Zebrafish anti-apoptotic protein zfBcl-x\textsubscript{L} can block betanodavirus protein \(\alpha\)-induced mitochondria-mediated secondary necrosis cell death

Horng-Cherng Wu\textsuperscript{a,b,c}, Chuan-Sheng Chiua, Jen-Leih Wud, Hong-Yi Gond, Ming-Chyuan Chen\textsuperscript{e}, Ming-Wei Lud, Jiann-Ruey Hong\textsuperscript{a,b,*}

\textsuperscript{a} Laboratory of Molecular Virology and Biotechnology, Institute of Biotechnology, National Cheng-Kung University, Tainan 701, Taiwan
\textsuperscript{b} Research Center of Ocean Environment and Technology, National Cheng Kung University, Tainan 701, Taiwan
\textsuperscript{c} Department of Food Science & Technology, Chia Nan University of Pharmacy & Science, Tainan 717, Taiwan
\textsuperscript{d} Laboratory of Marine Molecular Biology and Biotechnology, Institute of Cellular and Organismic Biology, Academia Sinica, Nankang, Taipei 115, Taiwan
\textsuperscript{e} Department of Marine Biotechnology, National Kaohsiung Institute of Marine Technology, Kaohsiung 811, Taiwan

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**Abstract**  Betanodavirus protein \(\alpha\) induces cell apoptosis or secondary necrosis by a poorly understood process. In the present work, red spotted grouper nervous necrosis virus (RGNNV) RNA 2 was cloned and transfected into tissue culture cells (GF-1) which then underwent apoptosis or post-apoptotic necrosis. In the early apoptotic stage, progressive phosphatidylserine externalization was evident at 24 h post-transfection (p.t.) by Annexin V-FLUOS staining. TUNEL assay revealed apoptotic cells at 24–72 h p.t., after which post-apoptotic necrotic cells were identified by acridine orange/ethidium bromide dual dye staining from 48 to 72 h p.t. Protein \(\alpha\) induced progressive loss of mitochondrial membrane potential (MMP) which was detected in RNA2-transfected GF-1 cells at 24, 48, and 72 h p.t., which correlated with cytochrome \(c\) release, especially at 72 h p.t. To assess the effect of zfBcl-x\textsubscript{L} on cell death, RNA2-transfected cells were co-transfected with zfBcl-x\textsubscript{L}. Co-transfection of GF-1 cells prevented loss of MMP at 24 h and 48 h p.t. and blocked initiator caspase-8 and effector caspase-3 activation at 48 h p.t. We conclude that RGNNV protein \(\alpha\) induces apoptosis followed by secondary necrotic cell death through a mitochondria-mediated death pathway and activation of caspases-8 and -3.

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Introduction

Apoptosis is a suicidal process triggered by a wide variety of stimuli in individual cells of multicellular organisms [1]. The process is genetically controlled and preprogrammed to eliminate redundant cells during development, and is used as an emergency response following radiation damage, viral infection, or aberrant cell growth induced by oncogenes [2,3]. Apoptosis and necrosis are two stereotyped mechanisms by which nucleated eukaryotic cells die [1,4–6]. Understanding these mechanisms may ultimately lead to novel therapeutic strategies.

Mitochondria function as central integrators of pro-death stimuli [7] by sequestering apoptogenic proteins such as cytochrome c, Smac/DIABLO, apoptosis inducing factor, and endonuclease G in the intermembrane space, and releasing these factors into the cytosol on exposure to proapoptotic signals [8,9]. Loss of mitochondrial membrane potential (MMP) leads to cytosolic binding of caspase-9, the downstream activator of apoptosis [8,10]. MMP loss can affect both the inner and outer mitochondrial membranes, and precedes the signs of necrotic or apoptotic cell death including the apoptosis-specific activation of caspases [7]. Hence, mitochondria integrate pro-death stimuli by joining together various types of proapoptotic signals into a common caspase-dependent pathway [10].

In contrast, little is known of cell death induced by aquatic viruses. Nodaviruses are small, non-enveloped, spherical viruses with bipartite positive-sense RNA genomes that are capped but not polyadenylated [11]. The family Nodaviridae contains two genera. Betanodaviruses predominantly infect fish, while alphanodaviruses mostly infect insects [12–14]. The betanodavirus is the causative agent of viral nervous necrosis (VNN), an infectious neuropathological disease characterized by necrosis of the central nervous system and retina. Its clinical symptoms include abnormal swimming behavior and, very often, darkening of the fish [14]. VNN kills massive numbers of larvae and juveniles of several marine teleost species globally [15].

The genome of fish nodavirus is bipartite, comprising two single-stranded molecules of positive polarity RNA (RNA1 and RNA2; about 3.1 and 1.4 kb in length, respectively) and no poly(A) extension at the 3’ end [16,17]. RNA1 encodes RNA-dependent RNA polymerase (RdRP; approximately 100 kDa), which is also named protein A, an enzyme that replicates the viral genome. RNA2 encodes the capsid protein of about 42 kDa [16,18]. According to the RNA1 sequences, betanodavirus isolates should be classified within four main groups named I, II, III and IV, which correspond to greasy groupy nervous necrosis virus (RGNNV), barfin flounder NNV (BFNNV), tiger puffer NNV (TPNNV), and striped jack nervous necrosis virus (SJNNV) [19]. These groups may in fact represent independent genotypes [19].

Despite their severe economic impact on the aquaculture industry, betanodaviruses have not been well studied. Characterization of the viral molecular regulation processes should help clarify the mechanism(s) of viral pathogenesis and infection. In a previous study, we demonstrated that RGNNV TN1 strain induces apoptosis followed by secondary necrosis in GL-av cells and loss of mitochondrial membrane potential (MMP) in the mid-apoptotic stage [20], and demonstrated that this death process is prevented by Bcl-2 member proteins zfBcl-xL and zfMcl-1a [21,22]. In addition, the GGNNV strain protein α is an apoptosis inducer [23]. Presently, we demonstrate that RGNNV protein α induces apoptotic loss of MMP for cytochrome c release and an increase in caspase-8 and -3 activation, and that zfBcl-xL can block these post-apoptotic necrosis processes thereby rescuing virus-infected cells.

Materials and methods

Cell culture and reagents

The grouper fin cell line GF-1 was grown at 28°C in Leibovitz’s L-15 medium (GibcoBRL, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum and 25 μg/ml of gentamycin. The annexin V-FLUOS Kit and In Situ Cell Death Detection Kit were purchased from Roche GmbH (Mannheim, Germany). Anti-NNV particle polyclonal antibody and the plasmids encoding zfBcl-xL and infectious NNV particle polyclonal antibody and the plasmids encoding zfBcl-xL and infectious NNV particle polyclonal antibody were gifts from Dr Jen-Leih Wu. The ECL Western blotting detection system kit was purchased from Amersham (Piscataway, NJ, USA). The apoptosis detection, mitochondria bioassay kit was purchased from USBiological (Jomar Diagnostics Pty. Ltd, Stepney, SA, Australia). PhiPhiLux-G2D2 cell-permeable fluorescent substrate was from OncolImmunin (Gaithersburg, MD, USA).

Cloning of RNA2 and construction of expression vector

Synthesis and amplification of cDNA was carried out using the SuperScript One-Step™ step reverse transcriptase-polymerase chain reaction (RT-PCR) system kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. RGNNV RNA2 primers P1 and P2 were each added to a final concentration of 0.2 μM. PCR cycling conditions were 54°C for 30 min, 2 min at 94°C (to inactivate the reverse transcriptase), 95°C for 30 s (DNA denaturation), 57°C for 30 s (annealing), and 72°C for 45 s (extension) for a total of 35–40 cycles. The RT-PCR primers NNV RNA2 P1 (5’-ACA ATg gTA CgC AAA ggt gAg-3’) and NNV RNA2 P2 (5’-TTA gTT TCC CgA gTC AAC CCT-3’) were used to amplify a fragment covering the variable region of RNA2. The purity and size of the amplified product was checked by 1.5% agarose gel electrophoresis after staining with ethidium bromide as described previously [24]. The 1017 bp, double-stranded cDNA was purified using the QIAquick™ gel extraction system (Invitrogen) and RGNNV RNA 2 subcloned using a pCDNA3.1 cloning system (Promega, Madison, WI, USA). The cloned PCR products were sequenced by the dye termination method using an ABI PRISM 477 DNA sequencer (Applied Biosystems, Foster City, CA, USA) and scanned against the GenBank database BLAST (http://www4.ncbi.nlm.nih.gov/) and PROSITE (http://psort.ims.u-tokyo.ac.jp/) programs.
Western blot analysis

GF-1 cells were cultured by seeding 10⁵ cells/ml in a 60 mm diameter Petri dish for 20 h prior to rinsing the monolayers twice with phosphate-buffered saline (PBS). In the RNA2-induced cell death group, cells were transfected with pCDNA3.1 (as negative control), pCDNA3.1-RNA2, and pCDNA3.1-VP5 plasmids [24], and exposed to lipofectamine. Transfected cells were incubated for 0, 24, 48, and 72 h. In the zfBcl-xL rescue group, cells were co-transfected with pCDNA3.1-RNA2 and zfBcl-xL (1:1 = 1 μg:1 μg), and the transfected cells were incubated for 0, 24, 48, and 72 h. At each time point, the culture medium was aspirated, cells were washed with PBS, and then lysed in 0.3 ml of lysis buffer (10 mM Tris base, 20% glycerol, 10 mM sodium dodecyl sulfate, and 2% β-mercaptoethanol, pH 6.8). An aliquot of the lysate was used for the separation by electrophoresis [25]. The gels were subjected to immuno-blotting [26]. Blots were incubated with a 1:3000 dilution of rabbit anti-fluorescence antibody conjugated with horseradish peroxidase (POD) to produce the signal conversion that was detected using phase-contrast microscopy. The slide was incubated in a humidified chamber for 60 min at 37 °C and rinsed with PBS. Finally, 50--100 μl of the substrate solution (diamino butanoic acid; DAB) were added for 10 min at room temperature. The slide was rinsed with PBS, mounted under a glass coverslip, and analyzed by light microscopy [24]. Identification of apoptotic and secondary necrotic cells was by acridine orange/ethidium bromide dual dye staining [28]. Cells (1 ml of 10⁵/ml of culture medium) were seeded into wells of a chamber slide (Nalge Nunc International, Rochester, NY, USA) at 28 °C for 20 h. The cells were transfected with pCDNA3.1 and pCDNA3.1-RNA2 plasmids, and exposed to lipofectamine. Transfected cells were incubated for 0, 24, 48, and 72 h at 28 °C. At each time point subsequent to a change of the culture medium, 1 ml was removed. The cells were washed with PBS, stained with 1 μl of acridine orange solution (100 μg/ml)—ethidium bromide (100 μg/ml) in 0.5 ml of PBS for 2--5 min at room temperature. The stained cells were placed on a glass slide, and covered with a 22 mm² coverslip. Slides were examined by fluorescence microscopy using an Olympus IX70 microscope equipped with a BP450--480 band-pass excitation filter and a BA515 barrier-emission filter to determine the proportion of dual dye-stained cells [28]. Each sample group (two wells) was counted three times, with at least 200 cells counted each time. Recorded cell characteristics included color and structure of the cell or chromatin. The mean of the three counts of each different cell characteristic was used to calculate the apoptotic and necrotic cell indices and their respective standard error bars.

Apopotic cells and secondary necrotic cells assay

The cells were transfected with pCDNA3.1and pCDNA3.1-RNA2 plasmids and exposed to lipofectamine. Transfected cells were incubated for 0, 24, 48, and 72 h. In the early apoptotic cell assay, which involved annexin V-FLUOS staining [27], exposure of phosphatidylserine on the outer leaflet of early apoptotic cell membranes was analyzed using annexin V-fluorescein (Boehringer Mannheim, Mannheim, Germany) to differentiate apoptotic from non-apoptotic cells. At 0, 24, 48, and 72 h post-transfection (p.t.), cells were removed from the medium, washed with PBS, and then incubated with 100 μl of annexin V-fluorescein in a HEPES buffer (Boehringer Mannheim) for 10--15 min. Evaluation was by fluorescence microscopy (Olympus IX 70; Halagaya Shibuta-ku, Tokyo, Japan) using a 488 nm excitation wavelength and 515 nm long-pass filter for detection [27]. Middle and late apoptotic cells were identified by TUNEL assay [27]. For TdT-dUTP labeling, GF-1 cells were seeded (10³ cells/0.1 ml) into wells of a chamber slide (Nalge Nunc International, Rochester, NY, USA) at 28 °C in Leibovitz’s L-15 medium (GibcoBRL, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum for 20 h. The cells were transfected with pCDNA3.1and pCDNA3.1-RNA2 plasmids and exposed to lipofectamine. Transfected cells were incubated for 0, 24, 48, and 72 h. At the end of the various incubation times, each sample was removed from the medium and washed with PBS, and then cell samples were fixed with a freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 30 min at room temperature. Slides were washed with PBS and incubated with blocking solution (0.3% H₂O₂ in methanol) for 30 min at room temperature. Slides were rinsed with PBS and then incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 5 min on ice. Slides were rinsed twice with PBS. Next, 50 μl of TUNEL reaction mixture (supplied as an in situ cell death detection kit; Boehringer-Mannheim) were added to the sample, and the slides were incubated in a humidified chamber for 60 min at 37 °C. Samples were either analyzed under a fluorescence microscope or 50 μl of anti-fluorescein antibody conjugated with horseradish peroxidase (POD) was added to the sample to produce the signal conversion that was detected using phase-contrast microscopy. The slide was incubated in a humidified chamber for 60 min at 37 °C and rinsed with PBS. Finally, 50--100 μl of the substrate solution (diamino butanoic acid; DAB) were added for 10 min at room temperature. The slide was rinsed with PBS, mounted under a glass coverslip, and analyzed by light microscopy [24]. Identification of apoptotic and secondary necrotic cells was by acridine orange/ethidium bromide dual dye staining [28]. Cells (1 ml of 10⁵/ml of culture medium) were seeded into wells of a chamber slide (Nalge Nunc International) and incubated at 28 °C for 20 h. The cells were transfected with pCDNA3.1 and pCDNA3.1-RNA2 plasmids, and exposed to lipofectamine. Transfected cells were incubated for 0, 24, 48, and 72 h at 28 °C. At each time point subsequent to a change of the culture medium, 1 ml was removed. The cells were washed with PBS, stained with 1 μl of acridine orange solution (100 μg/ml)—ethidium bromide (100 μg/ml) in 0.5 ml of PBS for 2--5 min at room temperature. The stained cells were placed on a glass slide, and covered with a 22 mm² coverslip. Slides were examined by fluorescence microscopy using an Olympus IX70 microscope equipped with a BP450--480 band-pass excitation filter and a BA515 barrier-emission filter to determine the proportion of dual dye-stained cells [28]. Each sample group (two wells) was counted three times, with at least 200 cells counted each time. Recorded cell characteristics included color and structure of the cell or chromatin. The mean of the three counts of each different cell characteristic was used to calculate the apoptotic and necrotic cell indices and their respective standard error bars.

Evaluation of MMP

GF-1 cells (10⁵/ml in a 60 mm diameter Petri dish) were cultured as monolayers for 20 h and then rinsed twice with PBS. To evaluate RNA2 induced cell death, cells were transfected with pCDNA3.1, pCDNA3.1-RNA2, or pCDNA3.1-VP5 plasmids, exposed to lipofectamine, and then incubated for 0, 24, 48, and 72 h. By contrast, to evaluate zfBcl-xL rescue cells were co-transfected with pCDNA3.1-RNA2 and zfBcl-xL (1:1 = 1 μg:1 μg) and then incubated for 0, 24, 48, and 72 h. For assessment of MMP (ΔΨm), pCDNA3.1-RNA2-transfected GF-1 cells were stained using 500 μl of Mitocapture reagent (BioVision, Mountain View, CA, USA) per dish followed by incubation at 37 °C for 15--20 min. This lipophilic cationic dye accumulates and aggregates in mitochondria when ΔΨm is normal and remains in the cytoplasm when it is not.
Loss of fluorescence intensity observed using fluorescence microscopy (488 nm excitation wavelength and 515 nm long-pass filter for detection of fluorescein, and using a 510 nm excitation wavelength and 590 nm long-pass filter for detection of rhodamine) was taken as a marker of mitochondrial membrane disruption and reduced potential.

### Preparation of mitochondria from RGNNV RNA2-transfected cells

The cells were transfected with pCDNA3.1 and pCDNA3.1-RNA2 plasmids and exposed to lipofectamine. Transfected cells were incubated for 0, 24, and 48 h. At each time point subsequent to a change of the culture medium, 1 ml was removed. Mitochondria were isolated by a modification of a previously described protocol [29]. Briefly, GF-1 cells (2 × 10^6) were washed with PBS and homogenized in 0.3 ml of mitochondria isolation buffer (0.35 M mannitol, 10 mM HEPES, 0.1% bovine serum albumin, pH 7.2) using a glass homogenizer. Unbroken cells and nuclei were pelleted by centrifugation (600 × g for 5 min at 4°C). The mitochondria pellet was isolated from centrifuged supernatant (10,000 × g for 10 min at 4°C) and supernatant was collected and mixed with 25 µl of 10× sodium dodecyl sulfate sample buffer. Samples (50 µl) were boiled and subjected to Western blot analysis [26].

### Cell counts and statistical analyses

Loss of MMP and percentage of annexin V-fluorescein positive cells were determined in each sample by counting 200 cells. Each result was expressed as the mean ± SEM. Data were analyzed using either paired or unpaired Student’s t-tests, as appropriate. A value of p < 0.05 was taken to represent a statistically significant difference between group mean values.

### Caspase activity assays

**In vitro assay**

About 10^5 GF-1 cells/ml were seeded in a 60 mm diameter Petri dish from (Nalge Nunc International) and cultured for 20 h. GF-1 cells, either transfected with pCDNA3.1 or pCDNA3.1-RNA2 or co-transfected with pCDNA3.1-RNA2 plasmids containing zfBcl-xL as described earlier in the text, were incubated for 24, 48, and 72 h at 28°C. Caspase-8 and 3 activation assays were performed using a kit purchased from Clontech (Palo Alto, CA, USA). Experiments were performed using 1 × 10^6 cells obtained at each time point. Cells were recovered by centrifugation at 2000 × g, supernatants were removed, and the cell pellets were frozen at −70°C until analyzed. Assays were performed in 96-well plates and using a fluorescent plate reader (CytoFluor 4000, PerSeptive Biosystems, Framingham, MA, USA). Cleavage of DEVD-AFC and IETD-ABC (which are synthetic caspase-3 and -8 substrates, respectively) was used to determine caspase activities. Cleavage after the second Asp residue produces free AFC [29,30]. The amount of fluorescence detected is directly proportional to amount of caspase-8 and -3 activities. Fluorogenic substrate assay experiments were performed at the same time. In cells transfected with pCDNA3.1-RNA2 alone and in cells co-transfected with zfBcl-xL, the caspase-8 and -3 activity profiles were the same in all experiments, and are included in each figure to facilitate comparison. Results of all experiments are reported as the mean ± SEM.

### Intact cell assay

The assay was performed as previously described [31]. Cells transfected with pCDNA3.1-RNA2 alone and cells co-transfected with zfBcl-xL were incubated for 0 h or from 48–72 h p.t. At each time point, substrate (PhosphoLux-G_3D_2) was added at 10 µM for 1 h at 28°C, prior to evaluation by fluorescence microscopy using a 510 nm excitation wavelength and 590 nm long-pass filter for detection of rhodamine. Each sample group (two wells) was counted three times, with at least 200 cells counted each time. Recorded cell characteristics included color and structure of the cells. The mean of the three counts of each different cell characteristic was used to calculate the caspase-3 activation cell indices and their respective error bars.

### Results

#### Viral protein expression in GF-1 cells

The smaller genomic segment of RGNNV contains RNA2, which encodes a capsid protein designated protein α [32]. RGNNV RNA2 over-expression was associated with the detection of protein α (about 42 kDa) at 24 h p.t. (Fig. 1A:a, lane 4). The protein content progressively increased at 48 and 72 h p.t. (Fig. 1A:a, lanes 5 and 6), as compared with protein content in pCDNA3.1 vector-transfected cells as a negative control (Fig. 1A:a, lanes 1–3). The internal control β-actin is shown in Fig. 1A:b. Similarly, the control protein VPS (17 kDa; 27) from IPNV progressively increased at 24, 48, and 72 h p.t. (Fig. 1B:a, lanes 2–4), as compared with cells at 0 h p.t. (Fig. 1B:a, lane 1). The internal control, β-actin, is shown in Fig. 1B:b.

#### Cloning of RGNNV RNA2

Two specific primers were used to clone capsid protein α in a one-step RT-PCR process from the RNA genome of RGNNV TN 1. The alignment of the RNA2 nucleotide sequence and the deduced amino acid sequence are indicated in Fig. 1C. The cDNA of RNA2 was 1017 bp in length and the molecular weight of protein α was approximately 42 kDa. When we compared the amino acid identity of the RGNNV TN1 strain (RGNNV TN1; EU391590) with other species, the match was 98% for DGNNV (AF245004), 98% for RGNNV (AAT35534.2), 86% for BFNNV (BAA07616.1), 82% for TPNNV (BAA07618.1), and 80% for SJNNV (BBA06491.1) (Fig. 1D).

#### Influence of protein α on post-apoptotic necrosis cell death

In GGNNV, protein α induces apoptosis [23]. Presently, we examined the protein’s role in secondary necrosis in GF-1 cells. In early apoptotic cells (EA), middle apoptotic cells (MA), and post-apoptotic cells (PN), apoptotic
characteristics were evident after transfection with RGNNV TN1 RNA2 as compared with normal control group (pCDNA3.1 vector). In Annexin V-FURO-stained samples, pCDNA3.1-RNA2-transfected cells exhibited phosphatidylserine externalized from EAs and MAs of the inner membrane, and morphology changes when examined at 24 h p.t. (Fig. 2A, a–d). In pCDNA3.1-RNA2-transfected cells, many TUNEL positive cells were evident at both 48 and 72 h p.t. (Fig. 2B, c and d) as compared with control cells (Fig. 2B, a and b). The TUNEL positive ratio progressively increased from 1.5% (0 h) to 28% (48 h p.t.) and 45% (72 h p.t.), as compared with pCDNA3.1 vector control cells (1.5%, 3%, and 4% at 0, 48, and 72 h p.t., respectively). Furthermore, dual staining with acridine orange and ethidium bromide to identify post-apoptotic cells [28] revealed that pCDNA3.1-RNA2-transfected cells underwent post-apoptotic necrosis, with loss of plasma membrane integrity and membrane dissolution (Fig. 2B, i) and nuclear leakage of chromatin (Fig. 2D, c and d) being evident, as compared to the vector control (Fig. 2D, a and b). In contrast, MA cells were more rounded and displayed prominent plasma membrane blebs (Fig. 2D, c and d). RNA2-induced secondary necrosis ratios which progressively increased from 1% at 0 h p.t. 8% and 22% at 48 h and 72 h p.t., respectively, as compared with pCDNA3.1 vector control cells (0.7%, 2.7%, and 3.7% at 0 h, 48 h, and 72 h p.t., respectively).

pCDNA3.1-RNA2-transfected cells exhibited phosphatidylserine externalization from the plasma membrane and a morphology that differed between the apoptotic and secondary necrotic forms of the cells (Fig. 3A, a–k), as compared to pCDNA3.1-transfected cells (Fig. 3A, a–c) and IPNV VP5-transfected cells (negative control; Fig. 2A, d and l). The phospholipid externalization and bleb formation were frequent in RNA2 transfected cells (Fig. 3A, n–o) but were relatively infrequent in pCDNA3.1-transfected cells (Fig. 3A, e and f) and in negative control IPNV VP5-transfected cells (Fig. 3A, h and p). The assay for annexin V positivity (Fig. 3B) revealed that the percentage of pCDNA3.1-RNA2-transfected cells with phosphatidylserine exposure was low at 24 h p.t. (8%), but progressively increased (20% and 40% at 48 h and 72 h p.t., respectively) as compared with pCDNA3.1 vector control cells (2%, 2.5%, and 5% at 24 h, 48 h, and 72 h p.t., respectively) and IPNV VP5-transfected cells (2%, 2%, and 4% at 24 h, 48 h, and 72 h p.t., respectively) (Fig. 3B). At all time points, the number of annexin V positive RNA2-transfected GF-1 cells was significantly greater than the number of apoptotic or pCDNA3.1 and pCDNA3.1-VP5 vector-transfected GF-1 cells. Inhibition of RGNNV-induced necrotic cell death

To determine whether MMP was affected by RGNNV death inducer protein α-over-expression, a mitochondrial function dye was used to determine change in the MMP of protein α-transfected GF-1 cells. The dye aggregates in the mitochondria of healthy cells and fluoresces red. In apoptotic cells, where the dye cannot accumulate in mitochondria, the dye molecules remain as cytoplasmic monomers and fluoresce green. The loss of red fluorescence was evident in RGNNV RNA 2-transfected GF-1 cells at 24 h p.t., and progressed gradually thereafter (Fig. 4A, panels d, h, and l; denoted by the arrows). The decreasing red fluorescence was concomitant with an increase in green fluorescence (Fig. 4A, panels c, g, and k; denoted by the arrows). In contrast, no such changes occurred in pCDNA3.1 vector-infected cells (Fig. 4A, panels b, f, and j for red fluorescence, and panels a, e, and i for green fluorescence) and pCDNA3.1-VP5-transfected cells (Fig. 4A, panels n and p for red fluorescence, and panels m and o for green fluorescence). Protein α effectively reduced MMP at 24 h p.t. (15%), 48 h p.t. (20%), and 72 h p.t. (45%) when compared to the corresponding reduction at 24 h p.t. (2%), 48 h p.t. (2%), and 72 h p.t. (3%) in pCDNA3.1 vector-transfected cells, and at 24 h p.t. (2%), 48 h p.t. (2%), and 72 h p.t. (2%) in pCDNA3.1-VP5-transfected cells (Fig. 4B). At all time points the number of cells with MMP loss was significantly greater in RGNNV protein α-transfected GF-1 cells than pCDNA3.1 vector-transfected cells and pCDNA3.1-VP5-transfected cells. It was appropriate to determine if protein α-induced MMP loss could induce cytochrome c release during cell death. Western blot analysis revealed that the membrane form of cytochrome c (Fig. 4C, a, lane 2) was released to the cytoplasm (Fig. 4C, a, lane 4) at 48 h p.t. as compared with vector control (Fig. 4C, a, lanes 1 and 3). Cytochrome c oxidase I internal controls are shown in Fig. 4C, b, lanes 1–4.

Effect of anti-apoptotic protein zfBcl-xL on protein α-induced loss of GF-1 MMP

RGNNV RNA2 over-expression was associated with the detection of an approximately 42 kDa protein, which was presumed to represent protein α, at 0 h, 24 h, and 48 h p.t. (Fig. 5A, lanes 2–5). The protein content progressively increased, as evident at 24 h p.t. (Fig. 5A, a, lanes 2 and 4),

Figure 1 Identification of viral protein expression in GF-1 cells. (A) Expression of protein α in GF-1 cells following incubation without or with RGNNV RNA2 transfection for 24 h, 48 h, and 72 h. (a) Protein α was detected by Western blots, and the gels were immunoblotted with a polyclonal antibody to whole particle NNV. Cells transfected with the pCDNA3.1 vector alone were incubated for 24 h (lane 1), 48 h (lane 2), and 72 h (lane 3). Cells transfected with pCDNA3.1-RNA 2 were incubated for 24 h (lane 4), 48 h (lane 5), and 72 h (lane 6). (b) Actin internal control for experiments whose results are depicted in panel (A). (B) Expression of IPNV VP5 in GF-1 cells following incubation with IPNV VP5 transfection for 0 h, 24 h, 48 h, and 72 h. (a) VP5 was detected by Western blots, and the gels were immunoblotted with a polyclonal antibody to N-terminus peptide of IPNV. Cells transfected with pCDNA3.1-VP5 vector alone were incubated for 0 h (lane 1), 24 h (lane 2), 48 h (lane 3), and 72 h (lane 4). (b) Actin internal control for experiments whose result is depicted in panel (B). (C) Alignment of nucleotide sequence and deduced amino acid sequence of RGNNV TN1 RNA2. (D) Alignment of the deduced amino acid sequence of RGNNV TN1 RNA2 with the corresponding sequences of other betanodavirus proteins. The conserved amino acids for residues common to at least five betanodavirus proteins are shown in black blocks.
Figure 2  Identification of RGNNV RNA2 induces apoptotic and post-apoptotic necrosis in GF-1 cells. (A) Annexin V-labeled (fluorescing) early apoptotic (EA) and middle apoptotic cells (MA; indicated by arrows) with pCDNA3.1-RNA2 (d, e and f) at 24 h p.t.; with pCDNA3.1 vector alone as normal control (a, b and c) at 24 h p.t. (Bar = 10 μm.). (B) TUNEL positive nuclei staining demonstrating that RGNNV RNA2 induces early, middle apoptotic (c and d) and post-apoptotic necrosis cell death (e; indicated by arrow) in GF-1 cells between 48 and 72 h p.t. as compared to cells with pCDNA3.1 vector control (a and b) at 48 and 72 h.
as compared with the protein content of pCDNA3.1 vector-transfected cells as a negative control (Fig. 5A, a, lane 1). EGFP-Bcl-xL over-expression was associated with the detection of fusion protein EGFP-Bcl-xL (about 60 kDa) at 24 h and 48 h p.t. (Fig. 5A, b, lanes 4 and 5). The protein content progressively increased, as evident at 24 h p.t. (Fig. 5A, b, lane 4), as compared with the protein content of non-EGFP-Bcl-xL transfected cells (Fig. 5A, b, lanes 1–3). The β-actin internal control is shown in Fig. 5C.

 zfBcl-xL effectively prevented the loss of MMP (Fig. 5B). In RGNNV RNA 2-transfected GF-1 cells at 48 h p.t. (Fig. 5B, d; denoted by the arrows), apoptotic cell numbers were elevated, as indicated by decrease in cellular red fluorescence that was concomitant with an increase in green fluorescence (Fig. 5B, c; denoted by the arrows). On the other hand, no changes in fluorescence were apparent in pCDNA3.1 vector-transfected cells (Fig. 5B, a; for red fluorescence, a for green fluorescence) and pCDNA3.1-RNA2 plus pEGFP-Bcl-xL-transfected cells (Fig. 5B, f for red fluorescence, e for green fluorescence).

 zfBcl-xL effectively prevented the loss of MMP at 24 h p.t. (2.2%) and 48 h p.t. (3%), when compared to untreated protein α-transfected cells at 24 h p.t. (15%) and 48 h p.t. (22%), and with pCDNA3.1 vector-transfected cells at 24 h p.t. (2%) and 48 h p.t. (3%) (Fig. 5C). At all time points, the number of cells with reduced MMP was significantly greater in RNA2-transfected GF-1 cells than either pCDNA3.1 vector-transfected cells or zfBcl-xL co-transfected GF-1 cells.

Effect of zfBcl-xL on protein α-induced caspase-8 and -3 activation

RNA2 transfection induced activation of caspase-8, and activation was blocked by co-transfection by zfBcl-xL (Fig. 6A). In RNA2-transfected cells, caspase-8 activity at 48 h p.t. was 1.8-fold that of pCDNA3.1 vector-containing control cells and 2.2-fold that of normal control (untransfected cells). In RNA2-transfected cells that were co-transfected with zfBcl-xL, caspase-8 activity was comparable to that of the vector control. Likewise, RNA2 transfection induced activation of caspase-3 (Fig. 6B). Activation was slower than for caspase-8; by 48 h p.t., caspase-3 activity was 2.2-fold that of pCDNA3.1-containing control cells. RNA2 co-transfection with zfBcl-xL also blocked caspase-3 activity, which was comparable to that in control cells. On the other hand, in RNA2 co-transfection with zfBcl-xL group, further examination of the caspase-3 positive cells directly in intact cells (Fig. 6C, c and f) revealed an apparently decreased caspase-3 positive red cells (indicated by arrows) as compared with RNA2 transfection group (Fig. 6C, b and e) and vector control group (pCDNA3.1; Fig. 6C, a and d). zfBcl-xL effectively prevented the RNA2-induced caspase-3 activation at 0 h (1%), 48 h p.t. (5%), and 72 h p.t. (8%) as compared with pCDNA3.1-RNA2-transfected cells at 0 h (1.%), 48 h p.t. (16%), and 72 h p.t. (35%), and with pCDNA3.1 vector control-transfected cells at 0 h (1%), 48 h p.t. (1%), and 72 h p.t. (3%) (Fig. 6D).

Discussion

In RGNNV-infected fish, histopathological changes include extensive cellular vacuolation and neuronal degeneration by necrosis in the central nervous system and retina [15,18,33,34]. The phenomenon of betanodavirus-induced neuronal degeneration, which is correlated with abnormal swimming, remains ill-defined. The present study demonstrates that the death inducer, protein α, causes post-apoptotic necrosis cell death from apoptosis by reducing MMP, and that death is blocked by the anti-apoptotic protein, zfBcl-xL.

The features of necrotic and apoptotic cell death are well described [4,5,35–38]. Apoptosis is a morphologically distinct form of cell death that spontaneously occurs in many different tissues under various conditions [1,36]. In our system, we found that RNA2 could induce apoptosis in the early stage, but that in the later stage RNA2 always triggered cells through post-apoptotic necrosis cell death. These observations may support results obtained during RGNNV infection in grouper cells [20–22]. The mechanisms of RNA2 induction of apoptosis that precede secondary necrosis in GF-cells can be summarized as follows. In early mid-apoptotic GF-1 cells, the previously observed exposure of phosphatidylserine [39–41] occurs rapidly on the outer leaflet of the plasma membrane and bleb-like vesicles develop. Subsequently, cells in mid-apoptotic phase round up, display plasma membrane blebbing, and nuclear destruction. Finally, at the late stage, post-apoptotic necrosis is evident by loss of plasma membrane integrity and membrane dissolution, and by continued nucleus break-down (evident as chromatin leakage).

Loss of MMP precedes signs of necrotic or apoptotic cell death [42–46]. MMP, which can affect both the inner and outer mitochondrial membranes, is mainly controlled by voltage-dependent anion channel proteins and adenine nucleotide translocase, two of the most abundant proteins of the outer and inner mitochondrial membranes. These
Figure 3  Phase-contrast and fluorescence images of RGNNV RNA 2-transfected GF-1 cells. (A) Annexin V-labeled (fluorescing) early apoptotic and middle apoptotic cells (indicated by arrows) induced by RGNNV RNA2 at 24 h p.t. (j and n), 48 h p.t. (k and o), and 72 h p.t. with pCDNA3.1-RNA2 (l and p); in cells at 24 h p.t. (a and e), 48 h p.t. (b and f), and 72 h p.t. as compared with pCDNA3.1 vector alone as a normal control (c and g); and in cells at 24 h p.t. (d and h) and 72 h p.t. and compared with pCDNA3.1-VP5 vector alone as negative control (i and m). (Bar = 25 μm.). (B) Percentage of annexin V-labeled pCDNA3.1-transfected, pCDNA3.1-VP5 transfected, and pCDNA3.1-RNA 2-transfected apoptotic GF-1 cells at different time points following transfection. Data were analyzed using either paired or unpaired Student t-tests as appropriate. *p > 0.01 was taken to represent a statistically significant difference between mean values of groups.
Figure 4  RGNNV RNA2-transfection induces loss of MMP in GF-1 cells. Lipophilic cationic dye labeling demonstrating that protein α induces the loss of MMP in RGNNV RNA2-transfected GF-1 cells (arrows). MMP loss is demonstrated by either strong green fluorescence or loss of red fluorescence. (A) Phase-contrast photographs showing (Left 1, a, e, and i) green fluorescence of pCDNA3.1-transfected GF-1 cells (normal control) stained with the lipophilic cationic dye, (Left 2, b, f, and j) red fluorescence of pCDNA3.1-transfected GF-1 cells (normal control) stained with the lipophilic cationic dye, (Left 1, m and Right 2, o) green fluorescence of pCDNA3.1-VP5-transfected GF-1 cells (negative control) stained with the lipophilic cationic dye, (Left 2, n and Right 1, p) red fluorescence of pCDNA3.1-VP5-transfected GF-1 cells (negative control) stained with the lipophilic cationic dye, (Right 2, c, g, and k) strong green fluorescence of pCDNA3.1-RNA2-transfected GF-1 cells (k, denoted by the arrows), and (Right 1, d, h, and l) red fluorescence of pCDNA3.1-RNA2-transfected GF-1 cells (l, loss of red fluorescence indicated by arrows). (B) Change in the percentage of dye-labeled pCDNA3.1, pCDNA3.1-VP5, and pCDNA3.1-RNA2-transfected GF-1 cells indicative of MMP loss. Data were analyzed using either paired or unpaired Student t-tests as appropriate. *P < 0.02 was taken to represent a statistically significant difference between mean values of groups. (C) Release of cytochrome c in GF-1 cells is shown 48 h p.t. with vector control pCDNA3.1 or pCDNA-RNA2 transfection. The release of cytochrome c from mitochondrial membranes was detected in gels using Western immunoblotting with polyclonal antibodies against mouse cytochrome c (a). (b) Cytochrome c oxidase I internal control.
Figure 5  Anti-apoptotic protein zBcl-xL blocks RNA2-induced loss of MMP in GF-1 cells. (A) Identification of protein α and anti-apoptotic proteins zBcl-xL expression in GF-1 cells. (a and b) Expression of protein α and zBcl-xL in GF-1 cells following incubation for 0 h, 24 h, and 48 h. Proteins were detected by Western blotting, and the gels were immunoblotted with a polyclonal antibody (to whole particle NNV with specificity for protein α) or monoclonal antibodies (against Bcl-xL and used for detection of EGFP-zBcl-xL protein). Lanes 1–3 correspond to pCDNA3.1-RNA2 transfected cells incubated for 0 h, 24 h, and 48 h, respectively. Lanes 4–5 correspond to pCDNA3.1-RNA2 plus EGFP-zBcl-xL transfected cells incubated for 24 h and 48 h, respectively. (c) Actin internal control. Lipophilic cationic dye labeling shows that protein α induces the loss of MMP in RNNS RNA2-transfected GF-1 cells (arrows). MMP loss is demonstrated by either strong green fluorescence or loss of red fluorescence (indicated by arrows). (a, c, and e) Phase-contrast images showing green fluorescence in GF-1 cells transfected with pCDNA3.1 vector (a), pCDNA3.1-RNA2 (c), pCDNA3.1-RNA2 + zBcl-xL (e). (b, d, and f) Phase-contrast images showing red fluorescence in GF-1 cells transfected with pCDNA3.1 vector (b), pCDNA3.1-RNA2 (d), and pCDNA3.1-RNA2 + zBcl-xL (f). (C) Change in the percentage of dye-labeled pCDNA3.1 or pCDNA3.1-RNA2-transfected GF-1 cells, or pCDNA3.1-RNA2 + zBcl-xL co-transfected GF-1 cells indicative of MMP loss. Data were analyzed using either paired or unpaired Student t-tests as appropriate. p > 0.01 or p < 0.02 were taken to represent a statistically significant difference between mean values of groups.
proteins interact with the Bcl-2 family of proteins to mediate mitochondrial damage during apoptosis [43–45]. We found that protein α induces MMP loss and triggers cytochrome c release (Fig. 4C), which induces secondary necrotic cell death in grouper fin cells. Moreover, MMP loss is apparently blocked by overexpression of the anti-apoptotic protein Bcl-xL (Fig. 5) in fish systems.

The molecular cornerstones of apoptosis are the family of cysteinyl aspartate-specific proteases, collectively known as caspases. At least 13 caspases have been identified [47]. Members of this family can be subdivided into two groups: initiators and executioners. Initiator caspases serve to relay death signals from pro-apoptotic signals to executioner caspases, which then cleave key proteins involved in cellular structure and function. Known initiators include caspase-8 and caspase-9, whereas known effectors comprise caspase-3 [48], caspase-6, and caspase-7.

In a previous study, the Singapore strain of GGNNV (Epinephelus tauvina) was demonstrated to induce apoptosis of SB cells through protein α, suggesting that caspase-8 and -3 activations may be an important element of GGNNV-mediated apoptosis [26]. In our system, protein α of RGNNV displayed a similar effect on GF-1 and activation of caspase-8 and -3 was evident. Furthermore, we found...
that protein α can activate the effector caspase-3, which also blocks its activity by zfBcl-xL, for enhancing cell viability. On the other hand, RGNNV TN 1 induces host secondary necrosis through a caspase-independent pathway [49,50] that is not consistently associated with caspase-8, and -3 activation by RNA2. But why RGNNVs act through the caspase-independent cell death pathway in preference to the caspase-dependent pathway is still unknown.

In summary, RGNNV RNA2 induces cell apoptosis through to post-apoptotic necrosis. The process involves phosphatidylserine exposure in the early apoptotic stage, and the loss of MMP for cytochrome c release in the mid-apoptotic stage. Subsequently, the triggers of post-apoptotic necrosis cell death are RNA2-activated caspase-8 and -3 at late apoptotic stage. Finally, the loss of MMP and caspase activation can be blocked by the over-expression of anti-apoptotic protein zfBcl-xL in GF-1 cells. Thus, these anti-apoptotic proteins may have potential as treatments for this viral infection.

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References


