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Identification and Functional Analysis of Salmon Annexin 1 Induced by a Virus Infection in a Fish Cell Line▽

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In this study, we investigated changes in protein expression of fish cells induced by infection of infectious pancreatic necrosis virus (IPNV) using two-dimensional electrophoresis and matrix-assisted laser desorption–time of flight proton motive force analysis and identified a novel type of salmon annexin 1 that is induced in fish cells by infection with IPNV. Northern blotting showed that this annexin is overexpressed in IPNV-infected cells compared to control cells, and further analysis revealed that it has a 1,509-bp full-length cDNA sequence with an open reading frame encoding 339 amino acids (GenBank accession no. AY944135). Amino acid sequence analysis revealed that this protein belongs to the annexin 1 subfamily. By applying RNA interference, the mRNA levels of salmon annexin 1 were suppressed and, under these conditions, apoptosis of IPNV-infected cells was significantly increased. While small interfering RNA (siRNA) treatment did not affect the levels of the viral proteins significantly until 10 h postinfection, it reduced the titer of extracellular virus to 25% of that of a scrambled siRNA-treated control. These data provide evidence of an antiapoptotic function for salmon annexin 1 that is important for IPNV growth in cultured cells.

Infectious pancreatic necrosis virus (IPNV) is a member of the genus *Aquabirnavirus*, *Birnaviridae* family, and has a bisegmented, double-stranded RNA genome. Two RNA genome segments encode a total of five proteins. The larger IPNV genome segment, A, encodes VP2 (major outer capsid), VP4 (protease), VP3 (submajor capsid), and VP5 (nonstructural protein), whereas segment B encodes the VP1 polypeptide, which is an RNA-dependent RNA polymerase. IPNV causes an acute and contagious disease in a number of economically important hatchery-reared trout and salmon (10). Infected cells display a marked necrosis, but induction of cellular apoptosis is initiated before cell death can occur by necrosis (18).

It is now apparent that apoptosis in virus-infected cells can induce premature death of the host cell, which would impair virus production, and apoptosis is clearly a mechanism used by the virus-infected host cell itself as part of the antiviral response. IPNV-infected cells induce apoptotic responses via Bad expression (20). However, a number of viruses encode proteins that suppress apoptosis in order to promote successful viral replication and pathogenesis (31). Additionally, virus-modulated expression of antiapoptotic proteins within host cells can also be used to delay cell death and ensure successful viral propagation (12, 27).

Annexins (also commonly called lipcocortins) are a family of structurally related proteins whose common properties are the binding of both phospholipids and cellular membranes in a calcium-dependent manner. Annexins have been found in many species of eukaryotes, including *Xenopus* (22), *Drosophila melanogaster* (23), *Dictyostelium* (11), *Caenorhabditis elegans* (8), *Neurospora* (1), *Giardia* (15), zebrafish (13), and all of the plant types (9) so far examined. Structurally annexins can be characterized as having a core of either four or eight conserved domains, each containing about 70 amino acids. Although the annexins contain highly conserved sequence, they have been divided into at least 13 subfamilies (A1 to A13) in the vertebrate species. This diversity within the annexin family of proteins is due to unique N-terminal domains, which convey specific and diverse biological functions to each of the different family members (17).

Human annexin 1, a 37-kDa species, mediates the anti-inflammatory actions of glucocorticoids that inhibit phospholipase A2. It is also involved in diverse cellular roles, including membrane fusion, differentiation, exocytosis, calcium channels, and interaction with cytoskeletal proteins (16). In addition, human annexin 1 has been reported as a stress protein induced by heat, oxidative stress, and a sulfhydryl-reactive agent (34).

In this study, we have adopted a proteomic method to identify alterations in protein expression patterns in fish cells undergoing apoptosis as a result of IPNV infection. From this screening, we have identified a novel annexin family protein, salmon annexin 1, that is overexpressed in IPNV-infected CHSE-214 cells. In addition, we determined the full-length cDNA sequences of salmon annexin 1 and its function in IPNV growth by using RNA interference (RNAi).

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MATERIALS AND METHODS

Cell and virus. CHSE-214 (Chinook Salmon Embryos) (26) cells were cultured at 20°C in Eagles' minimum essential medium containing 10% fetal bovine serum, 50 mg/liter streptomycin, and 80 mg/liter gentamicin. The virus used in these experiments was a Korean isolate, IPNV-DRT (7).

Protein extraction and 2-DE. The sample preparation of proteins in CHSE-214 cells and two-dimensional gel electrophoresis (2-DE) were described by Carroll et al. (3). Briefly, 2.5 mg of total proteins for preparative runs was mixed with a rehydration buffer to a total volume of 350 μl. The mixtures were pipetted into immobilized pH gradient (IPG) strip holder channels. Using the IPG dry strips at pH 4 to 7 (180 by 5 by 0.5 mm), isoelectric focusing (IEF) was run on an IPGphor isoelectric focusing system (Amersham Bioscience). The voltage was progressively increased from 500 to 5,000 V during the first 3 h, followed by 8,000 V for 80 to 100 kV · h. The temperature was maintained at 20°C. Then, IPG strips were run onto vertical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels in a Bio-Rad Protean XL electrophoresis. A 2-DE pattern obtained after Coomassie blue staining was scanned using an ImageScanner (Amersham Pharmacia Biotech). Spot detection and matching were performed using ImageMaster 2-D Elite (Amersham Bioscience) to normalize the protein spots of each gel. A selected spot was analyzed by matrix-assisted laser desorption–time of flight mass spectrometry (MALDI-TOF MS) (In2Gen Co., Korea), and acquired mass fingerprint data were analyzed with the MASCOT program (Matrix Science).

Construction of cDNA library. The CHSE-214 cells were harvested at 24 h after IPNV infection. Total nucleic acids were extracted using the guanidium thiocyanate-acid phenol-chloroform method, and mRNA was purified using oligo$(dT)_{30}$-latex suspension (QIAGEN). The cDNAs were synthesized using a cDNA cloning kit (Stratagene ZAP-cDNA synthesis kit). The cDNA was ligated into the EcoRI and XhoI sites of lambda ZAP vector and packaged into phage lambda. The resultant phage lambda was infected into the *Escherichia coli* XL1-Blue MRF′ strain according to the manufacturer's instructions.

Salmon annexin 1 cDNA isolation, sequencing, and characterization. Degenerate oligonucleotides were designed based on the amino acid sequence of annexin, which was acquired from MALDI-TOF MS analysis, and the homologous regions of annexin max 3 of *Oryzias latipes* (killifish medaka) and annexin 1 of *Homo sapiens*. The forward degenerate oligonucleotide Anx F (5′-GGTGT GGATGARRMMACY-3′) was designed based on amino acid sequence GVDENT, and the reverse degenerate oligonucleotide Anx R (5′-ACYAARG GAGAYTAYGAG-3′) was designed based on amino acid sequence TKGDYE. Degenerate sequences are denoted by "Y" for T or C, "R" for A or G, and "M" for A or C. PCR was carried out using as a template cDNA from IPNV-infected CHSE-214 cells. The gene amplification reaction conditions were as follows: 1 cycle of 94°C for 5 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and 1 cycle of 72°C for 5 min. A unique 843-bp product was isolated from the gene amplification reaction using a primer set, cloned into pEZ-T vector (RNA Inc., Suwon, Korea), and sequenced using the chain-terminating, dideoxy method. To obtain the 5′ end of the annexin gene, the T3 primer within lambda ZAP vector and the annexin-specific 20-bp primer Anx R2 (5′-AAACGTCTTC TTGTACTCCT-3′) were selected as forward and reverse primers, respectively. To obtain the 3′ end of annexin, the annexin-specific 20-bp primer Anx F2 (5′-AGAGACAGCAGATCAAAGCT-3′) and the T7 primer within the lambda ZAP vector were selected as forward and reverse primers, respectively. Reaction conditions were the same as described above. The PCR product was reamplified by a nested gene-specific primer of annexin: annexin forward primer Anx F3 (5′-AATATGACGCCCAACAGC-3′) and annexin reverse primer Anx R3 (5′-TCGACTTCAGACCTGCTC-3′). Reaction conditions were the same as described above. We cloned PCR products into pEZ-T vector and sequenced them using the chain-terminating, dideoxy method. Based on the obtained full-length annexin sequence, protein database searches were performed with the National Center for Biotechnology BLAST Network services. The comparison and alignment of the annexin translation product were performed using the ClustalW multiple-alignment program.

Northern blot analyses. Total RNA was extracted with the TRIzol reagent (Invitrogen) according to the manufacturer's instructions from mock- and IPNV-infected CHSE-214 cells at time points of 0, 5, 10, 15, and 20 h after infection. RNA samples (20 μl) were transferred to Hybond-N+ nylon membranes (Amersham Bioscience). A 571-bp PCR product using the Anx F3-Anx R3 primer set was used as a probe. This probe was labeled with [α-^{32}p]dATP (Amersham Bioscience) using the random prime labeling kit (Ambion).

Plasmid construction and transfection. CHSE-214 cells that overexpressed salmon annexin 1 were generated using the pCMV-Tag1 vector (Stratagene). Full-length human cDNA of salmon annexin 1 was cloned by reverse transcription-PCR (RT-PCR) from the RNA of CHSE-214 cells using the forward primer 5′-GGATCCATGTCCTTCATCGCAGCCTTC-3′ and the reverse primer 5′-C TCGAGGTTGTCACTTCCACACAGGGC-3′ and subcloned into the pCMV-Tag1 vector. A total of 1.5×10^7 cells were electroporated with 20 μg of pCMV-Anx1 at 500 V, 975 μF with a Gene Pulser electroporator II (Bio-Rad). After transfection, CHSE-214/Anx1 cells stably transfected with salmon annexin 1 were selected by adding 100 μg of G418 (Invitrogen)/ml 3 days after transfection and stably transfected cells were tested for an overexpression of salmon annexin 1 by RT-PCR using Anx F3 and Anx R3 primers. A control cell line, CHSE-214/pCMV, was generated by transfection with pCMV-Tag1 vector.

The sequence of salmon annexin 1 small interfering RNA (Anx1 siRNA [5′-AUUGAACACCGCGACAUCGdTdT-3′]) was derived from positions 54 to 34 upstream of the start codon of annexin mRNA. A scrambled siRNA (Sc siRNA) (5′-UCAGCAUACGAUCGCAGACdTdT-3′) with the same nucleotide composition as Anx1 siRNA, but which lacks significant sequence homology to the salmon annexin 1, was also designed as a negative control. Both siRNAs were purchased from Dharmacon Research (Lafayette, CO). A total of 1.5×10^6 cells were electroporated with 2 nmol of Anex1 siRNA or 2 nmol Sc siRNA at 500 V, 975 μF with a Gene Pulser electroporator II (Bio-Rad). After transfection, RNA was prepared from 24 h to 72 h and subjected to RT-PCR for salmon annexin 1 expression using Anx F3 and Anx R3 primers. β-Actin primers ACTB-F (5′-GGACTTCGAGCAAGAGATGG-3′) and ACTB-R (5′-AGCAC TGTGTTGGCGTACAG-3′) were used as controls for measuring cDNA synthesis efficiency by RT.

Cell viability and virus endpoint titration. At 36 h after siRNA transfection, cells were infected with IPNV, and at 36 h postinfection (p.i.), the viability of cells was measured by the 3-[4, 5-dimethylthiazolzyl]-2, 5-diphenyltetrazolium bromide (MTT) method according to the manufacturer's protocol (Sigma). At 36 h p.i., culture supernatants were collected, the virus titer in the supernatant was determined by the endpoint dilution technique, and the number of 50% tissue culture infectious doses ($TCID_{50}$)/ml was determined by the method of Reed and Muench (33).

Antisera and Western blotting. Rabbits were immunized with 100 μg of purified recombinant VP2 (29) with Freund's complete adjuvant. After two more immunizations with 100 μg of VP2 with Freund's incomplete adjuvant, the serum was collected after centrifugation and stored at −20°C. Cells were washed twice with cold phosphate-buffered saline, and 30 to 50 μg of protein was resolved by SDS-PAGE, transferred onto Hybond-P membranes (Amersham Biosciences, Inc.), and probed with appropriate dilutions of rabbit anti-VP2 antisera. Immunoreactivity was detected using the ECL enhanced chemiluminescence detection system (Amersham Biosciences). The films were exposed at multiple time points to ensure that the images were not saturated.

Semiquantitative RT-PCR for genomic RNA analysis. Semiquantitative RT-PCR was carried out to detect the genomic RNA. The following PCR primers were designed from nucleotide sequences in the GenBank/EMBL databases of IPNV-DRT (GenBank accession no. D26526): IPNV-F, 5′-GAGCCTGCAGG AGAATCAAG-3′; and IPNV-R, 5′-ATCGGTTTTCGCACGTAGTC-3′. cDNA was synthesized using IPNV-F primer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Semiquantitative RT-PCR was carried out using *Taq* polymerase (QIAGEN, Hilden, Germany) and IPNV-F and IPNV-R primers. The gene amplification reaction conditions were as follows: 1 cycle of 94°C for 5 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and 1 cycle of 72°C for 5 min.

TUNEL staining. Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining was conducted using an in situ cell death detection kit, TMR Red, according to the protocol supplied by the manufacturer (Roche Molecular Biochemicals). Briefly, cells were plated in a 24-well plate at 2×10^5cells/ml in Eagle's minimum essential medium. On the following day, the cells were infected with IPNV and, at the indicated time, cells were harvested and fixed with 2% paraformaldehyde solution and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After being washed twice with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.2), cells were incubated in a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and tetramethyl-rhodamine-dUTP. Cells were analyzed for fluorescence intensity using a fluorescence-activated cell sorting flow cytometer (Becton Dickinson, Inc.) and FluoView 500 confocal microscope (Olympus).

Measurement of intracellular calcium. The transfected cells were plated in Costar Black 96-well plates and treated with IPNV. Prior to the experiments, cells were loaded in the culture medium described above containing 2.5 mM probenecid, 4 μM Fluo-4 AM (Molecular Probes), and 0.01% pluronic acid at 18°C for 30 to 40 min. Plates were washed three times with Hank's basal saline solution ([Gibco]) containing 20 mM HEPES (Sigma), 1 mM Ca^{2+}, 1 mM Mg^{2+},

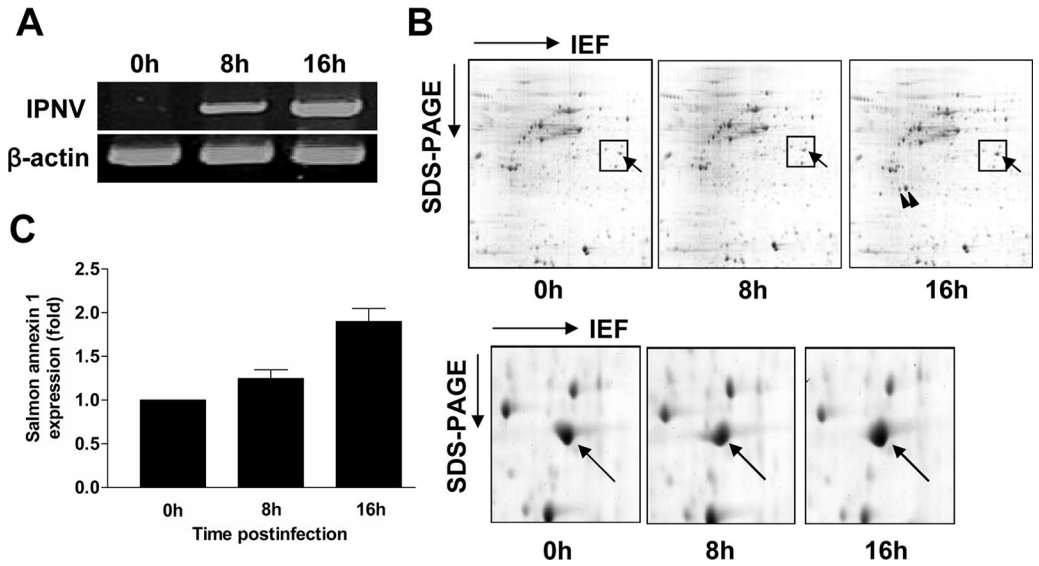


FIG. 1. Partial 2-DE gel image and histogram of salmon annexin 1 at different time points. CHSE-214 cells were harvested at 0, 8, and 16 h after IPNV infection. (A) Total RNA was extracted from IPNV-infected CHSE-214 cells. Genomic RNA replication of IPNV was determined by semiquantitative RT-PCR analysis. (B) Proteins were separated by 2-DE as described in Materials and Methods and stained with Coomassie blue. Arrows and arrowheads indicate salmon annexin 1 and IPNV proteins, respectively, induced in virus-infected CHSE-214 cells, and these spots were identified by MALDI-TOF MS. (C) Histogram showing the expression level of salmon annexin 1 in IPNV-infected CHSE-214 cells. The expression level of salmon annexin 1 at 0 h was defined as 1. The results are presented as means ± standard deviations of three independent experiments.

and 2.5 mM probenecid. Fluorescence was monitored in a Wallac Victor 2 plate reader (Perkin-Elmer Life Sciences) by excitation at 485 nm and reading the emission at 510 nm.

Statistical analyses. Experimental data are expressed as the mean ± standard error. Each experiment was performed independently at least three times. Statistical analyses using Student's *t* test were performed using Sigma Plot software (SPSS, Inc., Chicago, IL). The *t* test was used to compare the distribution of individual variables. A two-tailed *P* value of <0.05 was considered statistically significant.

RESULTS

Identification of the novel annexin protein. We identified a differentially expressed protein between mock-infected and IPNV-infected CHSE-214 fish cells using 2-DE and MALDI-TOF MS. Both mock- and IPNV-infected cells were harvested at 8-h intervals after infection, and IPNV genomic RNA replication was monitored by RT-PCR (Fig. 1A). The virus-infected cells showed cytopathic effects (CPE) at 12 h p.i. with a multiplicity of infection of 3, and all infected cells had CPE and eventually underwent cell death at 30 h p.i. The 2-D gel patterns obtained after Coomassie staining were scanned using an ImageScanner (Amersham Pharmacia Biotech). We used a housekeeping protein to normalize the intensity levels of the protein spots on each gel with ImageMaster 2-D Elite software (Amersham Pharmacia Biotech) and identified differentially expressed proteins. Figure 1B shows a partial 2-D gel image over the given time course, and proteins induced in IPNV-infected cells are indicated by arrows and arrowheads. The comparison of the intensities of these proteins between mock- and IPNV-infected cells showed that the arrow-indicated protein increased about 1.9-fold (Fig. 1C) and the arrowhead-indicated protein was detected only after IPNV infection.

The two proteins induced in IPNV-infected cells were analyzed by MALDI-TOF MS. While the proteins indicated by arrowheads were identified to be viral proteins of IPNV, the arrow-indicated protein was cellular protein, and, thus, we focused on this IPNV-induced cellular protein. The peptide sequence of the IPNV-induced protein was found to be "GVDENTIIEILVK" via MALDI-TOF MS analysis. A similarity search using "search for short nearly exact matches" of BLAST revealed that this peptide sequence showed the closest match with annexin max 3 of *O. latipes* and a significant match with human annexin A1.

Salmon annexin 1 cDNA isolation and cloning. Degenerate oligonucleotides were designed based on the amino acid sequences of the IPNV-induced annexin and of the homologous region of annexin max 3 of *O. latipes* and human annexin 1. PCR was then carried out using a cDNA library from IPNV-infected CHSE-214 cells as a template and generated a unique 843-bp product. To obtain the 5′ end of this putative annexin gene, we performed PCR analysis using a T3 forward primer, which is contained within the lambda ZAP vector of the cDNA library, and a reverse primer of 20 bp annealing a site located within the partial cDNA of the IPNV-induced annexin (5′-AACGTCTTCTTGTACTCCT-3′). To obtain the 3′ end of the IPNV-induced annexin, a 20-bp forward primer was selected from the partial cDNA of the annexin (5′-AGAGACA GCAGATCAAAGCT-3′) and a T7 reverse primer was chosen from the lambda ZAP vector.

We obtained a 1,509-bp full-length cDNA clone which contained an open reading frame (nucleotides 137 to 1156) encoding 339 amino acids with a calculated molecular mass of 37kDa (GenBank accession no. AY944135). In order to determine the relationship between the IPNV-induced annexin and previously reported annexins, we compared the amino acid sequences of the IPNV-induced annexin to those of 28 other annexins available in GenBank, including 12 human annexins

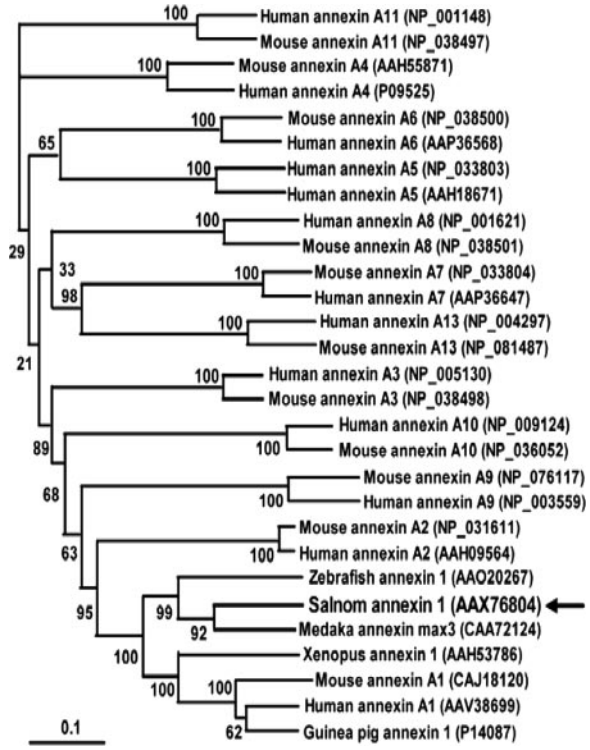


FIG. 2. Phylogenetic analysis of salmon annexin 1. The amino acid sequence of salmon annexin 1 was compared with those from 28 annexins. The GenBank accession numbers for proteins are indicated in parentheses. The multiple alignments of the annexin amino acid sequences were performed with ClustalW, and phylogenetic analysis was constructed using TreeView. The percentages of bootstrap values are indicated on the tree.

and 12 mouse annexins (A1 to A11 and A13). In the phylogenetic tree, the annexins used in the multiple alignments were divided into 12 groups. Among the 12 groups, the IPNV-induced annexin is clustered within a group which includes human annexin A1 and mouse annexin A1 (Fig. 2). This suggests the annexin induced in salmon cells by IPNV infection belongs to the annexin 1 subfamily, and we named it "salmon annexin 1." Human annexin 1 has four homologous repeats (numbered I to IV) and six calcium binding sites. Three type II calcium binding sites [(K,R)-(G,R)-G-T] are located at repeats II, III, and IV, and the other three type III calcium binding sites are at repeats I and IV (35, 41). Similar to the human annexin A1, the salmon annexin 1 has six calcium binding sites in four repeats (Fig. 3).

Induction of salmon annexin 1 transcription by IPNV infection. To determine whether the cloned gene showed an expression pattern matching that of the 2-D gels, the amount of mRNA was measured at various times after the virus infection by Northern blotting of RNA. In mock-infected normal cells, the transcripts of salmon annexin 1 were expressed constitutively at a negligible level throughout the incubation period of 20 h. However, in IPNV-infected cells, the expression level of the salmon annexin 1 significantly increased from 10 h p.i. (Fig. 4A and B). This result indicates that the expression pattern of the salmon annexin 1 gene coincided with that of protein determined by 2-DE. IPNV genomic RNA was detected at 10 h p.i., and after that its level increased (Fig. 4C). A one-step growth curve of IPNV revealed that there was no increase in the virus titer until 10 h p.i. and the virus titer rose from 15 h p.i. (Fig. 4D). These data suggested that the expression of salmon annexin 1 begin to increase before the release of viral progeny from IPNV-infected cells. However, it is not clear whether its expression begins ahead of IPNV genomic RNA replication.

Silencing of salmon annexin 1 expression using siRNA. To examine whether a reduction in salmon annexin 1 expression affects apoptosis in virus-infected fish cells, we designed a siRNA against salmon annexin 1. The Anx1 siRNA sequence was selected from positions 54 to 34 upstream of the start codon (Fig. 5A) and was subjected to a BLAST search to ensure exclusive targeting. An Sc siRNA was used as a control for nonspecific effects due to transfection of duplex RNA. The levels of inhibition of the salmon annexin 1 mRNA expression were demonstrated by RT-PCR from 24 h to 72 h posttransfection (Fig. 5B). The Anx1 siRNA treatment reduced the expression level of the salmon annexin 1 to less than 8% of that of the Sc siRNA-treated control at 48 h posttransfection. These results indicate that transient expression of Anx1 siRNAs confers specific inhibition of the expression of salmon annexin 1.

Treatment of salmon annexin 1 siRNA increases IPNV-induced cell death. To study the effect of Anx1 siRNA on the cell death induced by IPNV infection, Anx siRNA-transfected CHSE-214 cells were infected with IPNV. At 30 h p.i., CPE was determined by microscopic observation. Microscopic examination revealed that the CPE was accelerated in Anx1 siRNA-transfected cells relative to the control cells (Fig. 5C). To determine the percentage of cell death induced by Anx1 siRNA, Anx1 siRNA-treated and Sc siRNA-treated cells were stained by TUNEL, and apoptosis in cells was quantified by flow cytometry. At 10 h p.i., the apoptosis of IPNV-infected cells only slightly increased in both untreated and Sc siRNA-treated cells but significantly increased in Anx1 siRNA-treated cells (mock, 1.3% ± 0.5%; IPNV, 5.1% ± 1.0%; IPNV plus Anx1 siRNA, 20.7% ± 6.4%; IPNV plus Sc siRNA, 5.6% ± 1.6%) (Fig. 5D). At 30 h p.i., Anx1 siRNA treatment also increased the apoptosis of IPNV-infected cells compared with untreated and Sc siRNA-treated cells (mock, 3.1% ± 0.7%; IPNV, 43.0% ± 7.1%; IPNV plus Anx1 siRNA, 66.7% ± 5.9%; IPNV plus Sc siRNA, 48.3% ± 7.6%) (Fig. 5D). These data suggest that salmon annexin 1 mediates antiapoptotic function in IPNV-infected cells.

Treatment with salmon annexin 1 siRNA inhibits the growth of IPNV in CHSE-214 cells. To determine the effect of down-regulation of salmon annexin 1 on growth of IPNV, cells were treated with Anx1 siRNA and then infected with IPNV at a multiplicity of infection of 3 $TCID_{50}$ per cell. Culture fluids were collected at 72 h postinfection, and the amount of extracellular infectious virion was quantified using a $TCID_{50}$ assay (Fig. 5E). The Anx1 siRNA treatment reduced the virus titer to 25% of that of the Sc siRNA-treated control. These results suggest that salmon annexin 1 supports the growth of IPNV by prevention of apoptosis.

To test whether or not overexpression of salmon annexin 1 affected the growth of IPNV, a stable transfectant derivative of CHSE-214, CHSE-214/Anx1 was established. As a negative

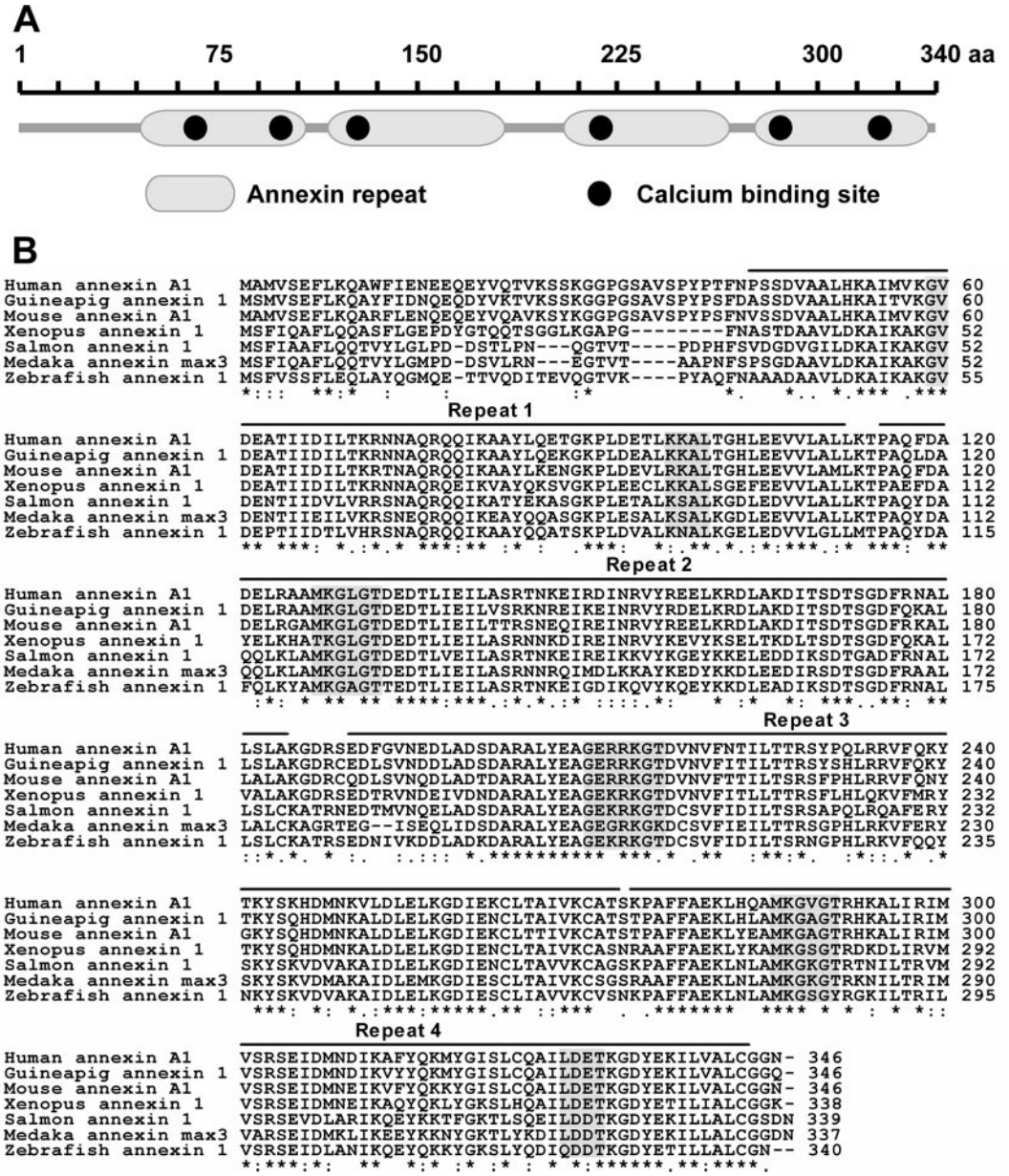


FIG. 3. Comparison of the amino acid sequence of salmon annexin 1 with those of other annexins. (A) Schematic representation of regions of annexin 1 showing locations of four annexin repeats and six calcium binding sites. (B) The amino acid sequence of salmon annexin 1 is aligned with those of six annexins that belong to the subfamily annexin 1. GenBank accession numbers for proteins are the same as in Fig. 2. The straight lines indicate the four homologous repeats, and the six calcium binding motifs are shaded. Dashes indicate gaps introduced to optimize the alignment. Asterisks denote identical residues in all sequences in the alignment, colons indicate conserved residues, and dots indicate semiconserved substitutions.

control, CHSE-214/pCMV cells stably transfected with empty vector were included. Overexpression of salmon annexin 1 in CHSE-214/Anx1 cells was confirmed by RT-PCR. Salmon annexin 1 expression of CHSE-214/Anx1 cells was increased by 1.7-fold compared to that in CHSE-214 cells (Fig. 5E). As shown in Fig. 5E, overexpression of salmon annexin 1 did not affect the virus titer.

It is possible that the Anx1 siRNA blocks the viral growth as a consequence of its reduction of expression levels of viral genes. To examine this possibility, CHSE-214 cells transfected with Anx1 siRNA were infected with IPNV and harvested at 10 h p.i. just before the progeny virions were released and expression levels of VP2 and VP3 proteins were determined using Western blot analysis, respectively. Anx1 siRNA treatment did not significantly affect the expression levels of VP2 and VP3 proteins (Fig. 5F). These results suggest that Anx1 siRNA does not inhibit the growth of IPNV through its reduction of expression levels of viral proteins.

Salmon annexin 1 alters intracellular Ca^{2+} concentration on viral infection. Annexin 1 is known to be involved in alter-

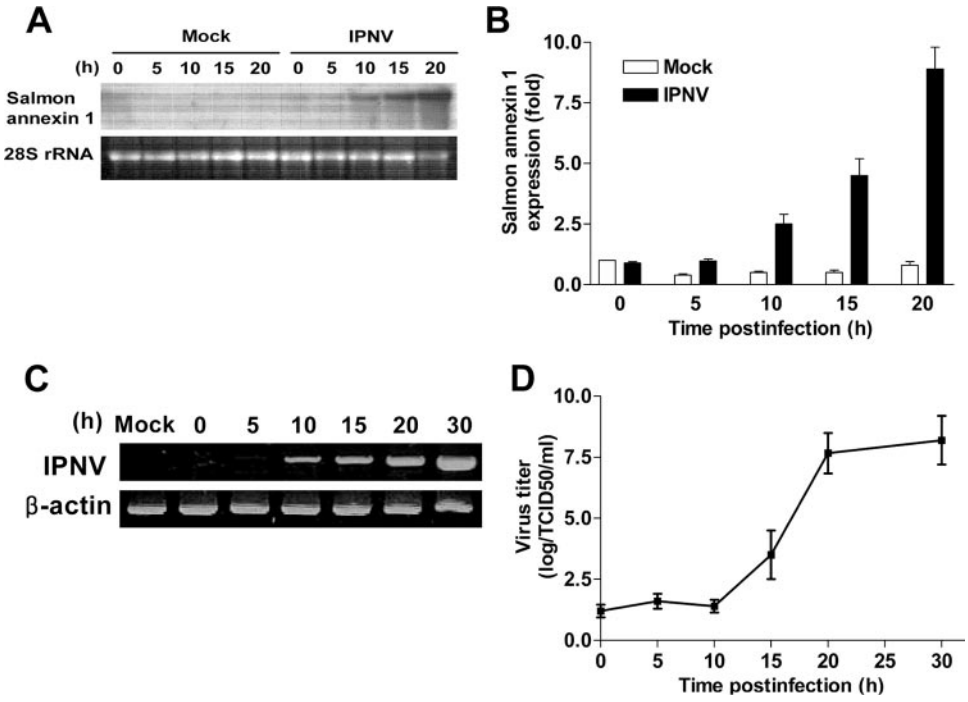


FIG. 4. Analysis of the salmon annexin 1 expression in IPNV-infected cells. CHSE-214 cells were harvested at 5-h intervals after IPNV infection. (A) Northern blot analysis of salmon annexin 1 expression. Total RNA was extracted from mock- or IPNV-infected CHSE-214 cells and hybridized with [α-^{32}P]dATP-labeled probe for salmon annexin 1. (B) Histogram showing the expression level of salmon annexin 1 in IPNV-infected CHSE-214 cells. The expression level of salmon annexin 1 in mock-infected cells at 0 h was defined as 1. The results are presented as means ± standard deviations of three independent experiments. (C) Semiquantitative RT-PCR analysis for IPNV genomic RNA. (D) One-step growth curve of IPNV. Culture supernatant was collected from IPNV-infected CHSE-214 cells at 5-h intervals. The virus titer in the supernatant was expressed as $TCID_{50}$/ml.

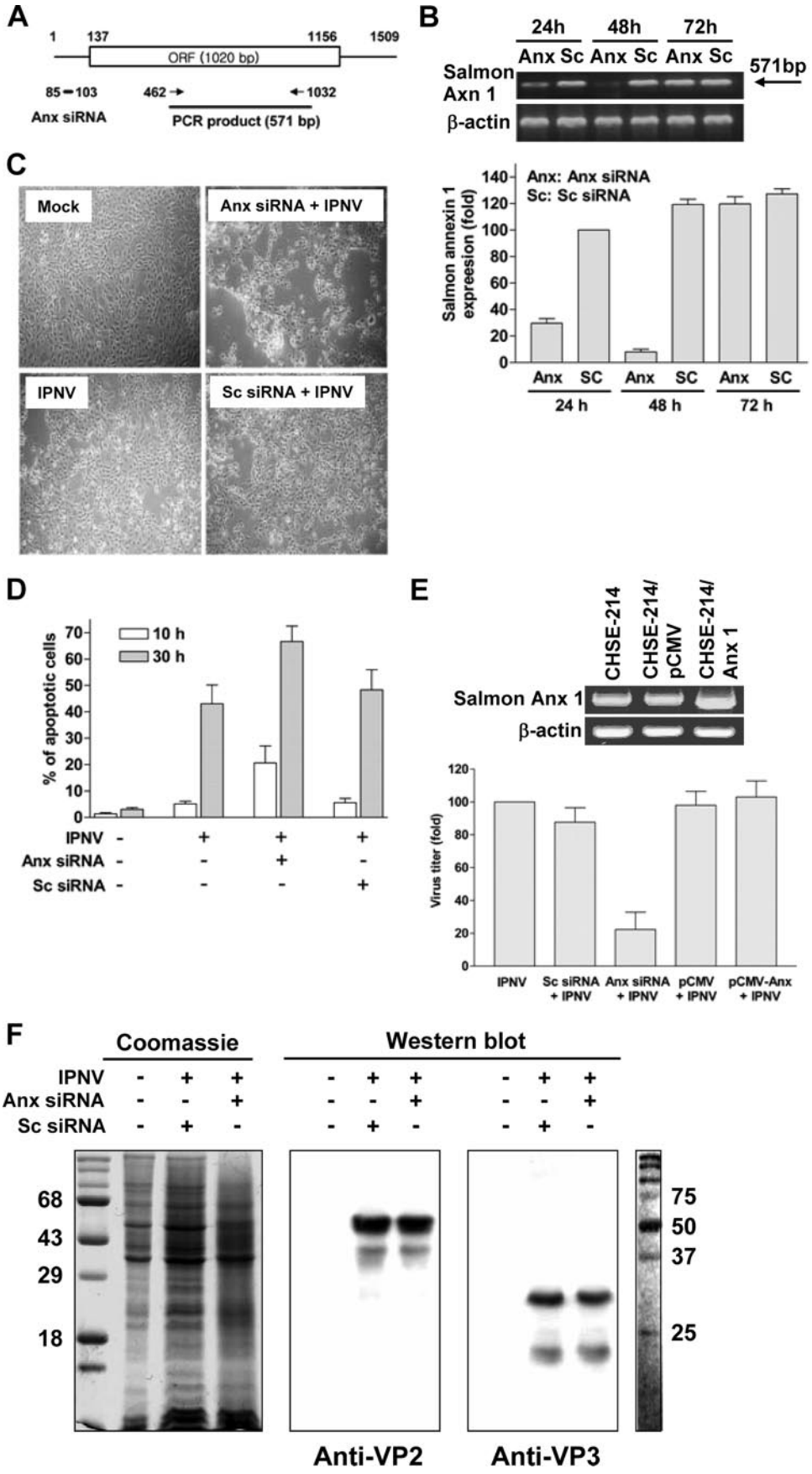
ation of intracellular Ca^{2+} concentration and in induction of apoptosis (38, 40). In addition, there are many reports on the alteration of intracellular Ca^{2+} concentration after virus infection (21, 39). In order to determine whether salmon annexin 1 is involved in the changes of intracellular Ca^{2+} concentration in IPNV-infected cells, the intracellular calcium level was determined in Anx1 siRNA-treated cells after IPNV infection. Mock-infected cells served as a negative control, and mock-infected cells exposed to calcium ionophore A23187 served as a positive control. The intracellular Ca^{2+} level began to increase progressively from 6 h p.i. ($P < 0.05$) and reached a steady state at 18 h p.i. (Fig. 6). Measurements beyond 24 h were not reliable because of cell membrane disruptions due to viral loads. Cell transfected with Anx1 siRNA exhibited a reduced intracellular Ca^{2+} concentration compared to the control cells. This result suggests that IPNV-induced salmon annexin 1 is associated with increase in Ca^{2+} concentration in IPNV-infected cells.

DISCUSSION

The purpose of our study was to identify and characterize novel proteins that are induced in fish cells by infection of IPNV. We found from our analyses using 2-DE and MS that a member of the annexin family is overexpressed in IPNV-infected CHSE-214 fish cells (Fig. 1). Using the information from the amino acid sequence, we have cloned a cDNA that corresponds to the annexin gene. The 339-amino-acid open reading frame of the cloned cDNA has the sequences of a peptide that was determined in the analysis of the IPNV-induced annexin. The expression pattern of the annexin gene as detected by cDNA hybridization to mRNA is consistent with the expression profile of the annexin protein established using the 2-DE gel. The amino acid sequence analysis of the annexin showed that the IPNV-induced annexin belongs to the annexin 1 subfamily, and we named it "salmon annexin 1."

Human annexin A1 has a core domain that contains four homologous repeats (numbered I to IV) of five α-helices each, namely A, B, C, D, and E. Helices A and B and helices D and E form two parallel helix-loop-helix folds. Helix C connects the C terminus of helix B to the N terminus of helix D. There are six calcium binding sites in human annexin 1; three type II and three type III calcium binding sites. Three type II calcium binding sites [(K,R)-(G,R)-G-T] are located at the AB loops in repeats II, III, and IV. Of the three type III calcium binding sites, two are at the DE loops in repeats I and IV and one is at the AB loop in repeat I (35, 41). As shown in Fig. 3, the salmon annexin 1 has six calcium binding sites in four repeats.

There are several reports supporting the roles of annexin 2 in viral infection. Annexin 2 promotes entry of human immunodeficiency virus (HIV) (28) and cytomegalovirus (32) into cells through an interaction with phospholipid membrane. Annexin 2 is involved in HIV Gag processing and is essential for the proper assembly of HIV in cells (36). However, even though Katoh (24) reported the detection of bovine annexins I



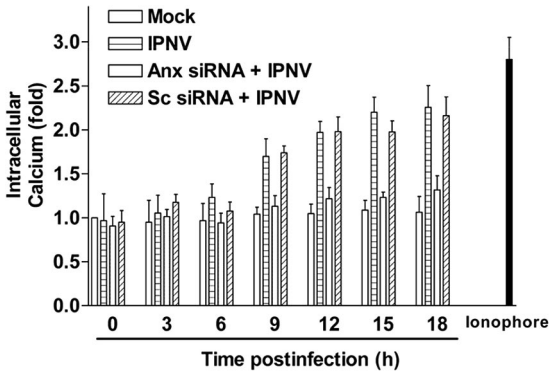


FIG. 6. Down-regulation of salmon annexin 1 by Anx1 siRNA decreases intracellular calcium concentration in IPNV-infected cells. CHSE-214 cells were transfected with 2 nmol of Anx1 siRNA or Sc siRNA, and then 36 h after the transfection, cells were infected with IPNV. Cells were collected at 3-h intervals, and intracellular calcium was determined as described in Materials and Methods. The Ca^{2+} concentration of untreated cells at 0 h was expressed as 1. Treatment with 100 nM calcium ionophore A23187 served as a positive control. The results are presented as means ± standard deviations of three independent experiments.

and IV in bronchoalveolar lavage fluids from calves inoculated with herpesvirus 1, there is no report on the role of annexin 1 in viral infection. To determine the roles of the novel salmon annexin 1 in IPNV infection, we analyzed the effect of salmon annexin 1 knockdown by siRNA on IPNV growth. The salmon annexin 1 knockdown did not affect the levels of IPNV proteins at the early stage of virus infection but decreased the titer of viral progeny to 25% of that of the untreated control. The siRNA treatment resulted in an increase of apoptosis of IPNV-infected cells. These results suggest that the increased expression of salmon annexin 1 decreased the apoptosis of IPNV-infected cells, which is not a host defense mechanism against IPNV infection but supports the growth of IPNV by delaying the apoptosis of IPNV-infected cells.

Apoptosis is an active process of cell death that serves diverse functions in multicellular organisms and can provide protection against viral infection by inducing premature death of virus-infected cells. Thus, inside the cells, viruses need to inhibit apoptosis of virus-infected cells in order to replicate efficiently. Failure to inhibit the apoptosis of virus-infected cells can restrict virus growth. A number of viruses depend on inhibition of apoptosis for normal replication, and consequently they encode potent cell death suppressors. For example, the adenovirus E1B 19-kDa protein suppresses apoptosis by heterodimerization with the death promoter of BAX, which is an activator of apoptosis (4). The Kaposi sarcoma-associated virus encodes a Bcl-2 homolog that does not interact with proapoptotic proteins such as Bax or Bad and inhibits programmed cell death (5). The CrmA of cowpox virus, SERP2 of the myxoma virus, and P35 of the baculovirus block apoptotic responses by inhibition of caspases (30). In case of IPNV, Hong et al. (19) reported that VP5, a nonstructural protein, inhibits the apoptosis of virus-infected cells. However, Santi et al. (37) reported that VP5 is dispensable for the growth of IPNV, which suggests the possibility that VP5 is not the only factor for the inhibition of apoptosis in IPNV-infected cells. In this study, we showed that IPNV infection increased the expression of a cellular protein, salmon annexin 1 and that the increased expression of salmon annexin 1 inhibits the apoptosis of IPNV-infected cells and supports the growth of IPNV in cells. These findings suggest that IPNV inhibits the apoptosis of virus-infected cells not only by using virus genome-encoded VP5 but also by increasing the expression of salmon annexin 1 for efficient replication.

Annexin 1 has been thought to be involved in the induction of apoptosis by a rapid increase in intracellular Ca^{2+} concentration followed by Bad dephosphorylation (38, 40). In IPNV-infected cells, knockdown of salmon annexin 1 resulted in a significant reduction of intracellular Ca^{2+}, which suggests that salmon annexin 1 is related to increase in the intracellular Ca^{2+} concentration. We do not know the precise mechanisms behind how the rise in intracellular Ca^{2+} concentration confers an antiapoptotic state to IPNV-infected cells. There are reports that the rise in cytoplasmic Ca^{2+} leads to the activation of calpains, Ca^{2+}-sensitive proteases that can exert antiapoptotic effects by cleaving caspases (6, 25). Other studies report that the Ca^{2+} fluxes from endoplasmic reticulum to mitochondria can lead to apoptosis by the release of proapoptotic molecules from mitochondria and, thus, the reduction of Ca^{2+} content of the endoplasmic reticulum and the resulting down-regulation of Ca^{2+} fluxes between this store and mitochondria

FIG. 5. Down regulation of salmon annexin 1 using siRNA increases the death of IPNV-infected cells and decreases the viral growth. (A) Schematic representation of regions of salmon annexin 1 showing the locations of siRNA and PCR primers. (B) Specific inhibition of salmon annexin 1 expression by Anx1 siRNA treatment. CHSE-214 cells were transfected with Anx1 siRNA. As controls for nonspecific effects, cells were transfected with Sc siRNA. After transfection, total RNA was prepared from 24 h to 72 h and subjected to RT-PCR for annexin expression. β-Actin was used as an internal control. (Top) Images of agarose gel electrophoresis. (Bottom) Histogram showing the expression level of salmon annexin 1. The expression level of salmon annexin 1 of Sc siRNA-treated cells at 24 h was defined as 100. The results are presented as the means ± standard deviations of three independent experiments. (C, D, E, and F) CHSE-214 cells were transfected with 2 nmol of Anx1 siRNA or Sc siRNA, and then 36 h after the transfection, cells were infected with IPNV. At 30 h after IPNV infection, the CPE was determined by microscopic observation (C). At 10 and 30 h after IPNV infection, cells were fixed with 2% paraformaldehyde, stained by TUNEL, and analyzed by fluorescence-activated cell sorting. The results are presented as the means ± standard deviations of three independent experiments (D). CHSE-214 cells were stably transfected with full-length cDNA of salmon annexin 1, and as a control, CHSE-214 cells were stably transfected with pCMV-Tag1 vector. The expression of salmon annexin 1 was determined by RT-PCR before the IPNV infection. Culture supernatant was collected at 30 h after IPNV infection from CHSE-214 cells; Anx siRNA-treated or Sc siRNA-treated CHSE-214 cells; and CHSE-214 derivatives stably transfected with pCMV-Anx, CHSE-214/Anx, or empty vector pCMV (CHSE-214/pCMV). The virus titer ($TCID_{50}$/ml) in the supernatant was determined. The virus titer in cells infected with IPNV only was defined as 100. The results are presented as means ± standard deviations of three independent experiments (E). At 10 h after IPNV infection, cells were harvested and expression levels of RNA transcript and proteins of IPNV VP2 and VP3 were determined by Western blotting (F) as described in Materials and Methods.

are the major components of the viral antiapoptotic program (2, 14). Further studies on the changes in the apoptotic pathways and on the intracellular Ca^{2+} compartmentalization in IPNV-infected cells will reveal the precise antiapoptotic mechanisms of increased Ca^{2+}.

We report here for the first time that salmon annexin 1 is overexpressed by IPNV infection and supports viral growth by inhibition of premature death of virus-infected cells. Our results provide information for better understanding of viral growth in host cells and a novel means of investigating cellular apoptosis regulatory systems.

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