Mapping a neutralizing epitope on the coat protein of striped jack nervous necrosis virus

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Striped jack nervous necrosis virus (SJNNV), a fish nodavirus, is the causative agent of viral nervous necrosis in marine fishes. The fish nodaviruses are divided into four different genotypes based on the nucleotide sequence of the coat protein gene. In the present study, partial coat protein genes of fish nodaviruses were expressed. This allowed the serological relationship among the different virus genotypes to be analysed and neutralizing epitopes on the coat protein to be mapped. Western blot analysis revealed that SJNNV and other fish nodavirus genotypes shared a significant number of common antigenic determinants, although SJNNV was serologically distinguishable. The results suggested that the SJNNV determinant for neutralizing MAb was a linear epitope, which consisted of a repeated amino acid sequence within the coat protein. One of the neutralizing epitopes of SJNNV was deduced to be PAN at aa 254–256 in the coat protein.

Fish nodaviruses are the causative agents of viral nervous necrosis (VNN) or viral encephalopathy and retinopathy in about 20 fish species in many countries in Asia and Europe (Munday & Nakai, 1997; Muroga et al., 1998). These viruses are isometric with a diameter of 25–30 nm and consist of a single coat protein and a bisegmented genome, RNA1 and RNA2 (Mori et al., 1992; Comps et al., 1994). Based on sequence analyses of RNA1 and RNA2, fish nodaviruses are clearly distinguishable from insect nodaviruses, and a new genus, Piscinodavirus, has been proposed in the family Nodaviridae (Nishizawa et al., 1999b; Delsert et al., 1997; and accompanying paper by Nagai & Nishizawa, 1999). Striped jack nervous necrosis virus (SJNNV), the causative agent of VNN in larval striped jack (Pseudocaranx dentex) (Mori et al., 1992; Arimoto et al., 1993), has an RNA2 of 1410 bases in length containing an ORF (nt 17–1036) encoding a coat protein (340 residues) with a predicted molecular mass of 37 180 Da. Based on the nucleotide sequence of SJNNV RNA2, five different PCR primers were designed and used for analysis of coat protein genes from other fish nodaviruses (Nishizawa et al., 1994, 1995b). A highly conserved region at aa 83–216 (more than 93% sequence identity) and a variable region at aa 235–315 (62% sequence identity) were identified among the amino acid sequences of the coat proteins. Molecular phylogenetic analysis of the partial coat protein gene showed that fish nodaviruses were classified into four different genotypes: SJNNV; tiger puffer NNV (TPNNV); barfin flounder NNV (BFNNV); and redspotted grouper NNV (RGNNV) types (Nishizawa et al., 1997). Although some serological investigations have been made using polyclonal antibodies (Nguyen et al., 1994; Nakai et al., 1994; Delsert et al., 1997; Grotmol et al., 1997) and MAbs (Nishizawa et al., 1995a), little is known about the serological relationships and neutralizing epitopes among fish nodaviruses. In the present study, the T2 (nt 115–1030 encoding aa 83–216) and T4 regions (nt 605–1030 encoding aa 204–331) of viral coat protein genes were expressed in bacterial cells for an analysis of the serological relationship among the different genotypes of fish nodaviruses. These studies also allowed for mapping of neutralizing epitopes on the SJNNV coat protein.

The isolates of fish nodaviruses, SJOri (DDBJ accession number, D30814), TP93Kag (D38637), BF93Hok (D38635) and RG91Tok (D38636), were used as representatives of four different genotypes, SJNNV, TPNNV, BFNNV and RGNNV types, respectively. Partial coat protein genes (T2 and T4 regions) of these isolates were sequenced in our previous work (Nishizawa et al., 1995b, 1997). The PCR primers, F1exp (5’ aacaatatggagtgttggacgtgccacaa 3’), F2exp (5’ aacaatatgtgcattactgtgtgctgctg 3’) and R3exp (5’ gtgaagccttcagactacaggttgcgtcag 3’) were used for PCR amplification of the T2 and T4 regions from the above four virus samples. The sense primers, F1exp (nt 115–174) and F2exp (nt 605–624), included an additional 9 bases of linker sequence as an Ndel recognition site. The antisense primer, R3exp (complementary to nt 1011–1030), has an additional 11 bases of linker sequence, representing a HindIII recognition site and termination codon. PCR amplification was performed under the conditions previously described (Nishizawa et al.,...
prepared from the cloned cDNAs of the T4 region of each fish nodavirus genotype with a PCR DIG labelling kit (Boehringer Mannheim) and PCR primers F2 and R3 (Nishizawa et al., 1994) according to the manufacturer’s instructions. The DIG-labelled SJNNV, TPNNV, BFNNV and RGGNNV T4 probes showed clear signals with the inserted DNAs in the clones obtained from respective homologous viruses, whereas very weak reactions were shown for heterologous virus DNA. These results confirmed that the T2 and T4 regions of each of the four different virus genotypes were cloned into the respective expression vector plasmids. Additionally, it was shown that rapid genotyping of fish nodaviruses was possible by Southern hybridization with the DNA probes against the T4 region of the viral coat protein gene.

Clones of transformed E. coli containing cDNA of the T2 or T4 region were induced by culture in LB medium (1% Bacto-trypthone, 0.5% Bacto-yeast extract, 1% NaCl, pH 7-4) with 50 µg/ml ampicillin and 1 mM IPTG at 37 °C for 3 h. The cells were resuspended in TE (50 mM Tris–HCl, pH 8.0, 2 mM EDTA) solution containing 100 µg/ml lysozyme and 0.1% Triton X-100 at 30 °C for 15 min. The cells were then sonicated until the viscosity of the solution was lost. The induced product from the target gene was found in the insoluble fraction and was washed twice by centrifugation (12,000 g, 15 min) and resuspended in TE solution. Purification of SJNNV particles was performed according to the procedure of Nagai & Nishizawa (1999).

SDS–PAGE analysis of the SJNNV coat protein and the induced products was performed according to Laemmli (1970) and proteins were electroblotted onto a nitrocellulose membrane by the procedure of Towbin et al. (1979). Proteins on the membrane were immunostained with an anti-SJNNV serum (A/S SJNNV) and two specific SJNNV MAbs, 102B and 204D (Nishizawa et al., 1995a), and then visualized with an immunoblot kit (Bio-Rad) according to the manufacturer’s instructions (Fig. 2). The coat protein of SJNNV had an apparent molecular mass of 40 kDa, which was in agreement with that estimated by Mori et al. (1992). The expressed proteins from the T2 region of SJNNV, TPNNV and BFNNV showed the same relative mobility in the gel, the apparent molecular mass being 32 kDa. The expressed product from the T4 region of each of the four fish nodavirus genomes. All of them ranged between 15-5 and 16-0 kDa (Fig. 2A, B). The sizes of the expressed proteins encoded by the T2 and T4 regions of the viral coat protein genes were in accordance with those estimated from the deduced amino acid sequences. In the immunoblot analyses, the A/S SJNNV showed positive cross-reactions with all of expressed proteins, although differences in the intensity of the antibody reaction were observed among them. In particular, the intensities with the products of homologous virus were much stronger than...
Fig. 2. SDS–PAGE of the expressed proteins from the T2 and T4 regions of the viral genomes and Western blot analyses with polyclonal and monoclonal antibodies against SJNNV. Polyacrylamide gel (12%) stained with Coomassie Brilliant Blue (A), immuno-stained with anti-SJNNV rabbit serum (B), immuno-stained with MAb 102B (C) and immuno-stained with MAb 204D (D). Lanes: M, molecular size marker; 1, coat protein of SJNNV; 2, the expressed SJNNV T2 protein; 3, SJNNV T4 protein; 4, TPNNV T2 protein; 5, TPNNV T4 protein; 6, BFNNV T2 protein; 7, BFNNV T4 protein; 8, RGNNV T4 protein.

those exhibited by heterologous viruses, indicating that the four different fish nodavirus genotypes share a significant number of antigenic determinants, although they are not identical to each other. This is in agreement with previous findings (Nguyen et al., 1994; Nakai et al., 1994; Grotmol et al., 1997). On the other hand, MAbs 102B and 204D reacted not only with a native coat protein and the expressed T2 and T4 proteins of SJNNV, but also with the expressed T2 proteins of TPNNV and BFNNV. However, no cross-reactions were observed with those from T4 regions of heterologous viruses (Fig. 2C, D). Both MAbs have a neutralizing activity against SJNNV (Nishizawa et al., 1995a), suggesting that SJNNV could be distinguishable serologically from the other genotypes of fish nodaviruses, and also that the antigenic determinant recognized by both MAbs has to be a linear epitope on the SJNNV coat protein.

The reactivities of MAbs with the expressed T2 and T4 proteins were considered to be useful for mapping the neutralizing epitope on the SJNNV coat protein. A total of 278 residues from aa 54 to aa 331 is encoded in the SJNNV T2 region, whereas the T4 region encodes 128 residues (aa 204–331), i.e. the expressed T4 protein has an amino acid sequence identical to 128 residues at the C terminus of the expressed T2 protein. In our experiments, both MAbs recognized the expressed T2 and T4 proteins of SJNNV but only the T2 protein of heterologous viruses. Therefore, it is unlikely that the epitope for these MAbs is present at only one site on the SJNNV coat protein. Thus, we deduced that the linear epitope for these MAbs should contain an amino acid sequence repeated in the SJNNV coat protein. Such a sequence is located between aa 54–203 (included in the expressed T2 protein) and another is located between aa 204–331 (included in both T2 and T4 proteins). This amino acid sequence also has to be present only in aa 54–203 of the T2 proteins of heterologous viruses.

A matrix plot analysis of amino acid sequences encoded in the T2 and T4 regions of the SJNNV coat protein was performed with a window of three residues and 100% for a minimum score (Fig. 3A). Four different repeated amino acid sequences were found on the SJNNV coat protein as follows: PAG (aa 69–71, 294–296 and 328–330); AGT (aa 70–72 and 329–331); LLP (aa 88–90 and 326–328); and PAN (aa 116–118 and 254–256). These elements, together with their flanking sequences, are displayed, after alignment, in Fig. 3(B) (Thompson et al., 1994). As described above, the epitope for the MAbs should be present within aa 54–203 as a common sequence among the four genotype viruses and also present within aa 204–331 as a specific sequence to SJNNV. Among the observed amino acid sequences, only AGT (aa 70–72 and 329–331) and PAN (aa 116–118 and 254–256) satisfied these conditions. However, an additional AGT sequence existed at aa 291–293 within the RGNNV T4 protein (Fig. 3B). Therefore, we conclude that the repeated sequence, PAN, is most likely a potential neutralizing epitope for both MAbs 102B and 204D. It was confirmed that a synthetic peptide with eight residues containing the PAN sequence reacts with A/S
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