



# Induction of caspase-dependent apoptosis by betanodaviruses GGNNV and demonstration of protein $\alpha$ as an apoptosis inducer

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## Abstract

Betanodaviruses, members of the Nodaviridae family, are the causative agents of viral nervous necrosis in fish and infection by which cause high mortality in larvae and juveniles in a wide range of marine fish species in Asia, Europe, Australia, Martinique, and Tahiti. Greasy grouper (*Epinephelus tauvina*) nervous necrosis viruses (GGNNV) were investigated for their apoptotic activity in culture cells. GGNNV infection of sea bass (SB) cells appeared to induce a typical cytopathic effect (CPE), i.e., cytoplasmic vacuolation, thinning, rounding up, detachment of infected cells from the cultured dish, and eventually cell lysis and death. The infected SB cells underwent DNA fragmentation and stained positive in terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay, suggesting that GGNNV infection induced apoptosis in SB cells. In addition, GGNNV-infected SB cells showed an increased activity of caspase-8-like proteases (IETDase) and caspase-3-like proteases (IETDase), whereas inhibitor of caspase-8 and caspase-3 reduced GGNNV-induced apoptosis. This suggests that GGNNV may promote apoptosis via the extrinsic pathway in SB cells. Protein  $\alpha$ , the precursor of GGNNV capsid proteins, was transiently expressed in SB and Cos-7 cells. The DNA fragmentation and TUNEL positive signal were apparent in SB and Cos-7 cells expressing protein  $\alpha$ , suggesting that protein  $\alpha$  may serve as an apoptotic inducer in these cells. Moreover, expression of protein  $\alpha$  resulted in the activation of caspase-3-like proteases in both cells, which could be inhibited by a caspase-3-like protease specific inhibitor DEVD-CHO peptide. These results suggest that fish caspases are important elements in GGNNV-mediated apoptosis.

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**Keywords:** Betanodavirus greasy grouper (*Epinephelus tauvina*) nervous necrosis viruses (GGNNV); Protein  $\alpha$ ; Apoptosis; Caspase-3-like protease (DEVDase) activity; Caspase-8-like protease (IETDase) activity; Sea bass cells; Cos-7 cells

## Introduction

Apoptosis or programmed cell death (PCD) is the process whereby an individual cell of multicellular organisms undergoes systematic self-destruction in response to a wide variety of stimuli. During this process, cells display characteristic morphological changes, including chromatin condensation, plasma membrane blebbing, cell shrinkage, and fragmentation into membrane-bound bodies (Majno and Joris, 1995; Vaux et al., 1994; Wyllie et al., 1980). There

are two different mechanisms by which a cell commits suicide by apoptosis. One is generated by signals arising within the cells, which involves B cell lymphoma-2 (Bcl-2) and subsequently leads to the release of apoptosis protease activating factor-1 (Apaf-1) and cytochrome *c*. Then the released cytochrome *c* and Apaf-1 bind to caspase-9 and other molecules to form the apoptosome. The sequential activation of one caspase by another creates an expanding cascade of proteolytic activity, which leads to digestion of structural proteins in the cytoplasm and degradation of chromosomal DNA. The other mechanism is triggered by death activators outside the cell, which bind to receptors at the cell surface, such as Fas (CD95 antigen) and tumor necrosis factor (TNF) receptors, and transmit a signal to the cytoplasm that leads to activation of caspase-8, which initiates a cascade of caspase activation to cause the formation of

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membrane-bound apoptotic bodies (Adams and Cory, 1998; Everett and McFadden, 2001; Jagus et al., 1999; Lee et al., 1997; Li et al., 1997; Teodoro and Branton, 1997).

In recent years, many viruses in different families have been found to induce apoptosis during their infection cycles. Host cells defend themselves from viral infection by apoptosis, but viruses have also developed a range of strategies to fight against the host immune response and apoptosis; they even make use of apoptosis to propagate. For some viruses, inhibition of apoptosis appears to be essential for the maintenance of viral latency. But for many other viruses, the careful induction of apoptosis during lytic infection may represent the basis for cytotoxicity and be an important outlet for dissemination of progeny virus (Hardwick, 1998; Roulston et al., 1999). The process of apoptosis is controlled by a large number of genes, many of which have been identified from viral genomes (Teodoro and Branton, 1997).

Nodaviruses are small, nonenveloped, spherical viruses with bipartite positive-sense RNA genomes, which are capped but not polyadenylated. Two genera have been distinguished within the Nodaviridae family: the alphanodaviruses that were originally isolated from insects and the betanodaviruses that infect a wide variety of larval and juvenile marine fish (Ball et al., 2000). The fish disease caused by betanodavirus is spreading worldwide, resulting in severe morbidity and mortality and significant economic losses to the aquaculture industry. Infected fish showed abnormal swimming behavior, such as erratic swimming in circles, or to the right, lying on their sides or belly up. Histopathological changes are characterized by extensive cellular vacuolations and neuronal degeneration in the central nervous system and retina (Delsert et al., 1997; Grotmol et al., 1999; Mori et al., 1992; Munday, 1997; Nakai et al., 1994). Despite their greater economic impact, the viruses in this genus are less studied. Therefore, it is essential to characterize their molecular biology to understand the mechanism of their infection.

GGNNV (greasy grouper (*Epinephelus tauvina*) nervous necrosis viruses) of the Singapore strain, a member of betanodaviruses, contain two genomic RNA segments: complete nucleotide sequences of RNA1, 3103 nt (GenBank Accession number AF319555), and RNA2, 1433 nt (GenBank Accession number AF318942), which have been determined in our previous work (Tan et al., 2001). RNA1 gene encodes a 110 kDa protein A, which is an RNA-dependent RNA polymerase and RNA2 encodes a 37 kDa protein  $\alpha$ , which is the precursor of capsid proteins  $\beta$  and  $\gamma$ . Two putative ORFs of protein B1 (111 aa) and protein B2 (75 aa) were also confirmed. Protein B1-encoding region is in the same reading frame as protein A. Protein B2-encoding region is in the +1 reading frame with respect to protein A and overlaps the C terminal of protein A almost entirely. The functions of putative 11 kDa protein B1 or 8.5 kDa protein B2 are still unknown. A sensitive method to diag-

nose GGNNV infection with recombinant protein  $\alpha$  has been established (Huang et al., 2001).

In this study, typical cytopathic effect (CPE), i.e., cytoplasmic vacuolation, fusion, rounding up, detachment of infected cells from the cultured dish, and eventually cell lysis and death were observed in GGNNV-infected SB cells. To understand if there is a relationship between GGNNV infection and apoptosis, a time course study of GGNNV infection was performed and GGNNV was demonstrated to be capable of inducing apoptosis in infected SB cells as indicated by the DNA ladder and TUNEL assays. Furthermore, transient expression of protein  $\alpha$  revealed that protein  $\alpha$  triggered apoptosis in transfected SB and Cos-7 cells. To further study the apoptotic signaling pathway utilized by GGNNV and protein  $\alpha$ , their effect on caspase-3-like protease (DEVDase) activity and caspase-8-like protease (IET-Dase) activity was examined.

## Results

### *Expression of GGNNV protein A and $\alpha$ in *E. coli* and characterization of antibodies against GGNNV viral proteins*

Protein A was expressed in *E. coli* using the pGEX-4T-2 vector which includes a glutathione *S*-transferase (GST) tag and is under the control of the tac promoter. Protein  $\alpha$  was expressed in *E. coli* using the pQE-30 vector which contains a 6X His-tag coding sequence and is under the control of the T5 promoter. The recombinant plasmids pGEX-4T-2/RNA1 and pQE30/RNA2 were transformed into *E. coli* JM 105, induced with 1 mM isopropyl-D-thiogalactopyranoside (IPTG) (final concentration) at midexponential growth phase, and incubated for 4 h. The recombinant proteins were efficiently synthesized and demonstrated to have the respective sizes of 136.4 (Fig. 1a, lane 2) and 37 kDa (Fig. 1a, lane 4) on Coomassie brilliant blue stained SDS-PAGE gel. The recombinant proteins of proteins A and  $\alpha$  were then purified and used to immunize guinea pig to obtain antibody against each protein. To confirm the monospecificity of each antibody, GGNNV-infected SB cells were used as viral antigens to perform Western-blotting analyses. Consistent with the reported sizes of protein A and  $\alpha$  (Tan et al., 2001), proteins of 110 and 37 kDa were detected by the two different antisera, respectively (Fig. 1b, lane 1 and 2), whereas no signals were detected when the control sera (Fig. 1b, lane 3) were reacted with extracts from the infected SB cells. In addition, no signals were detected when extraction from mock-infected SB cells (Fig. 1b, lane 4) was reacted with the mixture of both sera. These results indicated that the antibodies against proteins A and  $\alpha$  were monospecific and could be used as a tool to carry out the following experiments.

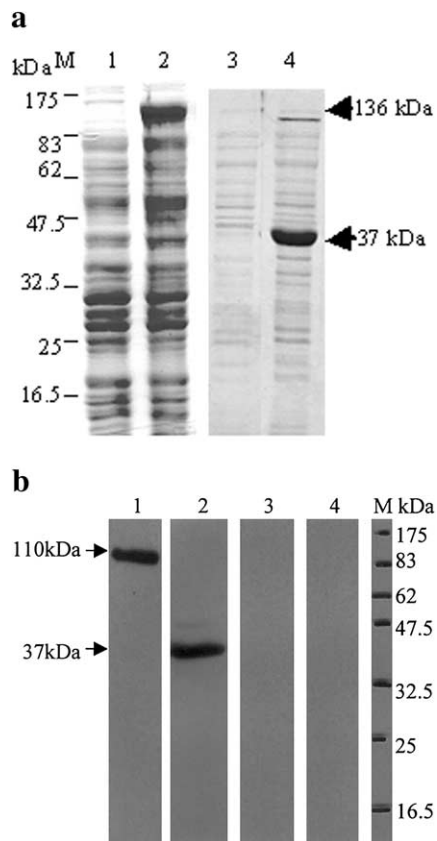


Fig. 1. (a) Expression of GGNNV proteins A and  $\alpha$  in prokaryotic expression system. Total cell lysates induced by IPTG showing the expression of recombinant protein A (lane 2) and  $\alpha$  (lane 4) were electrophoresed in a 12% SDS-PAGE and stained with Coomassie brilliant blue. Lanes 1 and 3 showed noninduced control. M indicates the molecular mass in kilodaltons. (b) Immunoblotting assay of GGNNV proteins A and  $\alpha$ . Total cell lysates of GGNNV-infected SB cells were transferred to nitrocellulose membrane and detected by the mono-specific antibodies against proteins A (lane 1) and  $\alpha$  (lane 2), respectively. Control sera (lane 3) and lysates from uninfected SB cells (lane 4) were used as negative control. M indicates the molecular mass in kilodaltons.

#### Induction of apoptosis by betanodaviruses (GGNNV) in cultured cells

The typical cytopathic effects (CPE) characterized by cytoplasmic vacuolation, fusion, rounding up, and detachment of infected cells from the cultured dish were first detected in betanodavirus (GGNNV)-infected SB cells at 24 h postinfection (PI) and the extent of CPE gradually increased by 48 h PI (Fig. 2, image C). To investigate if GGNNV kills cells by apoptosis and if there is a correlation between CPE and apoptosis, the membrane permeable DNA-binding dye Hoechst 33342 was used to stain the nuclei of the infected cells to see any gradual morphological change of the nuclei. As shown in Fig. 2, image D, the nuclei became distorted and condensed in GGNNV-infected cells at 48 h PI, whereas in mock-infected cells (Fig. 2, image B), the nuclei remained uniformly stained, suggesting

that apoptosis might be triggered in SB cells during GGNNV infection.

Cellular genomic DNA was extracted from the GGNNV infected SB cells at 24, 36, 48, 60, and 72 h PI. As shown in Fig. 3a, DNA fragmentation was observed as early as 24 h PI, and the ladder was not evident until 48 h PI; at 60 h PI and 72 h PI (Fig. 3a, lanes 3, 3–7), the number of fragments increased remarkably. Meanwhile, no ladder was observed in mock-infected cells (Fig. 3a, lane 2). These data demonstrated that extensive internucleosomal DNA cleavage occurred late in GGNNV-infected SB cells. The TUNEL assay was performed to further confirm DNA fragmentation in GGNNV-infected SB cells. TUNEL-positive cells began to appear at 24 h PI (Fig. 3b, image B), and the number of positive cells increased gradually. At 48 h PI, the positive signal was strongest (Fig. 3b, images C and D). No positive signal was found in mock-infected cells (Fig. 3b, image A). In addition, when the cells were treated with DEVD-CHO, a caspase-3 specific inhibitor, the number of TUNEL positive cells dramatically decreased at 48 h PI (Fig. 3b, image E).

#### Betanodavirus GGNNV-induced caspase-8 like protease activity in SB cells

Apoptotic death is known to involve a cascade of proteolytic events accomplished mainly by caspases. Caspase-8, the first caspase in the CD95 apoptotic pathway, directly or indirectly initiates the proteolytic activities of downstream effector caspases, such as caspase-3, caspase-6, and caspase-7. To determine if GGNNV induces apoptosis in fish cells through the CD95 apoptotic pathway, infected SB cells were harvested at 24 h PI and 48 h PI, and cell lysates were analyzed for IETDase activity, using IETD as the

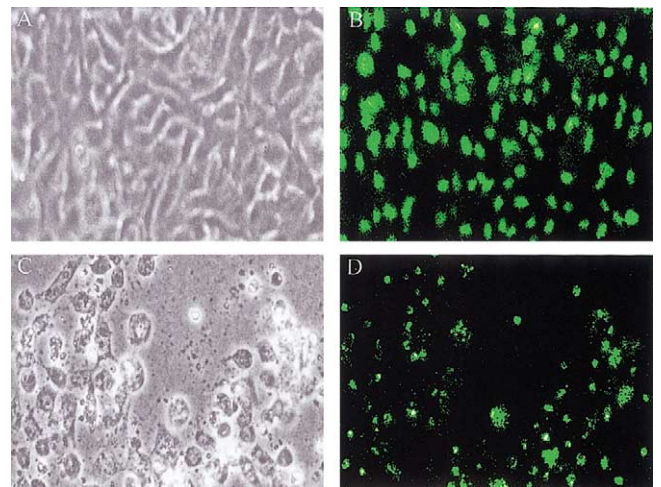


Fig. 2. CPE and nuclear morphology of GGNNV-infected SB cells. GGNNV infected (D) and mock-infected SB cells (B) were stained with Hoechst 33342 at 48 h post-infection to visualize nuclear morphology. C shows the CPE at 48 h PI. A refers to mock-infected cells at 48 h. Magnification, X200.

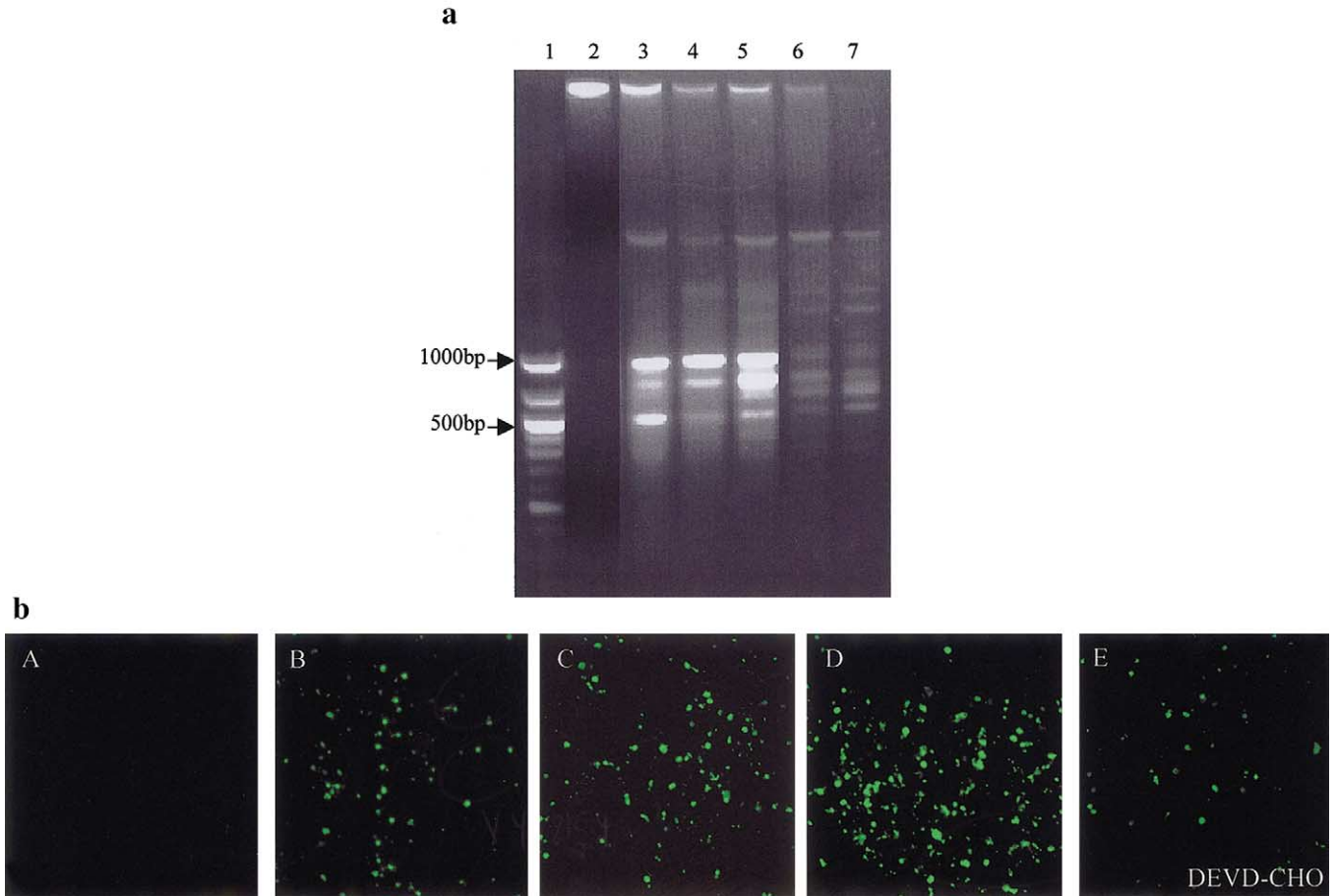


Fig. 3. (a) Time course study of betanodavirus GGNNV-induced fragmentation of cellular DNA in SB cells. DNA was extracted from GGNNV-infected SB cells and electrophoresed on 2% agarose gel. Lanes 3 to 7 indicated DNA extracted from cells at 24, 36, 48, 60, and 72 h PI, respectively. Lane 2 refers to mock-infected cells at 60 h PI. Lane 1 contains the 100 bp DNA size marker. (b) TUNEL assay showing apoptosis in GGNNV infected SB cells. TUNEL assay was performed at 24 h (B), 36 h (C), and 48 h (D and E) PI. Mock-infected cells (A) were checked at 48 h PI. Green indicates TUNEL positive cells. Image E shows that SB cells were treated with DEVD-CHO. Cells were observed under a confocal microscope and photographed in 250X magnification.

substrate for caspase-8. As shown in Fig. 4, the IETDase activity in lysates from infected SB cells was 6.5 nmol/h at 24 h PI and increased to 10.5 nmol/h at 48 h PI. No significant IETD activity was observed in mock-infected cells. Furthermore, GGNNV-mediated activation of caspase-8 was inhibited by IETD-fmk, a caspase-8 specific inhibitor.

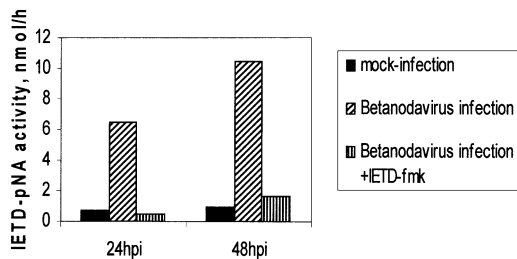


Fig. 4. Betanodavirus GGNNV infection induced activation of caspase-8-like proteases in SB cells. Cell lysates were harvested at 24 and 48 h PI and assayed for IETDase activity using caspase-8 colorimetric substrate IETD-pNA. Simultaneously, Mock-infected SB cells were used as a negative control. Values shown are means from duplicate experiments.

#### *Betanodavirus GGNNV-induced activation of caspase-3-like proteases in SB cells*

To evaluate the possible involvement of downstream effector caspases in apoptosis triggered by GGNNV infection in fish cells, infected SB cells were harvested at 24 PI and 48 h PI, and the cell lysates were analyzed for DEVDase activity, using DEVD as the substrate for caspase-3. As shown in Fig. 5, the DEVDase activity in lysates from infected SB cells was 3.2 nmol/h at 24 h PI and increased to 15 nmol/h at 48 h PI. There was no significant DEVDase activity in mock-infected cells. In addition, when infected cells were treated with DEVD-CHO, reduction of DEVDase activity in GGNNV-infected cells was observed.

#### *Identification of protein $\alpha$ as an apoptotic inducer*

To determine which protein encoded by betanodavirus GGNNV might be responsible for inducing apoptosis, the transient expression of viral proteins A and  $\alpha$  in SB and

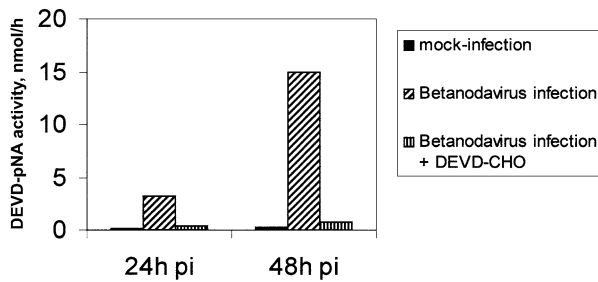


Fig. 5. Betanodavirus GGNNV infection induced activation of caspase-3-like proteases in SB cells. Cell lysates were harvested at 24 and 48 h PI and assayed for EDVDase activity using caspase-3 colorimetric substrate DEVD-pNA. Simultaneously, Mock-infected SB cells were used as a negative control. Values shown are means from duplicate experiments.

Cos-7 cells was carried out. pEGFP-RNA1 and pEGFP-RNA2 were transfected independently to SB or Cos-7 cells and the expression was confirmed by Western-blot assay using antibodies against protein A (Fig. 6, lanes 1 and 4) or  $\alpha$  (Fig. 6, lanes 2 and 5). Pooled guinea pig anti-protein A and  $\alpha$  antibodies failed to detect any viral proteins in cells transfected with pEGFP-C1 vector (Fig. 6, lanes 3 and 6). At 48 h post-transfection, the cellular genomic DNAs were extracted and analyzed on 2% agarose gel electrophoresis. Distinct DNA ladder was detected in DNA samples from SB and Cos-7 cells transfected with pEGFP-RNA2 (Fig. 7a, lane 2), but no DNA fragmentation could be observed from cells transfected with pEGFP-C1 or pEGFP-RNA1 (Fig. 7a, lanes 3 and 4, respectively). The apoptosis caused by protein  $\alpha$  was further demonstrated by TUNEL labeling. As shown in Fig. 7b, a great number of TUNEL-positive cells characterized by brown nuclei were detected in pEGFP-RNA2 transfected SB and Cos-7 cells at 48 h post-transfection (Fig. 7b, images A and C). In contrast, no brown nucleus signals were observed in the cells transfected with pEGFP-C1 vector (Fig. 7b, images B and D).

#### Protein $\alpha$ induced activation of caspase-3 like proteases in SB and Cos-7 cells

To determine whether the activation of caspase-3 like proteases occurred in the expression of protein  $\alpha$ , both SB and Cos-7 cells were transfected with pEGFP-RNA2 or pEGFP-C1, and lysates of transfected cells were assayed for DEVDase activity at 24 h post-transfection. As shown in Fig. 8, protein  $\alpha$  induced an activation of caspase-3-like proteases characterized by 2.8 and 4.2 nmol/h DEVDase activity in SB cells and Cos-7 cells, respectively. There was no significant DEVDase activity in SB and Cos-7 cells expressing EGFP. In addition, when protein  $\alpha$  transfected cells were treated with DEVD-CHO, reduction of DEVDase activity in protein  $\alpha$ -induced apoptosis in both cells was observed.

## Discussion

The interaction between viruses and host cells is very complex. Host defense mechanism attempts to limit the virus replication by killing infected cells (Mounira et al., 1998). On the other hand, many viruses have evolved mechanisms to cope with cellular antiviral responses. One of these viral strategies is triggering apoptosis during infection. In the case of virus-infected cells, apoptosis seems to represent a very efficient mechanism by which the virus can induce cell death and disseminate progeny while limiting induction of inflammation. The presence of progeny virions in membrane-bound bodies also protects them from contact with neutralizing antibodies. A lot of viruses, such as Adenoviridae, Papovaviridae, Herpesviridae, etc, have been shown to use apoptosis to kill the infected cells at the end of infection to spread the viral progeny to nearby cells (Krajcsi and Wold, 1998; Roulston et al., 1999; Teodoro and Branton, 1997).

This is the first report showing that GGNNV is capable of inducing apoptosis. This is demonstrated by several methods including analysis of total cellular DNA for low molecular weight DNA by agarose gel electrophoresis, TUNEL labeling for detection of DNA fragmentation in situ, and Hoechst 33342 staining for nuclear morphology. There was general agreement of the results: in the analysis of total cellular DNA, DNA fragmentation bands were detected at 24 h PI, which peaked at 48 h PI in GGNNV-infected cells. Also, the TUNEL positive signal was much stronger at 48 h PI than at 24 h PI. Similarly, IETDase and DEVDase activity induced by GGNNV infection was much higher at 48 h PI than at 24 h PI. These results suggested that there is a correlation between appearance of apoptosis and CPE that peaked at 48 h PI.

Caspases are the central players in apoptosis. At least two major pathways for caspase activation have been delineated, including a pathway linked to the TNF family of death receptors (“extrinsic”) and a pathway activated by mitochondria (“intrinsic”). The apical proteases in the ex-

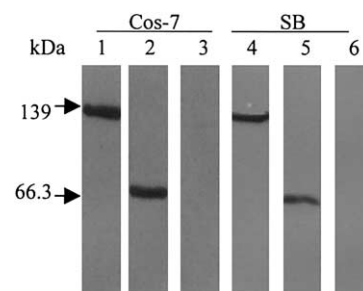


Fig. 6. Immunoblotting assay showing the expression of proteins A and  $\alpha$  in eukaryotic expression system. Cell lysates from Cos-7 and SB cells transfected with pEGFP-RNA1 and pEGFP-RNA2 were electrophoresed in a 12% SDS-PAGE, transferred to nitrocellulose membrane, and detected by the monospecific antibodies against proteins A (lanes 1 and 4) and  $\alpha$  (lanes 2 and 5), respectively. Lysates from cells transfected with pEGFP-C1 (lanes 3 and 6) were indicated as negative control.

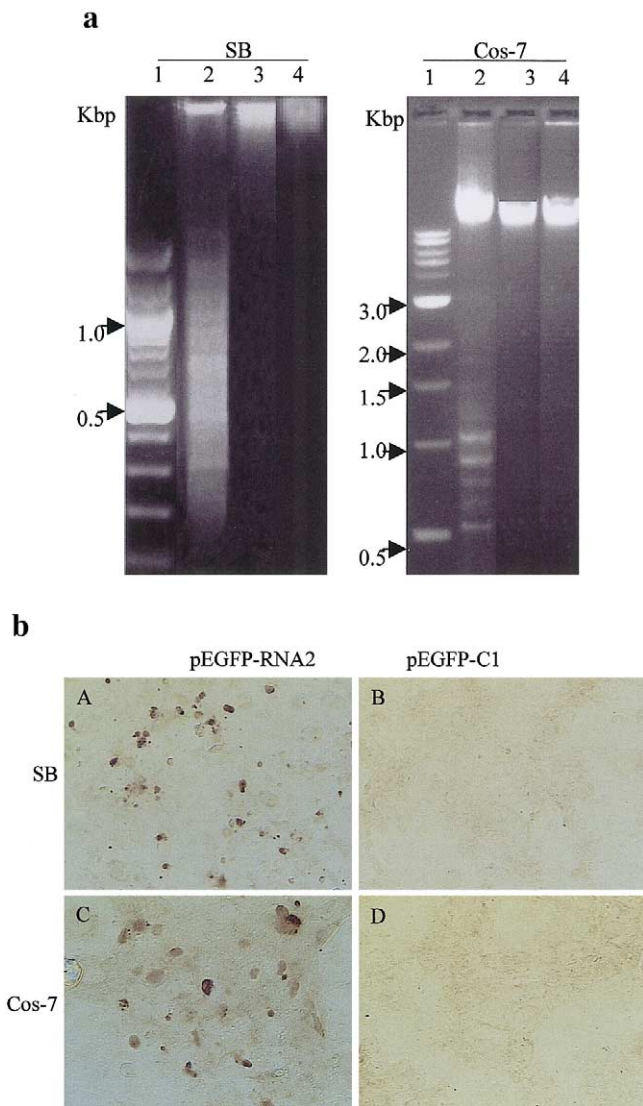


Fig. 7. (a) Cellular DNA fragmentation induced by transiently expressing protein  $\alpha$  in SB and Cos-7 cells. DNA samples were extracted from cells transfected with pEGFP-RNA2 (lane 2) pEGFP-RNA1 (lane 3) and pEGFP-C1 (lane 4) at 48 h post-transfection and DNA fragmentation were detected by 2.0% agarose gel electrophoresis. Lane 1 indicates the DNA size marker. (b) Detection of apoptosis in SB and Cos-7 by TUNEL-labeling. SB or Cos-7 cells were transfected with plasmid pEGFP-RNA2. At 48 h post-transfection, the cells were fixed and analyzed for detection of apoptosis by TUNEL staining, which detects the DNA breakage (brown color). A and C indicate TUNEL-labeling of Cos-7 and SB cells that were transfected with EGFP-RNA2 at 48 h post-transfection, respectively. B and D indicate Cos-7 and SB cells that were transfected with pEGFP-C1 at 48 h post-transfection, respectively. Magnification X200.

trinsic and intrinsic pathways are caspase-8 and caspase-9, respectively (Salvesen and Dixit, 1997). The results of high activity of IETD-pNA which can be inhibited by IETD-fmk in GGNNV-infected SB cells suggested that GGNNV triggers apoptosis of SB cells primarily through mechanisms that engage the extrinsic pathway at or above the level of caspase-8. Caspase-8 directly or indirectly initiates the proteolytic activities of downstream effector caspases, such as

caspase-3. When downstream “effector” caspase-3 is activated by apoptotic stimuli, it activates a caspase-activated DNase (CAD), which is present in the cytosol complexed with its inhibitor of CAD (ICAD). Caspase-3 cleaves ICAD and allows CAD to translocate to the nucleus and degrade DNA (Janicke et al., 1998). In fact, many viruses induce caspase-dependent apoptosis, such as murine coronavirus mouse hepatitis virus (MHV) (An et al., 1999), Langat flavivirus (LGTV) (Prihod’ko et al., 2001), and poliovirus (Agol et al., 1998). The results that GGNNV infection induced activation of caspase-3-like proteases and GGNNV-induced apoptosis could be inhibited by DEVD-CHO indicate that fish caspases are important mediators of virus-induced cell death.

It has been known that many viral proteins are involved in both the induction and suppression of apoptosis. Rubella virus capsid protein (Duncan et al., 2000), VP2 of IBDV (Fernandez-Arias et al., 1997), and E protein of LGTV (Prihod’ko et al., 2001) have been shown to induce apoptosis alone in cell culture and play an important role in viral pathogenesis. Other viruses, including adenoviruses, simian virus 40, and human papillomavirus, etc. have evolved a myriad of proteins to inhibit various steps in the inflammatory and apoptotic response pathways to protect infected cells from early death (Roulston et al. 1999). To localize possible inducers of apoptosis in betanodavirus GGNNV infection, we transiently expressed the viral protein A and protein  $\alpha$  in SB and Cos-7 cells and found that protein  $\alpha$  was capable of inducing apoptosis in both kinds of transfected cells. Protein  $\alpha$  is the precursor of capsid proteins and is important for viral assembly. Here, protein  $\alpha$  of GGNNV was demonstrated to be capable of inducing apoptosis by DNA fragmentation and TUNEL staining in transfected SB and Cos-7 cells at 48 h post-transfection. In addition, TUNEL positive signal was dramatically decreased when cells were treated with DEVD-CHO (data not shown). The expression of protein  $\alpha$  might trigger the in-

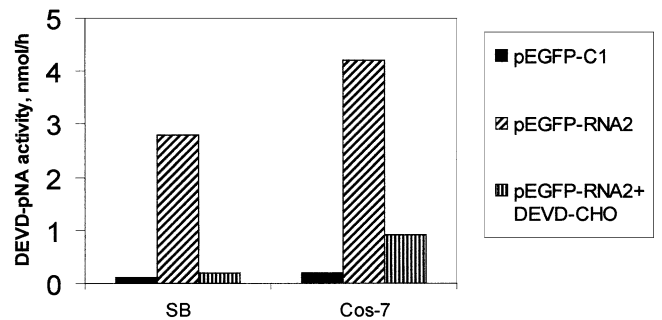


Fig. 8. Protein  $\alpha$  induces activation of caspase-3 proteases in both SB and Cos-7 cells. Cell lysates from SB or Cos-7 cells transfected with plasmids pEGFP-RNA2 or pEGFP-C1 were harvested at 24 h post-transfection and assayed for DEVDase activity using the caspase-3 colorimetric substrate DEVD-pNA. In addition, cells transfected with pEGFP-RNA2 were treated with DEVD-CHO. The pEGFP-C1 was used as a negative control and to maintain equal plasmid DNA concentration for each of the transfections. Values shown are means from duplicate experiments.

ternal death signal of cells to induce apoptosis, which serves as a host defense mechanism against viral proliferation. Further work needs to analyze its proapoptotic domain and subcellular localization in virus-infected cells to find its apoptosis mechanism.

In GGNNV-infected SB cells, high activity of caspase-8 and caspase-3 was detected. Meanwhile, the apoptosis was decreased by DEVD-CHO treatment demonstrated in TUNEL staining, which means the apoptosis induced by GGNNV was caspase dependent. Similarly, transient expression of protein  $\alpha$  from GGNNV in two cell lines; SB and Cos-7 cells, could activate caspase-3-like proteases and its activity could be inhibited by DEVD-CHO. Moreover, the reduction of TUNEL positive signal was observed in cells treated by DEVD-CHO. These results indicate that the apoptosis induced by protein  $\alpha$  in fish and mammalian cells possibly uses the same caspase-dependent pathway. But the function of apoptosis in the true life cycle of the GGNNV infection needs more investigation.

## Materials and methods

### *Cells and viruses*

Fish nodaviruses (GGNNV) and sea bass (SB) cell line (Chong et al., 1987, 1990) were obtained from AVA (Agriculture and Veterinary Authority of Singapore). The SB cells were grown in modified Eagle's medium (MEM; GIBCO, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO, USA), 0.34% NaCl, 0.12% HEPES, and 2 mM glutamine at 23°C. GGNNV was originally isolated in 1992 from brain, head, kidney, and liver of greasy grouper *Epinephelus tauvina* in Singapore. The SB cells were infected with the betanodaviruses GGNNV for propagation and inoculated cultures were harvested when 90% of cells in the monolayer showed specific CPE.

Cos-7 cells were maintained in Dulbecco modified Eagle's medium (DMEM; GIBCO, USA) supplemented with 10% FBS and grown at 37°C in 5% CO<sub>2</sub>.

The *E. coli* strain was grown in Luria Bertani (LB) broth. The medium was supplemented with 100  $\mu\text{g ml}^{-1}$  ampicillin or 30  $\mu\text{g ml}^{-1}$  kanamycin for selection of transformants and plasmid maintenance.

### *Preparation of monospecific antibodies against protein A and protein $\alpha$*

The protein A-encoding region in pCDNA3.1/RNA1 which contained the full-length cDNA of GGNNV RNA1 (Tan et al., 2001) was amplified by PCR using the pair of primers (5')ATGGATCCATGCGTCGCTTTGAGTTTGCA, and (3')GTTGTCGACTACACTTG AGTGCACGTCG and constructed into pGEX-4T-2 vector (Amersham, Pharmacia, Uppsala, Sweden) by digesting both vector and PCR fragment with BamHI/SalI to obtain plasmid pGEX-4T-2/RNA1. The

open reading frame of protein  $\alpha$  gene was obtained by PCR amplification with primers: (5')CGGGGTACCATGGTACGCAAGGGTGATAAG and (3')CCCAAGCTTTT-AGTTTCCCAGTCAACCC containing KpnI/HindIII restriction enzyme sites and cloned into pQE30 vector (Qiagen Hilden, Germany) to construct plasmid pQE30/RNA2. The fusion proteins were expressed and purified by standard techniques as recommended by the manufacturer.

To produce specific antibodies against protein A and protein  $\alpha$ , two female guinea pigs were administered 200–250  $\mu\text{g}$  of the purified proteins emulsified with adjuvant MONTANIDE ISA 70 (Seppic, France). Guinea pigs were boosted twice with the same quantities of antigen emulsion every other day for 14 days. Ten days after the final booster injection, the animals were bled and the antibody titers and specificity were determined by Western blot with GGNNV-infected SB cells.

### *Transient expression of protein A and protein $\alpha$ in vitro*

The coding sequence of proteins A and  $\alpha$  were amplified by PCR and inserted into pEGFP-C1 vector (Clontech) under the control of human cytomegalovirus (CMV) promoter to obtain pEGFP-RNA1 and pEGFP-RNA2. They were analyzed by sequencing to confirm that no errors were introduced as a result of PCR amplification. The primers used for these two gene amplification were as follows: RNA1-5': CCGAATTCTATGCGTCGCTTTGAGTTTGCAC (*EcoRI*); RNA1-3': GTGGATCCCTACACTTGAGTGCACGTCG (*BamHI*); RNA2-5': GC GAATTCTATGGTACGCAAAGGTGAGAAG (*EcoRI*); RNA2-3': GTGTGCGACTTAGTTTCCCGAGTCAACCCT (*SalI*).

In vitro expression of the pEGFP-RNA1 and pEGFP-RNA2 constructs was performed in transient expression experiments using SB and Cos-7 cells, and 60–80% confluent monolayers of SB or Cos-7 cells grown in 25 × 25 cm flask were transfected with a 2  $\mu\text{g/flask}$  of plasmid DNA (purified by Qiagen plasmid Midi kits, Chatsworth, CA, USA) mixed with lipofectamin plus reagent according to the instructions of the manufacturer (Life Technologies, Gaithersburg, MD, USA). Western-blot analysis was carried out to determine the expression of protein A and protein  $\alpha$ . Briefly, at 48-h post-transfection, the cells were washed twice with ice-cold PBS, harvested, and separated by 12% SDS-PAGE, then transferred to Hybond nitrocellulose membranes (Bio-Rad, Richmond, CA, USA). The membranes were blocked with 5% skimmed milk in phosphate-buffered saline with 0.05% Tween 20 (PBST) for 1 h, washed with PBST once, and incubated with guinea pig anti-protein A and protein  $\alpha$ , separately, at room temperature for 1 h, then washed with PBST three times and incubated with HRP-conjugated anti-guinea pig IgG secondary antibody at room temperature for 40 min. After rinsing three times, the specific proteins were visualized by

enhanced chemiluminescence (ECL) (Pierce, Rockford, IL, USA).

#### DNA fragmentation analysis

Low-molecular-weight nuclear DNA was isolated using apoptotic DNA-ladder kit (Promega, Madison, WI, USA) following the manufacturer's instruction. Briefly, the infected or transfected cells were harvested and resuspended in binding/lysis buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, and 20% Triton X-100, pH 4.4) and incubated for 10 min at RT. After incubation, isopropanol was added and the mixture was vortexed. The mixture was then placed in a filter tube and centrifuged for 1 min at 8000 rpm at RT. The upper reservoir was washed with washing buffer and centrifuged twice. After centrifugation for 1 min at 13,000 rpm, the nucleic acid was dissolved in prewarmed (70°C) elution buffer, centrifuged, and collected. Equal amounts of DNA were resolved on a 2% agarose gel containing ethidium bromide and the size of the oligonucleosomal DNA fragment was measured by comparison with a 100 bp DNA marker.

#### TUNEL assay

The terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was performed with apoptosis detection system fluorescein kit (Promega) and DeadEnd Colorimetric TUNEL System kit (Promega) according to the protocol of the manufacturer. Briefly, GGNNV-infected SB cells grown on 4-well chamber slides (IWAKI, Japan) were fixed with 4% paraformaldehyde for 25 min at 4°C RT at postinfection 24, 36, and 48 h and permeabilized with 0.2% Triton X-100 in PBS for 5 min at RT. After equilibration for 10 min at RT, cells were overlaid with 50  $\mu$ l TdT reaction buffer and incubated at 37°C for 60 min. The reaction was terminated by immersing slides in 2  $\times$  SSC for 15 min. After being washed three times with PBS, the slides were examined under confocal microscope LSM510 and photographed.

To perform TUNEL assay in transfected cells, SB or Cos-7 cells grown on chamber slides were transfected with 0.4  $\mu$ g of recombinant plasmid as described above. At 48 h post-transfection, cells were fixed with 4% paraformaldehyde for 25 min at 4°C RT and permeabilized with 0.2% Triton X-100 in PBS for 5 min at RT. After equilibration, cells were overlaid with TdT reaction buffer and incubated at 37°C for 60 min, then immersed in 2  $\times$  SSC for 15 min to terminate reaction. After being washed three times with PBS, the slides were immersed in 0.3% hydrogen peroxide for 5 min to block the endogenous peroxidases and washed three times with PBS. Streptavidin-HRP solution was then added to slides and incubated for 30 min at RT. After being washed three times with PBS, the slides were developed by 3,3'-diaminobenzidine tetrahydrochloride (DAB) components until there was a light brown reaction product and then

were rinsed several times in deionized water to stop the reaction.

#### Colorimetric assay of caspases activity

Colorimetric assay of caspase-8-like (IETDase) and caspase-3-like (DEVDase) proteolytic activity was performed using an ApoAlert Caspase-8 and Caspase-3 Colorimetric Assay Kit (Clontech Laboratories, Palo Alto, CA, USA). SB cells were cultured in 25  $\times$  25 cm flasks and infected with betanodavirus GGNNV. At 24 and 48 h postinfection, cells were centrifuged at 2000 rpm for 5 min to harvest. Cells (3–5  $\times$  10<sup>6</sup>) were then lysed in 50  $\mu$ l lysis buffer on ice for 10 min and centrifuged at 14,000 rpm for 3 min at 4°C and the supernatant was collected. Supernatant (50  $\mu$ l) was added to an equal volume of 2X reaction/DTT buffer supplemented with IETD-pNA (200  $\mu$ M) and incubated at 37°C for 2 h in the dark, and then the optical density was measured at 405. In the case of caspase-3-like proteases, supernatant (50  $\mu$ l) was added to an equal volume of 2X reaction/DTT buffer supplemented with DEVD-pNA (50  $\mu$ M) and incubated at 37°C for 1 h. The nanomoles of pNA released per hour were calculated from the standard curve.

To detect the caspase-3 like proteolytic activity in transfected cells, SB and Cos-7 cells were seeded in 25  $\times$  25 cm flasks on the previous day and transfected with 2  $\mu$ g of pEGFP-RNA2 and PEGFP-C1 plasmids. At 24 h post-transfection, cells were harvested at 2000 rpm for 5 min, then caspase-3-like protease (DEVDase) activity assay was carried out as described above.

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