Identification of B-cell epitopes on the betanodavirus capsid protein

J Z Costa, A Adams, J E Bron, K D Thompson, W G Starkey and R H Richards

Institute of Aquaculture, University of Stirling, Stirling, UK

Abstract

The pepscan procedure was used to identify betanodavirus B-cell epitopes recognized by neutralizing mouse monoclonal antibodies (MAbs) and serum samples obtained from sea bass, *Dicentrarchus labrax*, naturally infected with betanodavirus. Pepscan was performed with a panel of thirty-four 12-mer synthetic peptides that mimicked the entire betanodavirus capsid protein. Sea bass serum samples reacted strongly with three regions of the capsid protein comprising amino acid residues 1–32, 91–162 and 181–212. The latter region was also recognized by neutralizing MAbs and coincided with a region of high antigenic propensity identified by an antigen prediction algorithm. These data suggest that a region of the betanodavirus capsid protein spanning amino acid residues 181–212 may represent a neutralization domain that could potentially be used to inform the development of nodavirus vaccines and immunodiagnostic reagents.

Keywords: betanodavirus, epitope mapping, luminex, monoclonal antibodies, pepscan, xMAP technology.

Introduction

Within the last 20 years, betanodaviruses have emerged as major pathogens of teleosts, causing disease in several marine and freshwater species (reviewed in Munday, Kwang & Moody 2002). Betanodaviruses are the aetiological agents of viral nervous necrosis (VNN), a disease characterized by vacuolation of the encephalon and retina and the presence of virus particles within infected neurones. VNN is associated with extensive mortalities that may approach 100% in severe cases. Betanodavirus infection has been reported in more than 35 fish species belonging to 10 different orders including Atlantic cod, *Gadus morhua* L., European sea bass, *Dicentrarchus labrax* (L.), amberjack, *Seriola dumerili* (Risso), groupers, *Epinephelus* spp. and barramundi, *Lates calcarifer* Bloch (Glazebrook, Heasman & Beer 1990; Mori, Nagahara, Muroga, Mekuchi & Kanno 1991; Renault, Haffer, Baudin-Laurencin, Breuil & Bonami 1991; Nakai, Nguyen, Nishizawa, Muroga, Arimoto & Ootsuki 1994; Fukuda, Nguyen, Furushashi & Nakai 1996; Chi, Lo, Kou, Chang, Peng & Chen 1997; Starkey, Ireland, Muir, Jenkins, Roy, Richards & Ferguson 2001; Johnson, Sperker, Leggiadro, Groman, Griffiths, Ritchie, Cook & Cusack 2002; Ucko, Colorni & Diamant 2004). Many of the species affected by VNN are of economic importance to the aquaculture industry.

Betanodaviruses are icosahedral viruses with a diameter of 23 nm (Breuil, Bonami, Pépin & Pichot 1991). The betanodavirus genome is bipartite, comprising two single-stranded positive-sense RNA molecules with Mr of $1.01 \times 10^6$ Da (RNA1) and $0.49 \times 10^6$ Da (RNA2) (Mori, Nakai, Muroga, Arimoto, Mushiake & Furusawa 1992). RNA1 encodes a 100-kDa protein representing the viral component of the RNA-dependent RNA polymerase. RNA2 encodes the 42 kDa coat protein precursor (Nagai & Nishizawa 1999). A subgenomic transcript of RNA1, designated as RNA3, is also expressed in infected cells (Somerset & Nerland 2004). Betanodaviruses are genetically diverse, and have been classified into four genotypes based on the nucleotide sequence of the coat protein gene (Nishizawa, Furushashi, Nagai,
Nakai & Muroga 1997). A betanodavirus with a distinct coat protein nucleotide sequence potentially representing a novel genotype was isolated from sea bass in France (Thiéry, Arnauld & Delsert 1999).

The identification of epitopes on viral pathogens is of importance for the rational development of sub-unit vaccines and immunodiagnostic reagents. Relatively few studies have focussed on betanodavirus B-cell epitopes. Nishizawa, Takano & Muroga (1999) identified a putative B-cell epitope located at residues 254–256 of the coat protein, based on differential monoclonal antibody (MAb) binding patterns to recombinant proteins expressed in *Escherichia coli*. However, established mapping procedures have not been used to characterize epitopes in betanodaviruses. In this study, we have used the pepscan procedure (Geysen, Meloen & Barteling 1984) to identify betanodavirus B cells recognized by serum samples from betanodavirus-infected fish, and by anti-betanodavirus mouse MAbs.

**Materials and methods**

**Synthetic peptides**

The dragon fish nervous necrosis virus coat protein (Genbank protein access number AAG22496) was used as template for the production of the synthetic peptides, which were produced by Pepscan Systems (Leystad, the Netherlands) using solid phase synthesis and Fmoc chemistry. Peptides were 12 amino acid residues in length with an overlap of two residues and corresponded to the entire betanodavirus coat protein. All peptides contained an N-terminal amino hexanoic linker. The amino acid sequence of peptides is indicated in Table 1.

**Mouse monoclonal antibodies**

For production of MAbs, BALB/C mice were immunized with betanodavirus strain MT/01/Sba derived from European sea bass. Mice were immunized twice by intraperitoneal injection with a 1:1 dilution of virus in Titremax® Gold adjuvant (Titremax, Cyt Rx®; Stratech, Cambridge, UK). This was followed by a final intravenous immunization of virus without adjuvant. For each immunization 100 µL of virus (30 µg L⁻¹ stock) was used. The MAbs were produced using standard procedures (Campbell 1984) to promote fusion between SP2/0 cells and mouse splenocytes. Hybridomas were cloned by limiting dilution and selected by ELISA. Four MAbs were used for pepscan analyses: 3B10, 4A12, 4C3 and 5G10. These were purified using Protein-G columns (Hi Trap Protein G HP; GE Healthcare Life Sciences, Amersham, UK). MAb protein concentration was determined with the BCA protein kit (Pierce, Rockford, IL, USA). A MAb produced against *Tetracapsuloides bryosalmonae* was kindly supplied by Dr David Morris (Institute of Aquaculture, University of Stirling, Stirling, UK) for use as a negative control.

**Sea bass serum samples**

Serum samples were collected from European sea bass naturally infected with betanodavirus. The sea bass were raised in cages in Greece. The presence of antibodies against betanodavirus was assessed by an

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**Table 1** Sequences of the synthetic peptides (12-mers) used for epitope mapping. Sequences were derived from dragon fish nervous necrosis virus coat protein (Genbank protein access number AAG22496)
ELISA that utilized cell-culture propagated betanodavirus strain MT/01/Sba as antigen and an anti-European sea bass IgM MAb (Aquatic Diagnostics Ltd, Stirling, UK). Goat anti-mouse IgG conjugated to horseradish peroxidase and tetramethylbenzidine dihydrochloride were used for detection of bound antibodies. The sea bass serum sample used as a negative control was supplied by Dr W. Roy (Machrihanish Environmental Research Laboratory, UK) and was obtained from sea bass farmed in Wales, where nodavirus infection has never been recorded.

**Epitope mapping**

Synthetic peptides were coupled to polystyrene fluorescent microspheres (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s recommendations. Peptide-coupled microspheres were blocked with assay buffer [Dulbecco’s PBS containing 1% bovine serum albumin (w/v) and 0.02% sodium azide (w/v)] for 30 min at room temperature. The coupling procedure was performed by Pepscan Systems. Filter plates (MultiScreen HTS™ Millipore, Bedford, MA, USA) were blocked with assay buffer (two 30-min incubations at room temperature) to prevent nonspecific antibody binding.

Four types of microsphere, each with a unique spectral address, were used for pepscan analysis. Two thousand of each peptide-coupled microsphere were added per well and the assay buffer was removed using a manifold system (Bio-Rad). To each well, 50 µL MAb or fish serum was added and then incubated overnight at 4 °C with gentle agitation. MAbs were tested at a concentration of 100 µg mL⁻¹ in assay buffer. Fish serum samples were used at a dilution of 1:25 v/v in assay buffer. Peptide-binding antibodies were detected using 50 µL of reporter antibody (25 µg mL⁻¹ in assay buffer). Goat anti-mouse IgG conjugated with phycoerythrin (Molecular Probes, Eugene, OR, USA) was used as a reporter antibody and the incubation with the microsphere–antibody complex was performed at room temperature with gentle agitation for 30 min. For analysis of sea bass serum samples, 50 µL of mouse anti-sea bass IgM MAb (Aquatic Diagnostics Ltd) at a concentration of 20 µg mL⁻¹ was used as a primary antibody. Microspheres were resuspended in 100 µL of assay buffer, and sample fluorescence values were measured on a Bio-Plex™ (Bio-Rad) instrument programmed to analyse a 50 µL sample volume and to read a minimum of 100 events per each sample analysed. Results are expressed as mean fluorescence intensity (MFI).

Data analysis comprised three steps: (i) Subtraction of the background fluorescence from test sample MFI. Background fluorescence was determined by performing an experimental run in which antibody samples were replaced by assay buffer. (ii) Establishment of cut-off value. The mean and standard deviation of the 50% lowest MFI values were calculated. The cut-off value was then set at 5 × SD above the calculated mean MFI. (iii) To reduce the influence of nonspecific fluorescence when analysing sea bass serum samples, for each peptide the MFI value of a nodavirus-negative serum sample was subtracted from the corresponding MFI value for nodavirus-infected samples. The cut-off value was then established as described in (ii) above.

**Hydropathicity analysis of the betanodavirus capsid protein**

The hydropathicity profile of the betanodavirus coat protein was analysed in a seven amino acid window using the method developed by Kyte & Doolittle (1982). Analysis was performed using the Pepwindow algorithm (European Molecular Biology Open Software Suite; http://bioinfo.hku.hk/EMBOSS).

**Prediction of the betanodavirus coat protein structure**

The secondary structure of the betanodavirus coat protein was predicted using PredictProtein (McGuffin, Bryson & Jones 2000) (http://www.predictprotein.org/cgi).

The CPHmodels 2.0 homology-modelling server (Lund, Nielsen, Lundegaard & Worning 2002) was used to predict the 3D structure of the betanodavirus capsid protein. This algorithm is available on: http://www.ebs.dtu.dk/services/CPHmodels/ and produced a 3D structure of the capsid protein that incorporated amino acid residues 88–212.

**Antigenic prediction**

The prediction of potentially antigenic regions of the betanodavirus coat protein was performed using the Kolaskar & Tongaonkar (1990) method. Analysis was performed using the antigenic program (European Molecular Biology Open Software Suite; http://bioinfo.hku.hk/EMBOSS).
Results

Epitope mapping with sea bass serum samples

Nine serum samples from betanodavirus-infected sea bass were tested for binding to a panel of synthetic peptides (12-mers) that mimicked the entire betanodavirus capsid protein (Fig. 1). The peptides recognized most strongly by these serum samples were located within three regions of the capsid protein that corresponded to amino acid residues 1–32, 91–162 and 181–222 (Fig. 2). All of the serum samples tested recognized a region of the capsid protein represented by peptides 19–21, corresponding to residues 181–212.

Epitope mapping with mouse monoclonal antibodies

Four neutralizing anti-betanodavirus MAbs were tested for the ability to bind to a panel of synthetic peptides (12-mers) mimicking the betanodavirus capsid protein (Fig. 3). MAb 3B10 recognized peptide 20 (corresponding to capsid protein residues 191–202). MAb 4A12 recognized peptides 3, 16 and 19–21 (corresponding to residues 21–32, 151–162 and 181–212). MAb 4C3 recognized peptides 15–16 and 19–20 (corresponding to residues 141–162 and 181–202). MAb 5G10 recognized peptides 16 and 20 (corresponding to residues 161–162 and 191–202).
The identification of antigenic epitopes on viral pathogens is important for the rational design of vaccines and immunodiagnostic reagents. In this study, we have mapped neutralizing B-cell epitopes on the betanodavirus capsid protein using the pepscan procedure originally developed by Geysen et al. (1984). A panel of thirty-four 12-mer peptides mimicking the entire betanodavirus capsid protein was used to map the binding sites of neutralizing anti-betanodavirus MAbs, and serum samples from sea bass naturally infected with betanodavirus.

Serum samples obtained from betanodavirus-infected sea bass strongly recognized three regions of the betanodavirus capsid protein comprising amino acid residues 1–32, 91–162 and 181–212. The immunogenicity of the N-terminal region of the nodavirus capsid protein has previously been reported by Coeurdacier, Laporte & Pépin (2003). All of the sea bass serum samples recognized a region of the capsid protein spanning residues 181–212. This was also the region of the capsid protein recognized most frequently by neutralizing MAbs. The regions of the capsid protein recognized by MAbs and sea bass serum samples in the present study are distinct from the putative B-cell epitope located at capsid protein residues 254–256 identified by Nishizawa et al. (1999). This may be due to antigenic differences between the striped jack nodavirus isolate studied by Nishizawa et al. and the sea bass isolate studied in this report.

The region of the betanodavirus capsid spanning amino acid residues 181–212 is strongly hydrophobic (Fig. 4) and contains two potential

Figure 3 Epitope mapping of the betanodavirus coat protein with mouse monoclonal antibodies. Four monoclonal antibodies were studied, 4C3, 3B10, 4A12, 5G10. X-axis = peptide number; y-axis = fluorescence (MFI; see Materials and methods).

Figure 4 Hydropathicity plot of the betanodavirus coat protein showing the position of peptides 19–21. Positive scores indicate hydrophobic residues and negative scores indicate hydrophilic residues.
N-linked glycosylation sites at residues 187 and 193. The secondary structure of this region of the capsid protein was analysed using the Predict Protein algorithm (McGuffin et al. 2000), and contained two stretches of amino acids in strand conformation 5 and 14 residues in length, separated by seven amino acid residues (Fig. 5). Capsid protein amino acids 181–212 are exposed at the surface of the capsid protein (Fig. 6), based on a predicted 3D structure of the betanodavirus capsid produced using the algorithm of Lund et al. (2002).

Amino acid residues 181–212 contain the region of the betanodavirus capsid protein with the greatest antigenic propensity as defined by the antigenic prediction method of Kolaskar & Tongaonkar (1990). Correlation between B-cell epitopes identified in antibody-based mapping studies, and epitopes predicted by the method of Kolaskar & Tongaonkar has also been noted for SARS coronavirus (He, Zhou, Wu, Kou, Liu & Jiang 2004). The putative antigenic domain identified on the betanodavirus capsid protein by the method of Kolaskar & Tongaonkar spans residues 193–212 and comprises the amino acid sequence DVVNVSVLCRW-SVRLSVPSL. Interestingly, capsid protein residues 193–212 fall within the highly conserved region identified by Nishizawa, Mori, Furuhashi, Nakai, Furusawa & Muroga (1995), and are conserved in 69 of 78 betanodavirus capsid protein sequences deposited on the UniProt database (http://www.ebi.uniprot.org/index.shtml). These include viruses isolated from more than 25 species of fish including halibut, *Hippoglossus hippoglossus* (L.), Atlantic cod, *Japanese flounder, Paralichthys olivaceus* (Temminck & Schlegel), sea bass, dragon grouper, *Epinephelus lanceolatus* (Bloch), barfin flounder, *Verasper moseri* Jordan & Gilbert, turbot, *Psetta maxima* (L.), guppy *Poecilia reticulata* Peters, tiger puffer, *Takifugu rubripes* (Temminck & Schlegel), haddock, *Melanogrammus aeglefinus* (L.), and flathead mullet, *Mugil cephalus* L. Five other betanodaviruses on the Uniprot database contain a single amino acid substitution within residues 181–212 where the class of substituted amino acid is conserved. Three further viruses isolated from striped jack and sea bass contain a lys-met substitution at residue 200, and one virus isolated from sea bass contains an ala-arg substitution at residue 196. It is possible that immune selection has influenced sequence variation in the region of the nodavirus genome that encodes coat protein residues 181–212.

The results of the present study should be interpreted with caution when applied to the development of nodavirus vaccines. Firstly, the epitopes identified in pepscan analyses represent those recognized by MAbs or serum samples with the greatest MFI values. However, these epitopes...
could function as immunological decoys, such as those identified on human immunodeficiency virus envelope glycoproteins gp120 and gp41 (Neurath, Strick & Lee 1990) and glycoprotein gp5 of porcine reproductive and respiratory syndrome virus (Fang, Jiang, Xiao, Niu, Zhang & Chen 2006). Secondly, the short 12-mer peptides used for mapping mimic linear epitopes are formed by contiguous stretches of amino acids. Consequently, they cannot detect antibodies that recognize conformational epitopes formed by complex three-dimensional protein folding. Many neutralizing antibodies that recognize viral structural proteins are directed against conformational epitopes (Wolinsky, McCarthy, Allen-Cannady, Moore, Jin, Cao, Lovett & Simmons 1991). However, linear epitopes identified in mapping studies have successfully been used to inform the design of viral vaccines and immunodiagnostic reagents (Langeveld, Casal, Osterhaus, Cortes, de Swart, Vela, Dalsgaard, Puijk, Schaaper & Meloen 1994; Wang, Chang, Walfeld, Ye, Shen, Zhang, Lubroth, Chen, Li, Lin, Jong, Yang, Chyr, Kramer & Brown 2001). Thus, the results of the present study highlight regions of the betanodavirus capsid protein that might serve as vaccine components with the objective of inducing protective immune responses. This experimentally testable hypothesis will be the subject of future investigation.

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