Betanodavirus B2 Causes ATP Depletion-induced Cell Death via Mitochondrial Targeting and Complex II Inhibition in Vitro and in Vivo*

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The betanodavirus non-structural protein B2 is a newly discovered necrotic death factor with a still unknown role in regulation of mitochondrial function. In the present study, we examined protein B2-mediated inhibition of mitochondrial complex II activity, which results in ATP depletion and thereby in a bioenergetic crisis in vitro and in vivo. Expression of protein B2 was detected early at 24 h postinfection with red-spotted grouper nervous necrosis virus in the cytoplasm. Later B2 was found in mitochondria using enhanced yellow fluorescent protein (EYFP) and immuno-EM analysis. Furthermore, the B2 mitochondrial targeting signal peptide was analyzed by serial deletion and specific point mutation. The sequence of the B2 grouper nervous necrosis virus in the cytoplasm. Later B2 was mitochondrial targeting signal peptide (41RTFVISAHAA50) was identified and its presence correlated with loss of mitochondrial membrane potential in fish cells. Protein B2 also was found to dramatically inhibit complex II (succinate dehydrogenase) activity, which impairs ATP synthesis in fish GF-1 cells as well as human embryonic kidney 293T cells. Furthermore, when B2 was injected into zebrafish embryos at the one-cell stage to determine its cytotoxicity and ability to inhibit ATP synthesis, we found that B2 caused massive embryonic cell death and depleted ATP resulting in further embryonic death at 10 and 24 h post-fertilization. Taken together, our results indicate that betanodavirus protein B2-induced cell death is due to direct targeting of the mitochondrial matrix by a specific signal peptide that targets mitochondria and inhibits mitochondrial complex II activity thereby reducing ATP synthesis.

Betanodaviruses cause viral nervous necrosis, an infectious neuropathological condition in fish that is characterized by necrosis of the central nervous system, including the brain and retina and by clinical signs (e.g. abnormal swimming behavior and development of a darker body color) (1). This disease can cause massive mortality in larval and juvenile populations of several teleost species and is of global economic importance (2).

The family Nodaviridae is comprised of the genera Alphanodavirus and Betanodavirus. Alphanodavirus predominantly infects insects, whereas Betanodavirus predominantly infects fish (3–5). Nodaviruses are small, non-encapsidated, spherical viruses with bipartite positive-sense RNA genomes (RNA1 and RNA2) that are capped but not polyadenylated (3). RNA1 encodes an ∼110-kDa non-structural protein that has been designated RNA-dependent RNA polymerase or protein A. This protein is vital for replication of the viral genome. RNA2 encodes a 42-kDa capsid protein (6, 7), which may also function in the induction of cell death (8, 9). Nodaviruses also synthesize RNA3, a sub-genomic RNA species from the 3′ terminus of RNA1. RNA3 contains two putative open reading frames that potentially encode an 111-amino acid protein B1 and a 75-amino acid protein B2 (3, 10, 11). Recently, betanodavirus B1 was found to play an anti-necrotic death function in the early replication stages (10). In contrast, betanodavirus B2 was found to either be a suppressor of host siRNA silencing (12, 13) or a necrotic death factor (11, 14).

Mitochondria are organelles required for cellular energy production, programmed cell death regulation (15), reactive oxygen production (16), and intermediary metabolism (17). Changes in mitochondrial function, such as suppression of mitochondrial metabolism, accumulation of reactive oxygen species, loss of mitochondrial membrane potential, and reduced respiration have been shown to play a key role in induction of cell death (18, 19). Mitochondria produce the majority of cellular ATP via oxidative phosphorylation. The mitochondrial electron transport chain removes electrons from an electron donor (NADH for Complex I or O2 for Complex III) and passes them to a terminal electron acceptor (O2) via a series of redox reactions. These reactions are coupled to the creation of a proton gradient across the mitochondrial inner membrane. The resulting transmembrane proton gradient is used to make ATP via ATP synthase (20). Mitochondrial disorders are often present as neurological diseases such as Parkinson disease (21, 22), Alzheimer disease (23–27), and Huntington disease (28).

Several viruses and viral proteins can modulate the mitochondria-mediated death pathway in infected cells (29). Viral factors may be pro-cell death modulators, which when inserted into mitochondria trigger loss of mitochondrial membrane potential (MMP)2 or promote loss of MMP indirectly through activation of host factors, or anti-cell death modulators (which

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2 The abbreviations used are: MMP, mitochondrial membrane potential; EYFP, enhanced yellow fluorescent protein; EGFP, enhanced green fluorescent protein; RGNV, red-spotted grouper nervous necrosis virus; TRITC, tetramethylrhodamine isothiocyanate; m.o.i., multiplicity of infection; DPI, diphenyleneiodonium; SDH, succinate dehydrogenase; hpf, hours post-fertilization; 3-NP, 3-nitropropionic acid; aa, amino acid(s).
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The enzymatic cutting sites on nucleotides of primers are underlined.

have sequence and/or structural similarity to BH1–4 domain of the Bcl-2 family or inhibit cell death via other mechanisms (29). Mitochondrial membrane permeabilization culminates in the loss of mitochondrial transmembrane potential (ΔΨm), an arrest of mitochondrial bioenergetic and biosynthetic functions, in the release of mitochondrial intermembrane space proteins (including cytochrome c (30, 31) and Smac/DIABLO (32), apoptosis-inducing factor (33, 34), and endonuclease G (35)) into the cytosol, and then in exposure to pro-cell death signals (36, 37).

In our previous study of betanodavirus-induced host cell death, the red-spotted grouper nervous necrosis virus (RGNNV) TN1 strain induced apoptosis and post-apoptotic necrosis in a grouper liver cell line (GL-av) (38). The RGNNV infection also induced loss of the MMP, which was blocked by the MMP transition pore inhibitor BKA (38) as well as the Bcl-2 member protein zfBcl-xl (13). In addition, B2 protein (a novel necrotic cell death inducer translated from subgenomic RNA3) acts via a Bax-mediated pathway (14) and is prevented from acting via overexpression of the anti-apoptotic gene of zfBcl-xL (11, 14). However, the molecular mechanism of protein B2 induction of mitochondria-mediated necrotic cell death is still unclear and may provide insight into the molecular pathogenesis of RNA virus infection.

In the present study, we demonstrate that RGNNV B2 targets mitochondria using a specific signal peptide that directs B2 to the mitochondrial matrix, subsequently causing mitochondrial disruption and necrotic cell death in fish cells. Furthermore, we attempt to determine how B2 induces necrotic cell death.

EXPERIMENTAL PROCEDURES

Cells and Virus—The grouper cell line (GF-1) was obtained from Dr. Chi (Institute of Zoology and for the Development of Life Science, Taiwan). The GF-1 cells were grown at 28 °C in Leibovitz’s L-15 medium (Invitrogen) supplemented with 5% fetal bovine serum and 25 µg/ml of gentamycin. The human embryonic kidney 293T cells were grown at 37 °C in low glucose Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 5% fetal bovine serum and 5% CO2. Naturally infected red grouper larvae collected in 2002 in the Tainan prefecture were the source of red-spotted grouper nervous necrosis virus Tainan number 1 (RGNNV TN1), which was used to infect GF-1 cells. The virus was purified as previously described (7, 38) and stored at −80 °C until use.

Plasmid Construction and Cell Transfection—The B2 encoding sequence (14) and all deletion fragments were cloned into pcDNA3.1 vector (Clontech Laboratories, Palo Alto, CA), p3XFLAG-myc-CMV-26 vector (Sigma), or pEYFP-C1 vector (Clontech) in-frame with the EYFP, and sequenced to verify the reading frame. The primers and restriction enzyme sites used to construct different recombinant plasmids are shown in Table 1. Briefly, recombinant plasmid was amplified by Taq/Pfu DNA polymerase with the aid of the designed primers. After digestion with EcoRI and BglIII or BamHI restriction enzyme at 37 °C for 3 h, the PCR products were ligated with each vector to create recombinant plasmids in-frame with the EYFP, and sequenced to confirm the reading frame.

For cell transfection, 3 × 10^5 GF-1 cells were seeded in 60-mm diameter culture dishes. On the following day, 2 µg of recombinant plasmid was mixed with Lipofectamine 2000 (Invitrogen), and the transfection procedure was carried out according to the manufacturer’s instructions.

Immunofluorescence Assay—RGNNV-infected GF-1 cells with multiplicity of infection (m.o.i. = 5) were cultured on 35-mm Petri dishes. At 24, 48, and 72 h post-infection, cells were rinsed once with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min at room temperature,
and then permeabilized for 10 min with 0.2% Triton X-100 in PBS at room temperature. The immunofluorescence assay was performed by incubating these cells with primary polyclonal antibodies (1:50 dilution) against RGNNV protein B2 (14) for 1 h at room temperature, washing with PBS + 0.05% Tween 20 (PBST), incubating with secondary antibodies conjugated to TRITC or fluorescein isothiocyanate (FITC-conjugated goat anti-rabbit IgG; 1:100 dilution; Jackson Immunoresearch Laboratories) for 40 min at room temperature, and washing three times with PBST. Immunofluorescence was examined using an Olympus IX70 fluorescence microscope equipped with a 488-nm excitation and 515-nm long-pass filter for detection RGNNV B2-fluorescein.

**Western Blot Analysis**—GF-1 cells were seeded in 60-mm diameter Petri dishes with 3 ml of medium (10^5 cells/ml) for 20 h. The monolayers were rinsed twice with PBS and infected with RGNNV-TN1 (m.o.i. = 5; propagated and titrated in GF-1 cells with a TCID<sub>50</sub> of 10<sup>8</sup>/0.1 ml) for 0, 24, 48, and 72 h. In a transiently transfected assay, cells were transfected with different plasmids (pEYFP-B2, pEYFP, and different other deleted construction) into either GF-1 or 293T cells (14). At the end of each incubation, the culture medium was aspirated, the cells were washed with PBS and then lysed in 0.3 ml of lysis buffer (10 mM Tris base, 20% glycerol, 10 mM SDS, 2% β-mercaptoethanol, pH 6.8). An aliquot of each lysate with 30 μg of protein per sample was electrophoresed on an SDS-polyacrylamide gel to resolve the proteins. The gels were immunoblotted with either 1) primary antibodies (1:5,000 dilutions) of anti-EGFP and anti monoclonal antibodies followed by secondary antibody (1:10,000 dilution) of peroxidase-labeled goat anti-mouse conjugate (Amersham Biosciences); or 2) primary antibodies (1:1,000 dilutions) of anti-RGNNV B2, human SDHA, SDHB, SDHC, and SDHD polyclonal antibodies followed by a secondary antibody (1:5,000 dilution) of peroxidase-labeled goat anti-rabbit conjugate. Chemiluminescence indicative of antibody binding was captured on Kodak XAR-5 films (Eastman Kodak).

**Immunoelectron Microscopy**—GF-1 cells were transfected with pEYFP-B2 or pEYFP-C1, harvested 48 h after transfection, and then prepared for electron microscopy. Thin-section electron microscopy and immunogold labeling were carried out as described by McNulty et al. (39). The grids were stained with EYFP-specific monoclonal antiserum (1:1000) and then post-stained with 15-nm gold-labeled goat anti-rabbit immunoglobulin G conjugate (1:50).

**MitoTracker, Annexin V-Alexa 568 Staining, and Mitochondrial Membrane Potential Assay**—Live cells were labeled with the mitochondrial-specific dye (MitoTracker Red CMXRos) in accordance with the manufacturer’s instructions and as described previously (11, 38). For annexin-V-Alexa 568 staining, cells from the culture medium were washed with PBS, and incubated for 10–15 min with 100 μl of a HEPES-based annexin-V-Alexa 568 staining solution, according to the manufacturer’s instructions (Roche Applied Science). To assay the mitochondrial membrane potential, the culture medium was discarded from each dish, 500 μl of diluted MitoCapture reagent (Mitochondria BioAssay<sup>™</sup> Kit; BioVision, Mountain View, CA) was added, and the dishes were incubated at 37 °C for 15–20 min.

**ATP Assay**—The cellular ATP concentration was measured using an ATP Colorimetric/Fluorometric Assay Kit (BioVision). Cells (10^5) were lysed in 100 μl of ATP assay buffer, homogenized, and centrifuged (13,000 x g, 2 min, 4 °C) to pellet insoluble materials. The supernatants were collected and added to 96-well plates (50 μl per well) along with 50 μl/well of the reaction mixture (ATP probe, ATP Converter, Developer Mix in ATP assay buffer). The plates were incubated at room temperature for 30 min, while being protected from light, and absorbance in the wells was measured at 570 nm using a microplate reader. The absorbance of the no-ATP control was subtracted from each reading.

**NAD<sup>+</sup>/NADH Ratio Assay (Complex I Activity Assay)**—NADH concentration, NAD concentration, and their ratios were measured using an NAD<sup>+</sup>/NADH Quantification kit (BioVision). GF-1 and 239T cells were cultured in 35-mm Petri dishes for 24 h, transfected with pEYFP, EYFP-B2, and EYFP-B2(del) for 24 h, treated or not treated with the Complex I inhibitor diphenyleneiodonium (DPI, 100 μM) (Sigma) for 24 h, rinsed once with PBS, homogenized in buffer (10<sup>5</sup> cells in 400 μl of NADH/NAD extraction buffer), and centrifuged (13,000 x g, 5 min, 4 °C) to pellet insoluble materials. The supernatants were transferred to new labeled tubes and 50 μl of each was transferred to 96-well plates. NAD needs to be decomposed before NADH can be detected. To decompose NAD, 200 μl of each supernatant was transferred to Eppendorf tubes, heated to 60 °C for 30 min in a water bath, and cooled on ice. The resulting NAD-decomposed samples were transferred to 96-well plates (50 μl/well), treated with the NAD cycling mixture (100 μl/well), incubated at room temperature for 5 min to convert NAD to NADH, and treated with NADH developer (10 μl/well) at room temperature for 2 h. The OD at 450 nm of each well was read, and the NAD/NADH ratio was calculated as follows: (NAD<sup>+</sup> – NADH)/NADH.

**Succinate Dehydrogenase (SDH) Activity Assay (Complex II Activity Assay)**—GF-1 and 239T cells were cultured 24 h in 35-mm Petri dishes, transfected with pEYFP, EYFP-B2, or EYFP-B2(del) for 24 h, and treated or not treated with the Complex II inhibitor 3-nitropropionic acid (3-NP, 10 mM; Sigma) for 24 h. GF-1 cells and 239T cells (2 × 10<sup>5</sup>) were washed with PBS, homogenized in 0.1 ml of extraction buffer (20 mM Tris-HCl, pH 7.2, 250 mM sucrose, 2 mM EGTA, 40 mM KCl, 1 mg/ml of BSA) using a glass homogenizer, and centrifuged (2000 × g, 5 min, 4 °C) to pellet insoluble materials. The supernatants were placed into new labeled tubes, transferred to 96-well plates (90 μl/well), treated with a combination of 10× activity buffer (10 μl of 500 mM Tris HCl, pH 8.3, 5 mM EDTA, 100 mM succinate and 2-(p-iodophenyl)-3-(p-nitrophenoxy)-5-phenyltetrazolium chloride (20 μl of 10 mM), and incubated (room temperature, 90 min). The OD at 490 nm of each well was measured by a plate reader (39).

**Maintenance of Fish Embryos in Culture**—Techniques for the care and breeding of zebrafish have been previously described in detail (40). Embryos were collected from natural matings and maintained in embryo medium (15 mM NaCl, 0.5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 0.05 mM NaH<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 0.7 mM NaHCO<sub>3</sub>) at 28.5 °C. Embryos were staged according to standard morphological criteria (41).
RESULTS

Expression and Intracellular Localization of RGNNV B2—Western blot analysis detected expression of protein B2 in RGNNV-infected GF-1 cells at 24 h post-infection (Fig. 1A, lane 2) and increased expression at 48 and 72 h post-infection (Fig. 1A, lanes 3 and 4). Using immunofluorescence analysis (polyclonal rabbit antibody against protein B2), B2 (green fluorescence) was present in the cytoplasm of RGNNV-infected GF-1 cells at 24 (Fig. 1B, f), 48 (Fig. 1B, g, j, and k), and 72 h (Fig. 1B, h) post-infection but absent in the nucleus (Fig. 1B, j, indicated by arrows).

Cytosolic B2 was concentrated in mitochondria and appeared as dot-like structures (Fig. 1B, k, indicated by arrows). To track protein B2 directly, we transfected GF-1 cells with pEYFP-C1 vector containing the open reading frame of B2 cDNA and found transient expression of EYFP-B2 fused proteins. At 48 h post-transfection, Western blotting detected expression of the ~40.5-kDa EYFP-B2 fusion protein (Fig. 2A, lane 3) and 32-kDa EYFP (Fig. 2A, lane 2). Immunofluorescence revealed that EYFP-B2 fusion protein (Fig. 2B, b and d, indicated by arrows) but not EYFP alone (Fig. 2B, a and c) targeted mitochondria directly. Using immuno-EM, EYFP-B2 was found to be transiently expressed and to concentrate and aggregate in the mitochondrial matrix (Fig. 2B, f, indicated by arrows). EYFP-B2 and EYFP (Fig. 2B, e) were labeled with anti-EYFP IgG. Furthermore, we used MitoTracker Red CMXRsos to confirm the localization of protein B2 in mitochondria. Superimposition of the green fluorescent image of EYFP-B2 (Fig. 2B, h) with the MitoTracker Red dye image (Fig. 2B, i) resulted in a yellow green fluorescent image (Fig. 2B, j) and phase-contrast image (Fig. 2B, g) indicating colocalization in mitochondria. Taken together, these results demonstrated that EYFP-B2 targets mitochondria in either RGNNV-infected or EYFP-B2 overexpressing GF-1 cells.

B2 Has a Mitochondrial Targeting Peptide—Next, we determined the signal sequence used by B2 to target mitochondria.
during RGNNV infection. Signal sequence analysis, using two prediction databases (iPSORT and TargetP 1.1), identified the mitochondria target peptide of protein B2 (Fig. 3A). To locate the mitochondria targeting signal of protein B2, a sequential N-terminal to C-terminal deletion approach was used to construct EYFP-C1 vectors fused with different lengths of the B2 gene (Fig. 3B). Furthermore, we checked that the homology of the mitochondria-targeting motif of betanodavirus protein B2 were high (Fig. 3C), but very low homology in alphanodavirus (data not shown). The fusion proteins were expressed in GF-1 cells, and cellular expression of B2 protein fragments was assessed by Western blot analysis. Fig. 3D shows full-length B2 (lane 1), peptide containing aa 1–27 (lane 2), peptide containing aa 1–40 (lane 3), peptide containing aa 28–75 (lane 4), peptide containing aa 51–75 (lane 6), peptide containing aa 10–50 (lane 7), peptide containing a deletion of the aa sequence 40–51 (lane 8), and a peptide with 4 specific point mutations at V44VA, I45A, R52A, and R53A (lane 9).

Then, we examined the localization of these B2 fragments using MitoTracker (to detect mitochondrial localization) and fluorescence microscopy at 48 h post-transfection. EYFP-B2 (Fig. 3E, d–f), 1–50 (Fig. 3E, g–i), and 10–50 (Fig. 3E, j–l), but not EYFP (Fig. 3E, a–c) and B2-del41–50 (Fig. 3E, m–o), were detected in mitochondria as green, red, and yellow green fluorescence, respectively. Taken together, the data indicate that residues 41 to 50 of protein B2 have a mitochondrial targeting function.

Protein B2 Mitochondrial Targeting Can Induce Cell Death Mediated by Loss of MMP in Fish Cells—In our previous reports (11, 14), protein B2 was found to induce mitochondria-mediated cell necrosis and to target mitochondria. Here, we determined that the B2 targeting signal peptide (aa 41–50) is required for induction of MMP loss and necrotic cell death. Mitochondrial function was evaluated to determine whether mitochondrial targeting by protein B2 could induce the MMP loss. Using MitoCapture Reagent, mitochondria show red fluorescence (due to polymerization of green monomer) in healthy cells and green fluorescence in unhealthy cells. FLAG-B2 (Fig. 4A, d–f, indicated by arrows) but not FLAG (Fig. 4A, a–c, negative control) or FLAG-B2-del41–50 (Fig. 4A, g–i, function-less control) was able to induce MMP loss in trans-
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FIGURE 4. Protein B2 in mitochondria induces MMP loss and cell death. A, MMP is lost 48 h post-transfection with FLAG-B2 (d–f, indicated by arrows), but not with FLAG vector control (a–c) and truncated B2 (FLAG-B2-del; g–i). B, the percentage of cells showing MMP loss 48 and 72 h after transfection with FLAG vector control, FLAG-B2, and truncated B2. In this and all subsequent figures (unless otherwise noted) data are presented as the percentage of 200 cells (n = 200) at each time point determined in triplicate, with each point representing the mean of three independent experiments; error bars represent the S.E.; all data were analyzed using either paired or unpaired Student’s t tests as appropriate. *, p < 0.05 indicates a statistically significant difference between mean values of the groups. C, annexin-V staining showed that B2 specific targeting induces cell death in GF-1 cells transfected with (upper panel, phase-contrast images) EYFP (a), EYFP-B2 (d), EYFP-B2(1–50) (g), EYFP-B2(10–50) (j), and EYFP-B2(del) (m). Dead cells are red fluorescent (annexin-V positive; indicated by arrows) in the middle panel (EGFP, b; EYFP-B2, e; EYFP-B2(1–50), g and EYFP-B2(del), n). The merged yellow orange fluorescent images (lower panel) show the areas of colocalization (indicated by arrows). D, the death rate of GF-1 cells (n = 200) expressing various truncated forms of B2 at 48 h post-transfection.

Betanodavirus B2 Induces Novel Necrotic Cell Death—Mitochondria are the principal organelles involved in ATP production and regulate cell survival or death. To elucidate the cell death mechanism induced by protein B2, intracellular ATP levels were assayed in different cells including fish GF-1 cells and human 293T cells. Overexpression of EYFP-B2 in EYFP-B2-transfected cells (Fig. 5A) at 48 h post-transfection caused a marked decrease in intracellular ATP levels in GF-1 cells (61%) and 293T cells (45%) but not in GF-1 cells transfected with EYFP vector only. Transfection of EYFP-B2(del41–50) reduced ATP levels in both cell lines (40% for GF-1 cells and 30% for 293T cells) compared to EYFP-B2 (54% for GF-1 cells and 42% for 293T cells). These results suggest that B2-induced MMP loss and cell death are associated with the depletion of ATP levels.
levels slightly in GF-1 (91%) and 293T cells (93%). On the other hand, complex I inhibitor DPI (100 mM) and complex II inhibitor 3-NP (10 mM), used as positive controls, limited the amount of ATP depletion to 22% (GF-1 cells) and 31% (293T cells) for DPI and to 53% (GF-1 cells) and 56% (293T cells) for 3-NP.

Additionally, to determine whether B2 reduced ATP synthesis in the mitochondrial respiratory chain, we assayed the NAD⁺/NADH ratio for the NADH ubiquinone oxidoreductase activity of complex I. EYFP-B2 (0.95) and EYFP-B2 (del; 0.98) had no effect on the NAD⁺/NADH ratio in GF-1 cells at 48 h post-transfection (compare with EYFP (1.0) control, complex I inhibitor DPI (0.08) control, and complex II 3-NP (1.02)) (Fig. 5B). In the assay of SDH for complex II (complex II that consists of four protein subunits such as SDHA, SDHB, SDHC, and SDHD) activity, EYFP-B2 was found to markedly reduce complex II activity to 65% in GF-1 cells and 54% in 293T cells (compare with EYFP alone, all 100% in GF-1 and 293T cells; EYFP-B2(del) as a negative control, 92% in GF-1 cells and 93% in 293T cells; DPI control, 99% in GF-1 cells and 97% in 293T cells; 3-NP control, 35% in GF-1 cells and 43% in 293T cells; Fig. 5C). Then, we found that protein B2 targeting into mitochondria could induce complex II subunit proteins such as SDHB, SDHC, and SDHD partial degradation at 48 h in 293T cells. Western blot showing EYFP-B2 fusion protein (lane 2), EYFP (lane 1), and MCF-7 cell lysate as a positive control (lane 3) detected by anti-human SDHA, SDHB, SDHC, and SDHD polyclonal antibodies. Actin is the internal control.

**Protein B2 Can Deplete ATP Synthesis in Zebrafish Early Embryos (i.e. in Vivo)—**To examine whether protein B2 induces ATP depletion-mediated cell death, zebrafish embryos were injected with low (10 ng/µl) and high (30 ng/µl) doses of pEYFP-B2 and pEYFP and stained with acridine orange (1 µg/ml) for 10 h and assessed 24 hpf. In the results (Fig. 6A), EYFP-B2-injected embryos contained many dead cells at 24 hpf (low dose, Fig. 6A, b and e; high dose, Fig. 6A, c and f) and 24 hpf (low dose, Fig. 6A, h and k; high dose, i and l), as compared with EYFP-injected embryos at 24 hpf (high dose, Fig. 6A, a and d) and 24 hpf (high dose, Fig. 6A, g and j). Then, the percentage of dead embryos (Fig. 6B) indicated that
EYFP-B2 results in a high embryonic death rate at 10 hpf (10 ng/μl, 52.7%; 30 ng/μl, 86%) and 24 hpf (10 ng/μl, 76.3%; 30 ng/μl, 95.3%) when compared with embryos without transfection at 10 (1.6%) and 24 hpf (7.5%), and embryos transfected with EYFP only at 10 hpf (10 ng/μl, 16.8%; 30 ng/μl, 18.6%) and 24 hpf (10 ng/μl, 27.2%; 30 ng/μl, 23.5%). Furthermore, low and high doses of EYFP-B2 fusion proteins reduced intracellular ATP levels to 53 (low dose) and 50% (high dose) at 10 h post-infection and to 46 (low dose) and 31% (high dose) at 24 h post-infection as compared with wild type (WT) control at 10 (100%) and 24 h post-infection (105.8%); and EYFP at 10 h (low dose, 103%; high dose, 102%) and 24 h (low dose, 101%; high dose, 103%) post-infection. The results in Fig. 6, A and B, indicate that ATP depletion is required for B2-induced embryonic cell death.

**DISCUSSION**

Betanodavirus causes viral nervous necrosis and the infected fish to lie on its side, float belly up, or swim abnormally (such as in circles or to the right). Histopathological changes include extensive cellular vacuolation and necrotic neuronal degeneration in the central nervous system and retina (44). The molecular mechanisms involved in the pathogenesis of this disease are still unknown.

In the present study, the sequence (*RTFVISAHAA*) in RGNNV protein B2 was identified as the molecular signal used to target the mitochondrial matrix. Protein B2 induced MMP loss in both fish and human cells, followed by mitochondrial ATP depletion. Furthermore, this novel necrotic death factor triggered ATP depletion in embryos and consequently embryonic cell death. These results may be the first to elucidate the
mechanism of betanodavirus-induced neuronal degeneration in the central nervous system and retina (44).

A Motif of the RGNNV B2 Protein Is Required for Mitochondrial Targeting during the Early Replication Stage of Infection—
B2 (a 75-amino acid protein translated from subgenomic RNA3) (13) may either be a suppressor of host siRNA silencing (12, 13) or a necrotic death factor (11, 14). Mitochondria appear to be the targets of B2. In our system, B2 was expressed early during replication at 24 h post-infection (Fig. 1A, lane 2). Immuno-fluorescence assay showed that B2 targets mitochondria-like particles (Fig. 1B, j and k) and to some extent exists in the nucleus at 48 h post-infection, where it may act as a host siRNA silencing suppressor (Fig. 1B, j, indicated by arrows). Using tag EYFP tracing (Fig. 2A, f and h) and MitoTracker staining (Fig. 2B, i and j), protein B2 was shown to target mitochondria. Furthermore, we wanted to know whether B2 localized in the outer membrane or inner membrane of mitochondria because protein function differs between them (29). Immuno-EM staining showed that most protein B2 localized and formed small complexes in the mitochondrial matrix (Fig. 2B, f, indicated by arrows).

Most mitochondrial proteins are synthesized in the cytoplasm as precursors, which are post-translationally translocated into either the outer or inner membrane of the mitochondria (45–47). These proteins usually have an N-terminal cleavable sequence that is either positively charged or hydrophobic and usually forms an amphipathic helix (48–52). Proteins targeted to mitochondrial membrane often contain an N- or C-terminal targeting sequence and anchoring signal sequence (45, 53), but the midsequence location of the B2 targeting motif is not well known. In our study, the targeting motif (41RTFVISAHAA50) of protein B2 contained 10 amino acid residues and played a very important function early during RGNNV infection. Deletion of this sequence resulted in loss of MMP (Fig. 4A, g–i) and loss of necrosis-inducing activity (loss of SDH activity; Fig. 5C) in fish GF-1 cells. The positively charged and hydrophobic residues within the signal peptide are essential for mitochondrial targeting (45, 52, 54). When the positively charged residues (Arg52 and Arg53) and the two hydrophobic residues (Val44 and Ile45) near the signal sequence were changed to uncharged Ala residues (Fig. 3A), targeting to the mitochondrion was lost, implying that these 4 residues (Val44, Ile45, Arg52, and Arg53) of protein B2 are necessary for targeting (Fig. 3E, g–i) and necrosis induction (Fig. 4C, j–l).

Protein B2 Induces MMP Loss and Produces a Mitochondrial Energy Crisis—The mitochondria are vital cellular machines for maintaining cellular energy and use oxygen to produce ATP through a process known as oxidative phosphorylation (16). The inner mitochondrial membrane contains a respiratory chain of four multisubunit protein complexes that release energy used to pump protons across this membrane. The created electrochemical gradient of protons and resulting mitochondria membrane potential (MMP, ΔΨ) drives ATP formation from ADP and phosphate (16). Thus, damage to mitochondria plays an important role in a wide range of human diseases (20, 55). Mitochondrial disorders often present as neurological disorders such as Parkinson disease (21, 22), Alzheimer disease (23–27), and Huntington disease (28). A variety of studies have suggested that neural cell death could be due to mitochondrial energy deficits. Cell death could be mediated by loss of MMP, release of cytochrome c, and depletion of ATP (56).

In our system, protein B2 entered mitochondria to inhibit the activity of complex II (Fig. 5C) but not complex I (Fig. 5B), and thereby deplete ATP (Fig. 5A) and degrade the component proteins of complex II as SDHB, SDHC, and SDHD degradation, causing MMP loss and depletion of ATP. Finally, cells undergo a bioenergetic crisis, which triggers cell death through necrosis.

Viral Proteins Induce Mitochondrial Disruption and Cellular Death—Recently, some well known DNA and RNA viruses have been shown to cause mitochondria-mediated cell death. A number of viral polypeptides modulate apoptosis by either increasing or decreasing MMP through modification of the outer mitochondrial membrane or by acting on upstream/downstream steps of the cell apoptotic cascade (29). In contrast, only a few viral proteins (e.g. HCV NS4A (57) and HIV-1 Tat (58)) accumulate in mitochondria, induce MMP loss, and activate caspase-3-dependent cell death.

Chen et al. (38) showed that the RGNNV TN1 strain induced apoptosis and post-apoptotic necrosis in a grouper liver cell line (GL-av). RGNNV infection in fish cells induced loss of MMP, which was blocked by the mitochondrial membrane permeability transition pore inhibitor BKA (38) as well as the Bcl-2 member protein zBcl-xL (11). Moreover, they showed that cell death was dependent on viral RNA replication, indicating the need for the expression of the viral death factor(s), protein α/B2, before cyto-
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chromosome c could be released and caspase-3-independent signaling could be activated in mitochondria (59). Furthermore, in betanodavirus-infected cells, both the viral capsid protein α (8, 9) and non-structural protein B2 (11, 14) triggered loss of MMP and necrosis. Protein α induces MMP loss, cytochrome c release, and increased caspase-8 and -3 activation, and zfBcl-XL blocks these post-apoptotic necrosis processes thereby rescuing virus-infected cells (8, 9). On the other hand, protein B2 up-regulates the pro-apoptotic gene Bax to enhance MMP loss, but does not induce release of cytochrome c and activation of caspase-3-independent signaling (11). Thus, necrosis can be blocked by B2-specific siRNA and the anti-apoptotic protein zfBcl-XL (11, 14). In the present study, protein B2 disrupted mitochondria via targeting (Fig. 3E) and inhibition of complex II activity (Fig. 5, C and D). The resulting MMP loss (Fig. 4A) and ATP depletion (Figs. 5A and 6B) contributed to necrosis induction, which is a strategy used by viruses to modulate the rate of cell death. As summarized in Fig. 7, protein B2 is a non-structural protein expressed early in the infection cycle. It localizes primarily in the mitochondria and secondarily in the nucleus. A targeting signal sequence of 10 amino acids in the nucleus. A targeting signal sequence of 10 amino acids in the nuclear localization signal (30). Protein B2 inhibits the nucleus. A targeting signal sequence of 10 amino acids in the nuclear localization signal (30). Protein B2 inhibits the complex II activity leading to induction of MMP loss and ATP depletion culminating in a bioenergetic crisis and necrosis. These findings may provide new insights into the molecular pathogenesis of RNA viruses and suggest new clinical treatments.

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