Infection competition against grouper nervous necrosis virus by virus-like particles produced in *Escherichia coli*

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Dragon grouper (*Epinephelus lanceolatus*) nervous necrosis virus (DGNNV) comprises 180 copies of capsid protein that encapsulate a bipartite genome of single-stranded (+)-RNAs. This study reports that virus-like particles (VLPs) are formed in *Escherichia coli* expressing the full-length ORF encoding the DGNNV capsid protein. Two sizes of VLPs are observed. The heavier particles resemble the native piscine nodavirus in size and stain permeability, while the lighter ones are approximately two-thirds of the full size. The recombinant VLPs block attachment of native virus to the surface of cultured fish nerve cells, blocking infection by the native virus.

**INTRODUCTION**

Piscine nodaviruses infect more than 20 species of fish around Asia (Skiliris et al., 2001), Europe (Grotmol et al., 1997; Starkey et al., 2001) and Australia (Munday et al., 1994). Infection causes nervous necrosis, encephalopathy and retinopathy, accompanied by abnormal swimming behaviour, dark colouring, and massive mortality in hatchery-reared larvae and juveniles (Munday & Nakai, 1997).

The piscine nodaviruses possess two positive RNA strands, ~3·1 kb RNA1 and ~1·4 kb RNA2. The nucleotide sequences of both genomic RNAs of striped jack nervous necrosis virus (SJNNV) (Nishizawa et al., 1995; Nagai & Nishizawa, 1999) and greasy grouper nervous necrosis virus (GGNNV) (Tan et al., 2001) have been determined. The sequences of the RNA2 of *Dicentrarchus labrax* encephalitis virus (DIEV) (Delsert et al., 1997), Atlantic halibut virus (AHV) (Grotmol et al., 2000) and two grouper viruses [malabaricus grouper (MGNNV) and dragon grouper (DGNNV)] (Lin et al., 2001) have also been determined. The larger genome strand, RNA1, presumably encodes the viral replicase, while the smaller genome strand, RNA2, has been demonstrated to encode the capsid protein (Lin et al., 2001).

By using the baculovirus expression system in *Spodoptera frugiperda* cell line SF21, our previous study demonstrated that MGNNV VLPs are small, nonenveloped T = 3 quasi-symmetric particles (Lin et al., 2001; Tang et al., 2002). The capsid protein of MGNNV spontaneously assembles into recombinant VLPs when expressed in SF21 cells infected with a recombinant baculovirus (Lin et al., 2001). The VLPs were indistinguishable from the native virus particles by electron microscopy. The three-dimensional structure of the VLPs has been determined to a resolution of 2·3 nm by electron cryomicroscopy reconstruction imaging (Tang et al., 2002). The VLPs and related mutants can be used to understand the structure of the virus. High purity of virus or VLPs in quantities of about 100 mg is required for determination of crystal structure by X-ray diffraction. *In situ* hybridization has been employed to study the infection path of the virus in fish nerve cells (Comps et al., 1996). The labelled virus or structure-analogue VLPs can be used to study the receptor on the nerve cells. Nevertheless, the quantities of native virus and VLPs from SF21 cells are limiting for crystallization and receptor-binding experiments. In contrast, *Escherichia coli* is the simplest host system for gene manipulation and has also been employed to express several viral VLPs (Qi et al., 1996; Bragard et al., 2000). This report is the first attempt to express nodavirus VLPs in *E. coli* and characterizes the ability of the VLPs to compete against binding of and infection by native DGNNV.

**METHODS**

**Constructing the full-length ORF encoding DGNNV capsid protein.** Plasmid pCA3, bearing the capsid protein gene, was constructed using the RNA template from dragon grouper (*Epinephelus lanceolatus*) nervous necrosis virus. The sequence of the capsid protein gene has been documented in GenBank (AF245004) and described by Lin et al. (2001).

The viral capsid protein ORF was inserted by PCR into the vector pQE30 (Qiagen), to give pDA8. The annealing conditions for the PCR reaction were 49 °C for 2 min. The primers were NW9a (5′-CAC AGA ATT CAT TAA AGA GGA GAA ATT AAC CAT GGT ACG CAA AGG T-3′), and the template was pCA3. The PCR product was digested with EcoRI and KpnI and ligated into pQE30. The Ncol site of pQE30 was filled-in prior to ligation. This resulting plasmid, pD8, contained an *E. coli* ribosome-binding site (AGGAG) and a full-length DGNNV capsid protein ORF, flanked by an IPTG-inducible promoter (T5pr) and the
terminators of λpa and rrnB T1. The junction sequence downstream of the promoter is T5pr-ATT AAA GAG GAG AAA TTA ACC ATG, while the junction at the 3’ end is DGNNV 3’UTR-GGTTAC-cgg-T1. The JM109 strain of E. coli was used for cloning and expression of the capsid protein.

**VLP production and isolation.** A 150 ml volume of seed culture grown overnight at 30 °C was inoculated into a 15 l culture (New Brunswick Scientific; BioFlo IV). The medium was LB-broth supplemented with 100 µg ampicillin ml⁻¹. When the cell density reached an OD₆₀₀ of 0.2–0.3, 0.84 mM IPTG was added for induction. After induction for 2 h, cells were harvested by centrifugation (5150 g, 4 °C, 20 min), suspended in 100 ml 10 mM Tris buffer (pH 8.0) and flushed through a French press (Avestin EmulsiFlex-C5) three times. Cell debris in the lysate was immediately removed by centrifugation at 39 500 g for 20 min (Hitachi CR20BZ). Collected supernatant was ultracentrifuged at 300 000 g for 45 min (Hitachi SCP70G) against a 30 % sucrose cushion. The VLP pellet was suspended in 400 µl of 10 mM Tris buffer (pH 8.0) and further purified using a 20–38 % (v/v) CsCl gradient (Hitachi SCP70G), 100 000 g for 12 h. Fractions of various buoyancies were collected and examined by SDS-PAGE; bands were visualized with Coomassie brilliant blue.

**Electron microscopy.** Purified VLPs (4 µl: 1·5 mg ml⁻¹) or native DGNNV virus particles were applied onto Parafilm to produce a spherical drop. Carbon-coated nitrocellulose films were fabricated on copper grids and placed face down on the sample drops for 1 min to absorb the particles. After being briefly washed twice in 10 µl 10 mM Tris buffer (pH 8.0), the samples were then rinsed twice in 1 % sodium phosphotungstate (PTA), and negatively stained using 1 % PTA for 1·5 min. The grids were blotted with filter paper until they were almost dry following each step of absorption, washing and PTA staining. The finished grids were dried in vacuo for 6 h. Micrographs were obtained using an electron microscope (JEOL JEM-1200EX, Japan).

**RNA extraction from native virus particles and VLPs.** RNA was extracted from purified particles using acidic phenol/chloroform in the presence of 0·2 M NaCl and 0·1 % SDS. RNA in the aqueous phase was precipitated in the presence of 0·3 M sodium acetate (pH 5.2) and 1 µg glycogen, washed with 70 % ethanol, dried and resuspended in nuclease-free water. Purified RNA (1–1·5 µg) was electrophoresed through a 1·3 % agarose/formaldehyde gel in 1 x MOPS buffer (20 mM MOPS, 8 mM sodium acetate, 1 mM EDTA, pH 7·0) at 40 V for 2·5 h, and visualized with ethidium bromide. The RNA marker was purchased from Gibco BRL (cat. #15620-016).

**Inhibition of CPE by VLPs.** SSN-1 cells were cultured in an 8–8 cm² dish (Nunc) at 27 °C using Leibovitz L-15 medium (Gibco BRL), supplemented with 10 % FBS (foetal bovine serum). Monolayer cells (80–90 % confluent) were washed with D-PBS (2·67 mM potassium chloride, 1·47 mM potassium phosphate, 138 mM sodium chloride, 8·1 mM sodium phosphate, pH 7·3) and incubated with 5 ng VLPs at 27 °C for 30 min. The cells were infected with virus (m.o.i. 10, 27 °C for 30 min) and cultured in L-15 medium supplemented with 2 % FBS at 27 °C. CPE was monitored daily for 4 days.

**Labelling virus and VLPs with FITC.** The virus and VLP Fraction II were suspended in 50 mM sodium carbonate and 100 mM sodium chloride containing 0·5 mg FITC ml⁻¹ (pH 8·0) (Sigma) as described by Weingart et al. (1999). FITC labelling was carried out in the dark at room temperature for 1 h. The FITC-conjugated particles were pelleted at 300 000 g for 1 h and washed twice in D-PBS (2·67 mM potassium chloride, 1·47 mM potassium phosphate, 138 mM sodium chloride, 8·1 mM sodium phosphate, pH 7·3) supplemented with 0·25 % bovine serum albumin (Gibco BRL).

**Attachment competition at the cell surface between VLPs and virus.** Competition of VLPs with virus was determined as described by Georgi et al. (1990) and White et al. (1996). SSN-1 cells (10⁵) were attached overnight onto a coverslip in a 24-well culture plate. The samples were chilled on ice briefly and the medium was removed. The cells were washed with ice-cold Dulbecco’s (D)-PBS and incubated with VLPs (~10⁵ particles per cell) at 4 °C for 1 h. After washing three times in ice-cold D-PBS, the cells were treated with FITC-labelled virus at 4 °C for 1 h, respectively. All samples were washed three times in ice-cold D-PBS to rinse off unbound particles and then transferred to 3 % glutaraldehyde in D-PBS for 15 min. The fixed samples were washed with D-PBS and observed by fluorescence microscopy (Olympus BX40).

## RESULTS AND DISCUSSION

**Formation of virus-like particles**

VLP formation from various viral capsid proteins expressed in E. coli has been reported elsewhere (Wizemann & Brunn, 1999; Bragard et al., 2000). To examine the possibility of forming DGNNV VLPs in E. coli, cDNA of the DGNNV capsid protein was cloned using standard protocols. The resulting DNA plasmid, pDAB, is described in Methods. The VLPs in the cell lysate were purified by a series of centrifugations and fractionated in a CsCl gradient. Two visible bands appeared at a density of 1·27 g cm⁻³ (Fraction I) and 1·34 g cm⁻³ (Fraction II) (Fig. 1A). The concentration of Fraction I was approximately 5 % of Fraction II, estimated by UV absorbance (A₂₈₀). The molecular mass of the proteins (~37 kDa) in both fractions was apparently the same as the native virus in SDS-PAGE (Fig. 1B). Bands of ~110 kDa appeared in all samples of native virus and VLPs, suggesting that small amounts of putative trimers still survived the SDS treatment. Heterogeneous VLPs existed in both fractions. Electron microscopy of VLPs in Fraction I showed that the small particles were about 23 nm in diameter, a few of which were irregular (Fig. 2A). In contrast, Fraction II consisted of equal amounts of intact and stain-permeable particles with a diameter of about 30 nm (Fig. 2B). Comparing these particles revealed that the heavy VLPs had a similar size and geometry to the native piscine nodavirus (Fig. 2C) and SS21-expressed VLPs (Fig. 2D). Interestingly, the surfaces of both native particles and VLPs are rougher than that of the insect nodavirus flock house virus (Lin et al., 2001). Similarly two types of Dicentrachus labrax encephalitis virus from the brain of diseased D. labrax have been reported (Breuil et al., 1991; Comps et al., 1994).

**RNA contents in VLPs**

Piscine and insect nodavirus VLPs are known to package random cellular RNAs when assembled in SS21 cells (Schneemann et al., 1993; Lin et al., 2001). E. coli-expressed VLPs were assumed to behave similarly. To examine this possibility, RNA from particles in Fractions I and II was extracted with acidic phenol and chloroform and analysed on a 1·3 % agarose/formaldehyde gel.

Native DGNNV virus contains two unique RNA species, RNA1 and RNA2, whereas the VLPs encapsulate variant
sizes of RNAs. The VLPs in Fraction I contained a smear of RNAs, ranging in size from about 200 to 1300 bases (Fig. 3, lane 2). VLPs in Fraction II contained a highly heterogeneous mixture of RNAs, ranging in size from about 200 to 4000 bases (Fig. 3, lane 3). Similar observations have been reported for insect and piscine nodavirus VLPs.

Fig. 1. Different types of VLPs produced in E. coli expressing the full-length capsid protein of DGNNV. (A) Two fractions of VLPs were observed in CsCl gradient purification of the bacterial cell lysate. (B) SDS-PAGE analysis revealed that the VLPs in Fractions I (lane 1) and II (lane 2) were packaged by a single ~37 kDa protein – the same molecular mass as the native DGNNV particles (lane 3). The capsid protein monomer is marked ‘CP’ and the putative trimer ‘Tri’.

Fig. 2. Electron micrographs of VLPs that assembled in E. coli expressing the full-length capsid protein of DGNNV. (A) Fraction I contains two small types of VLPs – irregularly shaped (top panel) and intact icosahedrons (bottom panel). (B) Fraction II contains two larger types of particles (indicated by arrows) – stain-permeable and intact. (C) The native DGNNV particle has a vivid rough surface. (D) VLPs obtained from Sf21 cells expressing MGNNV capsid protein on a baculovirus vector (Lin et al., 2001). Bars represent 50 nm.
expressed in the baculovirus system (Schneemann et al., 1993; Dong et al., 1998; Lin et al., 2001). The size limit of RNAs in VLPs probably corresponds to the largest single RNA molecule that can be packaged in the native virus particles. The heterogeneous RNA packaging might reflect the electrostatic requirements of assembly, which is mediated by the basic N-terminal residues of the capsid protein. The space inside the capsid protein shell restricts size and quantity of the occupants. Small VLPs in Fraction I indicate that the coat protein formed $T=1$ VLPs with short RNAs (Dong et al., 1998). The content of packaged RNAs results in different sedimentation density in Fractions I and II.

**VLPs compete against infection by native DGNNV**

The surface properties of VLPs can be characterized by their ability to compete with infection by the native virus. The first step of infection is attachment to the cell membrane. CPE is then observed when the virus multiplies in the host cells. As a viral structure analogue, VLPs should be able to specifically occupy virus receptor sites and block entry of the virus into the cell. Therefore, occurrence of CPE and attachment to the cell surface were employed to test the structural properties of the DGNNV VLPs.

CPE resulting from native virus infection was characterized by the formation of a vacuole in the cytoplasm and the disintegration of cells 4 days post-infection (p.i.) (Fig. 4B). In the presence of VLPs, cells were protected from invasion by the virus and retained almost complete integrity at 4 days p.i. (Fig. 4C). As observed in a fluorescent microscope, the cell surface did not bind FITC-labelled virus in the presence of VLPs, while FITC-labelled virus and VLPs alone attached to the cell surface (Fig. 5). These results suggest that the VLPs formed in *E. coli* can bind to the surface protein of cell membrane, and block early virus–cell recognition and the effective internalization of the virus. The virus receptor on the cell membrane can specifically bind both native virus and VLPs. The outer shell of DGNNV VLPs expressed in *E. coli* is thus structurally indistinguishable from native virus.

To our knowledge, this is the first report that the piscine nodavirus capsid protein can form VLPs in *E. coli*. The VLP
expression system in *E. coli* will be used for structural comparison to elucidate the correlation between the size/shape and the RNA content. These mutant VLPs will be employed to study their structure variation by using electron cryomicroscopy and X-ray diffraction. High similarity in structure implies that the VLPs could induce a strong immune response against virus. In addition to their structural and immunological properties, DGNNV VLPs with no virus genome are not infectious, therefore qualifying as a safe and efficient vaccine (Conner et al., 1996; Roy, 1996). Future experiments on VLPs with a variety of mutant RNA1 and RNA2 species will also help further understanding of the recognition and binding between the RNAs and capsid protein.

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