)		No. of animals/treatment	Blood sampling at day 66	IPNV challenge at day 69	Tissue sampling at day 79
	Initial (Day)	Booster (Day)				
Unimmunized	None (1)	None (32)	10	Yes	No	Yes
Adjuvant control	Injection (1)	Injection (32)	8	Yes	Yes	Yes
IPNV rVP2 SVP	Injection (1)	Injection (32)	13	Yes	Yes	Yes
c-myc SVP	Injection (1)	Injection (32)	9	Yes	Yes	Yes

2.2. Western blotting, purification, and transmission electron microscopy (TEM) of c-myc SVP

The western blotting, purification, and transmission electron microscopy (TEM) of *c-myc* SVP were performed following essentially the same protocols as described for IPNV VP2 SVP (Allnutt et al., 2007). Briefly, recombinant yeast (*Saccharomyces cerivisiae* strain YH501; Stratagene, La Jolla, CA) clones were grown in autotrophic SG-ura medium containing galactose, yeast extract without amino acids, and amino acid dropout mixture (all amino acids plus adenine, no uracil) at 30 °C for 4 days. Cells were collected by centrifugation, and crude protein extracts were prepared using Y-PER yeast breaking buffer (Pierce Biotechnology, Rockford, IL). An aliquot of total protein was electrophoresed in a 12% SDS-polyacrylamide gel (Bio-Rad, Richmond, CA) and transferred to nitrocellulose membrane by electroblotting. The blots were probed with sheep-anti-IPNV polyclonal antibody (Microtek International Inc., Saanichton, B.C., Canada) or anti-*c-myc* monoclonal antibody (Covance, Emeryville, CA) and detected with rabbit-anti-sheep polyclonal antibody conjugated to HRP (Bethyl Laboratories, Montgomery, TX). Detection was obtained using the colorimetric substrate tetramethyl benzidine (TMB) in a one step solution as described by the manufacturer (Pierce, Rockford, IL).

In order to purify *c-myc* SVPs, recombinant yeast were grown as described above. Cells were then collected by centrifugation, lysed by freeze thaw (3 cycles), and then sonicated by 60 s pulses with 20 s intervals (5 cycles). The cellular lipids were removed by performing two successive freon extractions. SVPs were then purified by passing them through a 26% sucrose cushion at 82,705 × *g* (average) for 4 h at 4 °C in a swinging bucket rotor (Beckman SW28), followed by CsCl-gradient centrifugation overnight at 115,584 × *g* (average) in a swinging bucket rotor (Beckman SW41). Bands were withdrawn with a syringe and dialyzed overnight at 4 °C in TN buffer (50 mM Tris and 100 mM NaCl, pH 8.0) before using for western blot analysis and transmission electron microscopy (TEM). Western blot analysis was also performed using purified *c-myc* SVPs following the method described above. TEM of the *c-myc* SVP was performed according to previously published protocols (Dykstra, 1992).

2.3. Immunization and sampling of rainbow trout

Rainbow trout (*Oncorhynchus mykiss*; ~25 g juveniles), originating from the Clear Springs Foods Inc. (Buhl, Idaho) and known to be free of IPNV, were used for this immunization protocol. Briefly, the fish were anesthetized and injected intraperitoneally (IP) with 100 μL of vaccine (50 μL of purified SVPs containing 100 μg of antigen and 50 μL of Freund's Complete Adjuvant; Sigma Fluka Aldrich, St. Louis, Missouri). Immunization was performed using *c-myc* SVP or IPNV rVP2 SVP. There were four groups of fish: naive fish ($n = 10$), fish injected with adjuvant only (sham-injected treatment; $n = 8$), a treatment group that was injected with IPNV rVP2 SVPs ($n = 13$) and a group injected with *c-myc* SVPs ($n = 9$) (Table 1). Vaccinations were performed at days 1 and 32. At 66 days post-vaccination, blood samples were drawn from vaccinated and control fish, serum was separated from the cells, and then stored at −75 °C until further use.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Immuno Breakapart microplates (Nunc, Rochester, NY) were coated with purified IPNV rVP2 SVPs at 150 μg/mL or *c-myc* peptide at 100 ng/mL in a 50 mM carbonate coating buffer (pH 9.6) at 4 °C for 16 h. Plates were washed 3 times in TBST (1× Tris Buffered Saline (TBS) + 0.05% Tween 20) for 5 min each wash. The plates were blocked with 1× TBS containing 3% BSA at room temperature. Test sera were diluted 1:32 and 1:64, 150 μL of the diluted sera were added per well, and then the plates were incubated for 1 h at room temperature. Following incubation with the test sera, the microplates were washed again 3 times with TBST for 5 min per wash. The secondary antibody (rabbit-anti-rainbow trout IgG; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was diluted 1:1000 and added to all wells (150 μL/well). The plates were incubated for 1 h at room temperature and then washed 3 times in TBST, 5 min each wash. Horseradish peroxidase-conjugated goat anti-rabbit IgG (BioSource International, Camarillo, CA) was added at a 1:1000 dilution and then detected by addition of the colorimetric substrate tetramethyl benzidine (TMB; Pierce, Rockford, IL). The absorbance was read at 450 nm using a Spectrafluor Plus fluorescent plate reader (Tecan, Salzburg, Austria). Negative controls consisted of wells that were coated as above, but a 3% BSA solution was added instead of the fish serum during the capture step. Positive controls consisted of wells coated with purified IPNV rVP2 SVPs and detected using IPNV polyclonal antibody (made against whole IPNV, Strain N1; Microtek International, B.C., Canada). A subset of samples was placed on each ELISA plate for normalization, giving us a scaling factor to allow plate-to-plate comparisons. Vaccinated fish were considered seropositive if their A_{450} values were above the mean adjuvant control + 2 standard deviations. Comparisons among A_{450} values of different treatments were performed using a factorial ANOVA test followed by Fisher's PLSD test.

2.5. IPNV challenge and sample collection

Three days after collecting the blood samples (*i.e.*, at 69 days post-vaccination) for the ELISA assays, IPNV challenge was performed by injecting each fish with approximately 250 μL of 10^7 $TCID_{50}$/mL of IPNV (Buhl strain) (Table 1). Naive fish injected with buffer served as the negative control for the IPNV challenge. Ten days post-injection, animals were sacrificed, spleen samples collected in TRI reagent (Molecular Research Center, Cincinnati, OH), and then stored at −75 °C until RNA isolation was performed.

2.6. Determining IPNV load by real-time RT-PCR

Total RNA was isolated from spleen tissue of control and IPNV-injected rainbow trout using TRI reagent following the manufacturer's protocols (Molecular Research Center). The RNA samples were treated with DNase I (Ambion Inc., Austin, TX) then the RNA quality assessed by running the samples on a 1% formaldehyde agarose gel (Sambrook et al., 1989). The cDNA synthesis was carried out in a 40 μL reaction volume containing 1 μg total RNA, 1× RT-PCR buffer, 1 mM dNTPs, 0.75 μM oligo dT, 4 U of RNase inhibitor,

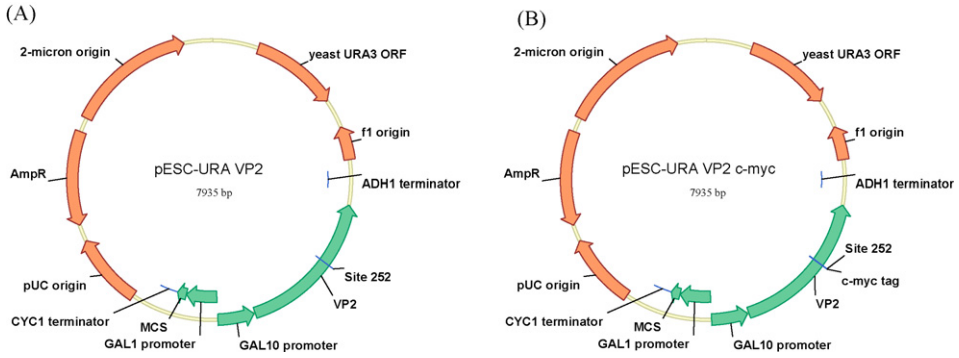

Fig. 2. Vector map of IPNV rVP2 SVP (A) and IPNV *c-myc* SVP (B) constructs in a yeast expression vector. The IPNV VP2 gene was first cloned into pESC-URA vector to generate pESC-URA VP2 (A) and *c-myc* epitope was introduced at amino acid position 252 in the VP2 gene to generate pESC-URA VP2 c-myc (B).

and 5 U of MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA) at 42 °C for 1 h. The cDNA was diluted 1:10 using DNase and RNase free molecular biology grade water and 2 μL of the diluted cDNA used per reaction in real-time RT-PCR.

IPNV real-time RT-PCR was performed using a Bio-Rad iCycler iQ™ (Bio-Rad Laboratories Inc., Richmond, CA), and following a previously published protocol (Bowers et al., 2008). The primers for real-time RT-PCR were designed based on the nucleotide sequence of Segment A of IPNV genome (GenBank accession number NC_001915), and the internal control genes, β-actin (AF157514) and elongation factor 1-α (EF-1α, AF498320). The primer sequences for IPNV were 1916F: 5′-AGGAGATGAC ATGTGCTACA CCG-3′ and 1999R: 5′-CCAGCGAATA TTTTCTCCAC CA-3′, for β-actin were 1301F: 5′-TGCTTCACCG TTCCAGTTGT G-3′, and 1413R: 5′-TGCTTCACCG TTCCAGTTGT G-3′ and for EF-1α were 136F: 5′-TGATCTACAA GTGCGGAGGC A-3′ and 236R: 5′-CAGCACCCAG GCATACTTGA A-3′.

The optimized reaction mixture for real-time RT-PCR contained 12.5 μL of 2× SYBR Green Supermix (iQ SYBR Green Supermix), 300 nM each of forward and reverse primers, and 2 μL of the 1:10 diluted cDNA in a 25 μL reaction volume. The amplifications were carried out in a 96 well plate and each sample had 3 replicates. The thermal profile for real-time RT-PCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min.

After each run, data acquisition and subsequent data analyses were performed using the iCycler iQ Real-Time PCR Detection System (Bio-Rad iQ Software Version 1.3). The relative IPNV load in a sample was determined by subtracting the mean C_t values for β-actin and EF-1α from the mean C_t values of the IPNV amplicon. The differences in the C_t value of the viral genes and the corresponding internal controls were expressed as ΔC_t. The difference in the ΔC_t for one vaccine group compared to the ΔC_t of the corresponding control was expressed as $\Delta\Delta C_t$, and $2\Delta\Delta^{C_t}$ represented the difference in the viral load between the two treatments.

3. Results and discussion

3.1. Identification of insertion site in IPNV VP2 gene and cloning of c-myc epitope in IPNV rVP2 SVP vector

The VP2 gene from IPNV was cloned previously and shown to form SVPs in a yeast expression system. The SVPs also induce an immune response in juvenile rainbow trout (Allnutt et al., 2007). Such VP2 SVPs have been previously isolated while purifying the whole IPNV from rainbow trout gonad-2 cell line (Galloux et al., 2004). It has also been demonstrated that truncated forms of VP2 (*e.g.*, for amino acids 147–307, Labus et al., 2001; and amino acids 79–359, Moon et al., 2004) when expressed in yeast (Labus et al., 2001) or bacteria (Moon et al., 2004) were able to induce an immune response in salmon and rainbow trout. These results indicate that the full VP2 protein is not required to induce an effective immune response, and perhaps the expression of another epitope on the VP2 could be unlikely to render the VP2 protein non-immunogenic. The full-length IPNV VP2 construct expressed in yeast (Allnutt et al., 2007) was used as the starting point in the current study.

Based on our VP2 amino acid sequence analysis, the amino acids between the positions 241 and 288 were found to be highly hydrophilic and probably represent the surface of the VP2 capsid protein. Using the Parker antigenicity, Hoops and Woods antigenicity, Goldman/Engleman/Steitz hydrophilicity, and Karplus and Schulz flexibility data, a composite score was assigned to each of the amino acids at positions 241–288 that reflects the degree of predicted surface exposure. A linear graph based on the composite score for these amino acids is presented in Fig. 1. A total of five amino acids had moderate to high scores. These sites are labeled as I through V and potentially represent sites that may accommodate foreign epitope (Fig. 1). Three of these sites (labeled I, Glu at position 249; labeled II, Arg at position 255; and labeled III, Arg at position 286, Fig. 1) were reported to play a role in IPNV anitigenicity as determined by an antibody escape assays (Heppell et al., 1995). Heppell and colleagues showed that a single nucleotide mismatch in these three sites causes one of two neutralizing antibodies to no longer prevent infection (Heppell et al., 1995). These three loci also showed high composite scores in our sequence analysis, since they are in the hypervariable, antigenic region of VP2 and are probably facing the outside of the virus-particle since they react with antibodies. However, these sites are turn indifferent and therefore, may or may not serve as potential insertion sites for heterologous epitope. Two other sites, Asn at position 252 and Ser at 276 (labeled IV and V, respectively, Fig. 1), had not only high scores but were also turn favorable predicting that these sites could be very flexible to accommodating a foreign epitope. One of these sites, amino acid position 252, was selected for inserting human oncogene epitope *c-myc* (Fig. 2).

3.2. Expression and detection of c-myc SVP

The human oncogene *c-myc* epitope was inserted into IPNV VP2 backbone plasmid pIPA1-Z1 and was used to transform yeast (Fig. 2). Recombinant yeast were grown on galactose containing medium to induce expression of the *c-myc* SVPs. Cells were pelleted by centrifugation and crude protein extracts were tested by western blot analyses using both anti-*c-myc* monoclonal and anti-IPNV polyclonal antibodies. Positive immunodetection was obtained with both anti-IPNV and anti-*c-myc* antibodies indicating that the *c-myc* linear peptide was successfully inserted into the chimeric SVPs; both the IPNV epitopes on the VP2 pro-

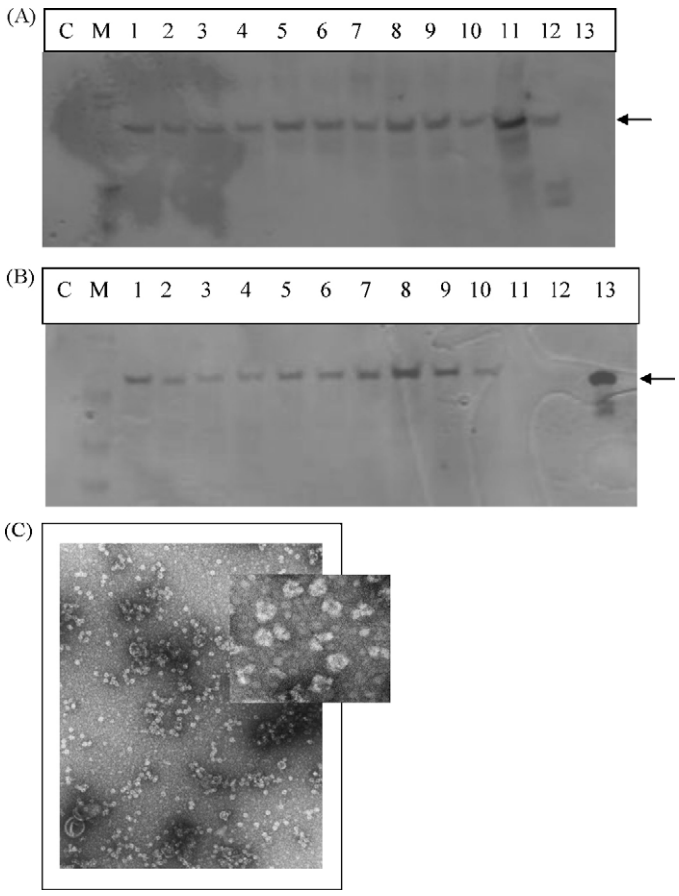

Fig. 3. Characterization of the *c-myc* SVPs by western blot analysis and transmission electron microscopy. Total proteins from recombinant yeast clones expressing *c-myc* SVPs were electrophoresed on 12% SDS-polyacrylamide gels, transferred to nitrocellulose membrane and western blot analyses were performed using anti-IPNV polyclonal antibody (A) or anti-*c-myc* monoclonal antibody (B). The arrow indicates *c-myc* VP2 SVP band or the positive control band. C = cell lysates from wild type yeast, M = Pre-stained SDS-PAGE low range protein standard, lanes 1–10 = cell lysates from *c-myc* SVP recombinant yeast clones, lane 11 = cell lysates from a recombinant IPNV rVP2 yeast clone, lane 12 = Cell culture supernatant from IPNV infected fish cells, and lane 13 = *c-myc* control protein, Posi-tag Control Protein, Covance Inc. (C) Transmission electron micrograph of *c-myc* SVPs isolated from recombinant yeast using cesium chloride centrifugation as described in Section 2.

tein as well as the *c-myc* epitope retained their antigenicity (Fig. 3A).

The chimeric SVPs were purified using a cesium chloride gradient centrifugation method. The purified *c-myc* SVP gave positive reactions in western blot analyses against anti-*c-myc* and anti-IPNV antibodies. When an aliquot of *c-myc* SVP preparation was examined by transmission electron microscopy, ~20 nm diameter particles were formed (Fig. 3C). The morphology and size of the *c-myc* SVPs was similar under electron microscopy to that produced by the IPNV rVP2 SVPs (Allnutt et al., 2007). Both of *c-myc* SVPs and the IPNV rVP2 SVPs particles were considerably smaller than the IPNV VLPs previously expressed in insects, which were made up of both VP2 and VP3 and assembled into a 60 nm VLP (Shivappa et al., 2005).

These data, therefore, suggest that IPNV rVP2 SVPs can tolerate the insertion of a foreign epitope of at least 10 amino acids long, the size of *c-myc* epitope, without affecting the morphology of the SVPs and the antigenicity of the epitopes on the backbone protein or the foreign epitope. This opens up the possibility of using IPNV rVP2 SVPs as a platform to develop multivalent vaccines containing at least one, and possibly more than one, epitope from one or multiple viruses. Such vaccines would potentially provide broader immune coverage for a vaccine or be used as a marker vaccine allowing one to differentiate vaccinates from infected animals by testing for both the foreign and viral epitopes.

Table 2
Determining the levels of anti-IPNV antibody in rainbow trout vaccinated with *c-myc* SVP and IPNV rVP2 SVP by enzyme-linked immunosorbent assay. *Fish considered positive if A_{450} was above 2×'s the mean adjuvant control.

Treatments	Serum dilution	Mean ELISA value ± 2 SD	
		Against IPNV rVP2 SVP	Against *c-myc* peptide
Unimmunized	1:32	0.304 ± 0.169	NT*
Adjuvant control	1:32	0.395 ± 0.141	0.352 ± 0.162
IPNV rVP2 SVP	1:32	0.996 ± 0.426	NT*
c-myc SVP	1:32	1.201 ± 0.352	0.967 ± 0.343
Unimmunized	1:64	0.279 ± 0.079	NT*
Adjuvant control	1:64	0.304 ± 0.169	0.241 ± 0.075
IPNV rVP2 SVP	1:64	0.694 ± 0.261	NT*
c-myc SVP	1:64	1.095 ± 0.442	0.682 ± 0.287

NT*, not tested.

3.3. Immunization of rainbow trout using c-myc SVP

Chimeric *c-myc* SVPs were purified as described in Section 2 then used to immunize juvenile rainbow trout by injection. Fish injected with IPNV VP2 SVPs were used as positive controls for the immunization assay. The experimental design and various treatments are outlined in Table 1. The *c-myc* SVPs were evaluated for the ability to induce an immune response against IPNV and *c-myc* epitopes. Previous research demonstrated that the administration of IPNV rVP2 SVPs, either via injection or orally, led to the induction of an antibody response in rainbow trout (Allnutt et al., 2007). Both the *c-myc* SVPs and IPNV rVP2 SVPs induced the production of anti-*c-myc* and anti-IPNV antibodies in the rainbow trout tested (Fig. 4A, Table 2). Although there was no significant difference between these two treatments (at 95% confidence level $p = 0.1294$), there was a trend in higher anti-IPNV antibody titer in *c-myc* SVP injected fish compared to IPNV rVP2-SVP injected fish. The anti-IPNV antibody response in the *c-myc* SVP injected group was roughly three times that observed for both control groups (adjuvant only and naïve fish, $p \leq 0.001$ at 95% confidence level).

When the sera of the rainbow trout injected with the *c-myc* SVPs were analyzed for antibodies against *c-myc* epitope, there was a significant difference ($p \leq 0.001$ at a 95% confidence level) between the *c-myc* SVP injected sera and those from the adjuvant only control (Fig. 4B, Table 2). The ELISA absorbance from the *c-myc* SVPs were three times that observed for the adjuvant only controls. Due to the lack of serum, we could not perform the ELISA test for the serum of IPNV rVP2 SVP injected and healthy control groups. Nevertheless, the present study clearly demonstrated that the *c-myc* epitope in chimeric SVP was recognized by the host immune system and antibody response was elicited against the foreign epitope.

3.4. IPNV challenge and viral load analysis in vaccinated fish

Juvenile rainbow trout were injected with purified *c-myc* SVPs with adjuvant and this was boosted with a second injection (Table 1). Injection with adjuvant only was used as the negative control and injection with IPNV rVP2 SVPs was used as positive control. Once the vaccination trial and blood sampling were completed, the vaccinated fish were virus challenged by injection with IPNV (Buhl strain) and the viral load followed for progress of the infection. IPNV was successfully detected in spleen tissues of virus-challenged rainbow trout but not in the healthy control group fish by real-time RT-PCR. Depending on the internal control gene used to calculate the viral load, the IPNV load in *c-myc* SVP injected

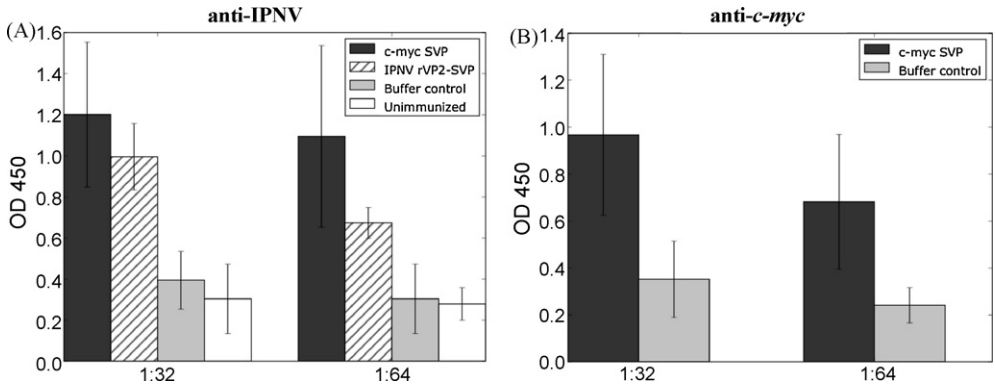

Fig. 4. Immunoassay of sera for anti-IPNV and anti-*c-myc* response in immunized rainbow trout using enzyme-linked immunosorbent assay. (A) The ELISA plate was coated with purified IPNV rVP2 SVP and mean ELISA values (A_{450}) of anti-IPNV titer were detected in rainbow trout serum. (B) The ELISA plate was coated with *c-myc* peptide and mean ELISA values (A_{450}) of anti-*c-myc* titer was detected in rainbow trout serum.

Table 3
Relative IPNV load in *c-myc* SVP and IPNV rVP2 SVP vaccinated rainbow trout as determined by the real-time RT-PCR.

Treatments	Average ΔC_t (IPNV-β actin) ± SD	$\Delta\Delta C_t$ (treatment –adjuvant control)	IPNV fold reduction ($2\Delta\Delta^{C_t}$)	Average ΔC_t (IPNV-EF-1α) ± SD	$\Delta\Delta C_t$ (treatment –control)	IPNV fold reduction ($2\Delta\Delta^{C_t}$)
Healthy	None	None	None	None	None	None
Adjuvant control	10.88 ± 4.34			9.27 ± 4.65		
IPNV rVP2 SVP	14.90 ± 5.24	4.02	16	12.60 ± 4.38	3.33	10
c-myc SVP	16.66 ± 0.77	5.78	55	14.74 ± 0.89	5.47	44

fish was 44–55-fold lower than the fish in the adjuvant only control group (Table 3). Similarly, the viral load in IPNV rVP2 SVP injected fish was 10–16-fold lower compared to the adjuvant control group (Table 3). There was a noticeable reduction in the IPNV load provided by the *c-myc* SVP as compared to the IPNV rVP2 SVP. Therefore, the expression of the *c-myc* epitope on the IPNV rVP2 SVP did not affect the ability of this vaccine to induce an immune response to IPNV in juvenile rainbow trout nor affect the subsequent clearing of the viral infection when challenged with infectious virus. In addition, the viral load in the *c-myc* SVP injected group was 3.5 ($2^{16.66-14.90}$ using β-actin as internal control, Table 3) to 4.5 ($2^{14.74-12.60}$ using EF-1α as internal control, Table 3) fold less than the IPNV rVP2 SVP injected group. The reduced viral load in *c-myc* SVP injected fish compared to IPNV rVP2 SVP injected fish mirrors a higher anti-IPNV antibody titer trend observed in the *c-myc* SVP vaccinated compared to IPNV rVP2 SVP vaccinated fish. It has been reported that when foot-and-mouth disease virus (FMDV) epitopes were expressed in a hepatitis B virus (HBV) VLP, the chimeric VLPs showed a significantly higher HBV immunogenicity compared to the HBV VLPs in immunized mice (Zhang et al., 2007). In addition, the chimeric FMDV HBV VLP immunized mice had elevated IFN-γ, and interleukin-4 production compared to HBV VLP immunized mice (Zhang et al., 2007). The data from the present study bear similarity to the HBV VLP study. It is, therefore, possible that the chimeric IPNV VLP carrying epitopes of other fish viruses, such as infectious salmon anemia virus (ISAV), may be more immunogenic than the IPNV VLP alone.

The results from this study demonstrate that the IPNV rVP2 proteins are flexible molecules that can tolerate the insertion of linear foreign epitopes without affecting either the antigenicity of the epitopes of the backbone capsid protein (VP2) or that of the expressed foreign epitope (*c-myc*). This opens the possibility of using chimeric IPNV rVP2 SVPs as a molecular scaffold to express epitopes of other important viruses (*e.g.* infectious hypodermal necrosis virus, infectious salmon anemia virus) leading the way to develop multivalent subunit vaccines or novel marker vaccines. Future studies need to be performed to determine if more complex epitope(s) can be expressed and maintain their antigenicity.

A conundrum of vaccination work has been the inability to tell vaccinated animals from infected or previously infected animals. In some cases this has led to laws or standard practices that prohibit animal vaccination against devastating diseases, such as foot-and-mouth, for the sole reason of allowing rapid detection of disease and subsequent containment of the disease after detection of any immune response to the causative viral antigen. The use of a marker vaccine would allow one to rapidly discern vaccinates from previously infected individuals by immunoassay to better manage contagious viral diseases. This approach would be enabled by a genetically engineered VLP or SVP expressing an epitope not likely to be found in the target animal as well as the disease related epitope(s). Expression of the human *c-myc* epitope on IPNV could provide such a vaccine, since this work has shown that the surface expression of the *c-myc* did not decrease the effectiveness of the immune reaction to the IPNV capsid genes while maintaining the *c-myc* antigenicity. Therefore, after vaccination, animals that are seropositive for both IPNV and *c-myc* would be vaccinates while those seropositive for IPNV only would either be chronic carriers, actively diseased animals, or animals that have recovered from the disease. Viral diseases, such as those caused by ISAV and IPNV, threaten salmon aquaculture worldwide (Roberts and Peason, 2005; Mardones et al., 2009). As the oceans have been progressively overfished, farmed fish and other aquatic organisms are more intensively cultivated. With this more intense aquaculture and its associated stress on the animals, the health of both wild and farmed organisms becomes harder to assure. Methods to reduce viral diseases in aquaculture fish will improve both the quality of life of the animals and make the industry more sustainable.

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