IPNV VP5, a Novel Anti-apoptosis Gene of the Bcl-2 Family, Regulates Mcl-1 and Viral Protein Expression

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VP5, a 5′-terminal, small open reading frame in segment A of the aquatic birnavirus (infectious pancreatic necrosis virus, IPNV) genome, encodes a 17-kDa nonstructural protein. We previously reported apoptosis induced by IPNV in a fish cell line. In the present study, we cloned and identified VP5 and tested its function. Comparisons of the amino acid sequence of VP5 with well-known Bcl-2 family member proteins showed that the VP5 protein contains Bcl-2 homology (BH) domains BH1, BH2, BH3, and BH4 but without the transmembrane region. VP5-stable clones enhanced viability, prevented membrane blebbing, delayed DNA internucleosomal cleavage, and decreased virus titer during IPNV infection but, when deleted, BH domains 1 and 2 could lose the preventable ability. In addition, VP5 was demonstrated to be able to enhance or assist in maintaining the functional half-life of survival factor Mcl-1 and regulate specific viral protein expression during the early replication cycle. Finally, we found that VP5 was capable of enhancing cell viability when cells were exposed to UV irradiation. In summary, these results suggest that the aquatic birnavirus may utilize a notable strategy via VP5 to regulate the host apoptosis-off system for enhancing progeny production.

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Key Words: infectious pancreatic necrosis virus; apoptosis; Bcl-2; Mcl-2; VP5; virus replication.

INTRODUCTION

Apoptosis plays a protective role systemically by eliminating cells that might prove harmful if allowed to survive. For example, those cells harboring mutations following irradiation or chemical insult and which could lead to cancer are eliminated by apoptotic processes. Further, some cells may even be eliminated in order to protect against virus infection (O’Brien, 1998). Thus, understanding the biochemical and molecular events that control apoptosis may ultimately lead to a novel means of dealing with disease.

Bcl-2, a proto-oncogene originally identified as an overexpressed membrane protein in follicular lymphomas carrying a t(14;18) chromosomal translocation (Cleary et al., 1986), has been shown to delay or even block apoptosis induced by numerous, often unrelated physiological and pathological stimuli (Reed, 1994). Bcl-2, through its hydrophobic C-terminus, is anchored to the outer membranes of mitochondria (Kroemer et al., 1997; Thomson, 2001), the endoplasmic reticulum (ER), and the nucleus (Krajewski et al., 1993), where it may form a cation-selective ion channel (Minn et al., 1997), block the release of cytochrome c from mitochondria into the cytosol (Kroemer, 1997; Thomson, 2001), and attract cytosolic adapter molecules (Reed, 1997). The multiple functions of Bcl-2 probably serve to prevent the activation of the ced-3/caspase-3 subfamily in response to apoptotic stimuli and thus suppress the death effector machinery (Boulakia et al., 1996; Monney et al., 1996). To date, more than a dozen cellular and viral Bcl-2 homologues have been identified (Reed, 1997; Kelekar and Thompson, 1998). There are many examples of viruses that kill cells apoptotically; in most cases, apoptosis is a defense mechanism beneficial for the host cell because it curtails the infection cycle and prevents neighboring cells from being infected with progeny virions (Vaux et al., 1994). At one extreme, the host defense system is circumvented by the presence of anti-apoptotic proteins, such as bcl-2 (Levine et al., 1993), that either exist in host cells or are brought into the cells by viruses, such as viral latent membrane protein-1 (LMP-1) of Epstein–Barr virus and E1-B of adenovirus (Hendersen et al., 1991; Rao et al., 1992). Subsequent to viral infection, host cells typically survive and persistently produce progeny virions (viral persistence). At the other extreme, viruses may provoke pathological lesions because they benefit from the anti-apoptotic properties of the host cells for reproduction but then kill these cells by overcoming their survival potential at a later stage (Ubol et al., 1994; O’Brien, 1998).

Infectious pancreatic necrosis virus (IPNV) is a fish pathogen and is the prototype of the Birnaviridae virus family (Dobos et al., 1979). Birnaviruses possess a bi-segmented (A and B), double-stranded RNA genome contained within a medium-sized, unenveloped, icosahedral capsid. Birnavirus gene expression involves the pro-
duction of four unrelated major genes that undergo various posttranslational cleavage processes in order to generate three to five structural proteins (Dobos, 1995). The largest protein, VP1, is encoded by the smaller segment B genomic RNA, and the larger genome segment A encodes VP2, VP4, and VP3 proteins. Genome segment A contains an additional small, open reading frame (ORF) that overlaps the amino-terminal end of the polyprotein from the reading frame (Duncan et al., 1987). This small ORF encodes a 17-kDa arginine-rich minor polypeptide, VP5, which is produced in small quantities and is synthesized during the early replication cycle (Magyar and Dobos, 1994). Our particular interest is in unveiling the molecular mechanisms that govern the survival and death of cells infected by IPNV. We previously reported that apoptosis may be induced by IPNV infection in a fish cell line (Hong et al., 1998, 1999a) and subsequently found that viral proteins contribute to the down-regulation of survival factor Mcl-1 during IPNV-induced apoptotic cell death (Hong et al., 1999b). In the present study, we cloned and characterized the nonstructural viral protein VP5, which may either regulate specific viral protein expression or delay apoptotic cell death in the early replication cycle of IPNV infection. To the best of our knowledge, this is the first study to demonstrate that a novel Bcl-2 family member can arise from a double-stranded RNA virus, IPNV having been shown to prevent down-regulation of host Mcl-1 to enhance cell survival. This finding might provide an important insight into a Bcl-2 family member, VP5, that uses a novel strategy against host defense response in a virus' early replication cycle from a double-stranded RNA virus.

RESULTS

The early expression of VP5 protein in IPNV-infected CHSE-214 cells

This small ORF encodes a 17-kDa arginine-rich minor polypeptide, VP5, which is produced in small quantities and is synthesized during the early cell replication cycle (Magyar and Dobos, 1994). In our system, the pattern of VP5 in uninfected and IPNV-infected CHSE-214 cells was labeled by a continuous-labeling process using [35S]methionine. The data indicated that the major proteins pVP2, pVP3, and pVP4 were readily distinguished in infected cells (data not shown) as early as 3 h subsequent to infection, and no polypeptides of comparable sizes became labeled in uninfected cells. On the other hand, the nonstructural protein VP5 in IPNV-infected CHSE-214 cells was labeled (Fig. 1, lanes 8–14) such that it was readily distinguishable in infected cells as early as 3 h subsequent to infection, the degree of expression increasing rapidly at around 6 h postinfection (p.i.; the VP5 was finally completely degraded at 24 h p.i. (Fig. 1, lane 13).

Cloning of VP5

We used two specific primers to clone the nonstructural protein VP5 using a one-step RT-PCR process from total RNA extracted from cells at 6 h p.i. The alignment of the nucleotide sequence of VP5 and the deduced amino acid sequence are indicated in Fig. 2, the cDNA of VP5 being 444 bp in length. The molecular weight of VP5 is 218 HONG, GONG, AND WU

![FIG. 1. Identification of VP5 expression pattern in CHSE-214 cells. Both the uninfected and IPNV-infected cells were labeled with [35S]methionine at different times and analyzed using a 12% SDS–PAGE assay. The lanes correspond to labeled polypeptide released by virus-infected cells lysed after 3, 4, 6, 8, 10, 24, and 36 h, respectively.](image-url)
about 17 kDa and the interval contains a 16-arginine residue as shown by the underlined section in Fig. 2.

Bcl-2-related proteins that share homology in one to four regions, designated Bcl-2 homology (BH) domains BH1, BH2, BH3, and BH4, are depicted in Fig. 3A. VP5 contains the conserved BH1, BH2, BH3, and BH4 domains, which are indicated in boxes (Fig. 3A). According to the domain organization, the anti-apoptotic members of the Bcl-2 family proteins are classified into class I and class II (10). We found that VP5 belongs to a new class of anti-apoptotic Bcl-2 members, because its domain organization [i.e., lacking a transmembrane region (TM) but including the BH1 to BH4 domains] may allow it to be classified into class III (Fig. 3B).

Enhancing viability and reducing the virus titer of VP5 during IPNV E1S infection

The stable transfectants containing the CHSE-Neo were of two types, those without the VP5 gene (designated the negative control) and those with the VP5 gene present, these being designated CHSE-VP5-1 and CHSE-VP5-2. The VP5 gene was identified from stable transfectants by a one-step RT-PCR process. As indicated in Fig. 4A, a high level of VP5 expression occurred (lanes 3–4) as compared to CHSE-Neo cells (lane 2). Following this, we then tested the prevention of the cell death function in the overexpression of the VP5 cell line. Figures 4B–4G depict the phase-contrast micrographs of cell viability for cells experiencing and those not experiencing IPNV infection [multiplicity of infection (m.o.i.) = 1] at 24 h.p.i. The viability of VP5 transfectants and Neo transfectant cells was measured at serial time points subsequent to IPNV infection using a trypan blue exclusion technique (Fig. 4H). For the m.o.i. 1 infection group, at various time points including 0, 4, 8, 12, and 24 h, in the control group (CHSE-Neo cell) the majority of cells were viable from 100, 110, 63, 45, and 10%, respectively. By contrast, in the group containing VP5 the viability of the majority of CHSE-VP5-1 cells was enhanced 100, 133, 120, 115, and 38%, respectively, and in the CHSE-VP5-2
Figure 4H demonstrates that both the CHSE-VP5-1 and the CHSE-VP5-2 cell lines revealed greater cell viability than the CHSE-Neo cell line, when assessed at 12 h p.i. On the other hand, for the m.o.i. 10 infection group, the containing-VP5 group also consistently showed that VP5 could enhance the cell viability from 46% (CHSE-Neo) to 80% (CHSE-VP5-1) and 68% (CHSE-VP5-2) at 12 h p.i. (Fig. 4I). At 24 h p.i., for the m.o.i. 1 group, their cell supernatants’ titers were estimated according to the TCID$_{50}$ (Table 1). We found that the presence of VP5 could decrease the TCID$_{50}$ from a value of 7.0 to 6.0 (for CHSE-VP5-1) and 6.25 (for CHSE-VP5-2).

Prevention of morphological changes in virus-infected cells

Apoptosis is characterized morphologically by cell shrinkage and the presence of hyperchromatic nuclear fragments and biochemically by chromatin cleavage into nucleosomal oligomers (Wyllie et al., 1980). We compared the CHSE-Neo and CHSE-VP5-1 cells with a m.o.i. value of unity at 12 h p.i. Figure 5A shows that almost 50% of the cells are blocked from the early apoptotic stage (cell round-up) to the middle stage (membrane blebbing) and finally enter the late stage to form the apoptotic body. These sequential morphological changes having been previously described (Hong et al., 1999a). By contrast, the viability of the CHSE-VP5-1 cells appeared to be 10% below the value for the cell rounding-up stage and about 2–3% for the entrance to the middle apoptotic stage (Fig. 5B).

Delayed induction of internucleosomal cleavage in CHSE-VP5 cells

DNA fragmentation is a well-defined biochemical marker of apoptosis (Wyllie et al., 1980). We therefore examined the ability of VP5 to delay the induction of DNA fragmentation in a fish embryonic cell line. Intense internucleosomal fragmentation of DNA was observed in IPNV-infected CHSE-Neo cells at 8 h p.i., but not among IPNV-infected CHSE-VP5-1 cells at up to 12 h p.i. (Fig. 5C).

**TABLE 1**

Effect of VP5 Expression on Virus Progeny Infectivity$^a$

<table>
<thead>
<tr>
<th>Time (p.i.)</th>
<th>CHSE-Neo</th>
<th>CHSE-VP5-1</th>
<th>CHSE-VP5-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>7.0</td>
<td>6.0</td>
<td>6.25</td>
</tr>
</tbody>
</table>

$^a$ Results are representative of three independent experiments. Determined at 24 h p.i. with IPNV E1-S (m.o.i. = 1).

**FIG. 4—Continued**

FIG. 4. Expression of VP5 delayed the cell death of CHSE-VP5-1 and CHSE-VP5-2 stable clones during IPNV infection. (A) Characterization of VP5 gene expression from a VP5 stable cell line. The VP5 from a stable transfectant was amplified by one-step RT-PCR (see Materials and Methods). Lane 1 is a 1-kb ladder marker (BRL); lanes 2–4 are from CHSE-Neo, CHSE-VP5-1, and CHSE-VP5-2, respectively. (B–D) Phase-contrast images of uninfected CHSE-Neo, CHSE-VP5-1, and CHSE-VP5-2 cells, respectively, at 24 h p.i., as a negative control; (E–G) phase-contrast images of IPNV-infected CHSE-Neo, CHSE-VP5-1, and CHSE-VP5-2, respectively, at 24 h p.i. Bar, 20 μm. (H) Viability of CHSE-Neo (open squares), CHSE-VP5-1 (open diamonds), and CHSE-VP5-2 (open circles) clones expressing VP5 subsequent to IPNV infection (m.o.i. = 1). (I) Viability of CHSE-Neo (open squares), CHSE-VP5-1 (open diamonds), and CHSE-VP5-2 (open circles) clones expressing VP5 following IPNV infection (m.o.i. = 10). Cell viability at each time point for each cell line was determined in triplicate; each point represents the mean cell viability of three independent experiments ± SEM.
VP5 could regulate the viral protein expression and maintain the half-life of Mcl-1 levels

From the data revealed in Fig. 1, it would appear that the polypeptide VP5 is an early expression protein, and from the results presented in Figs. 4 and 5 it appears that overexpression of VP5 could enhance the cell viability and delay the DNA internucleosomal cleavage at a late virus-replication cycle. It thus is of interest to be able to determine whether VP5 could regulate other viral proteins or, in fact, even host survival factor expression. The expression patterns of the major capsid protein VP2 and the submajor capsid protein VP3 in CHSE-VP5-1 and CHSE-Neo clones that were examined using Western blot analysis at serial time points subsequent to IPNV infection are indicated in Fig. 6A. The precursor expression of major capsid protein VP2 (pVP2-1) and submajor protein VP3 in CHSE-VP5-1 and CHSE-Neo cells was traced by personal densitometer (Molecular Dynamics). We found that the pVP2-1 expression in CHSE-VP5-1 could be limited by at least 30% in the whole replication cycle (as shown in Fig. 6D) compared with CHSE-Neo cells at 6, 12, and 24 h p.i. In contrast, we observed the very conflict-expression pattern of VP3 in CHSE-VP5 cells compared with pVP2-1 protein (Fig. 6E). Briefly,
during the overexpression of VP5 a resultant delay to the major peak of VP3 protein expression could be observed at about 6 h postinfection. The details are as follows: at 6 h p.i., the VP3 protein level in CHSE-VP5-1 cells appears to decrease by 52% compared with CHSE-Neo cells. At 12 h p.i., VP3 in CHSE-VP5-1 cells appears to be rapidly increasing the expression dose by about 18% compared with CHSE-Neo cells. Finally, for CHSE-VP5-1 and CHSE-Neo cells there appears to be a virtually equivalent level of VP3 expression at 24 h p.i. As regards the survival factor Mcl-1 expression profile (as shown in Figs. 6B and 6F), in total, we found that VP5 could maintain the Mcl-1 expression level, possibly by enhancing its protein half-life. Specifically, VP5 could maintain the Mcl-1 expression level at 6, 12, and 24 h at 127, 70, and 82.5% compared with 0 h, respectively. But without overexpression of the VP5 group (CHSE-Neo cell) the MCL-1 is quickly down-regulated so that its expression level dropped to 42.5, 12.5, and 8% compared with 0 h at 6, 12, and 24 h p.i., respectively. These data appear to be consistent with the results indicated in Fig. 4, thus suggesting that VP5 could maintain the MCL-1 expression...
level necessary to enhance the host cell viability during IPNV infection.

Decreasing cell viability of VP5dBH1 and VP5dBH2 during IPNV E1S infection

When deleted, the BH1 and BH2 domains may be able to influence the prevention of the cell death function. In this regard, Fig. 7A demonstrates a deletion of a VP5 construction map. In an anti-apoptotic death functional assay, the CHSE-Neo, CHSE-VP5-1, CHSE-VP5dBH1-2, and CHSE-VP5dBH2 cell lines were used. From the results presented in Fig. 7B it would appear that the anti-apoptotic function may be maintained from VP5 or deleted VP5 transfectants at 6 h p.i., although at 12 h p.i., the anti-apoptotic death function is apparently lost from CHSE-VP5dBH1-2 cells (41.6%) and CHSE-VP5dBH2 cells (41%) compared to CHSE-Neo cells (40%, as a negative control) and cells containing the VP5 gene (CHSE-VP5-1 cells, 80%).

Enhancing cell viability of VP5 with UV irradiation

Here, we tested the anti-apoptotic death function of VP5 using a classical stimulus such as UV irradiation. The stable transfectants containing CHSE-Neo and CHSE-VP5-1 were used for this investigation. The phase-contrast micrographs of cell viability with UV irradiation for 0.5 and 3 Gy at 16 h p.i. are shown in Fig. 8A. The viability of VP5 transfectants and Neo transfectant cells was measured subsequent to such UV irradiation using a trypan blue exclusion method (Fig. 8B). We found that VP5 was capable of enhancing the cell viability (95%) when compared with Neo vector cells (70%) subsequent to UV irradiation of 0.5 Gy. At the higher UV irradiation level (3 Gy), the CHSE-VP5-1 cells were able to maintain a high viability level (70%) compared to CHSE-Neo cells (40%). Such consistent results from both the lower and the higher UV-irradiation groups suggest that VP5 could enhance cell viability from apoptosis.

DISCUSSION

The present study shows that a novel Bcl-2 family member, VP5, can regulate the cellular survival factor Mcl-1 and viral proteins through apoptosis in a fish cell line. Analysis of the structural features of the VP5 protein revealed that VP5 contains putative domains BH4, BH3, BH1, and BH2 from the N-terminal to the C-terminal
region (Fig. 1), which would appear to be characteristic of Bcl-2 family members.

A number of Bcl-2-related proteins have been previously identified (Kelekar and Thompson, 1998). While some reveal anti-apoptotic activity similar to that of Bcl-2, such as Bcl-xL (Boise et al., 1993), Mcl-1 (Kozopas et al., 1997), and A1 (Lin et al., 1993), others appear to promote apoptosis, such as Bax (Oltvai et al., 1993), Bak (Chit-
tend et al., 1995), and Bad (Yang et al., 1995). Most proteins in the Bcl-2 superfamily also harbor C-terminal signal-anchor sequences that target these proteins predominantly on the outer mitochondrial membrane, endoplasmic reticular membrane, and outer nuclear envelope (Krajewski et al., 1993; Boyed et al., 2001; Thomson, 2001). This new class member, VP5, however, appears to be absent in the transmembrane domain in the C-terminal region of the VP5 protein. It could thus belong to a new class of Bcl-2 family members, termed class III; such an anti-apoptotic member has been previously described (Kelekar and Thompson, 1998).

According to its amino acid sequence, VP5 is a novel Bcl-2 family member, hence it appears appropriate to determine the role that the VP5 protein plays in the host cell. Overexpression of VP5 in CHSE-214 cells enhanced host viability during IPNV infection (Fig. 3) and delayed the induction of internucleosomal cleavage by IPNV (Fig. 4). On the other hand, we observed consistent results regarding the influence of the deletion of the BH2 domain alone or the deletion of both the BH2 and BH1 domains, in causing VP5 to lose its cell viability-enhancing function at 6 h p.i. (as shown in Fig. 6). Thus it appears that the BH1 and BH2 domains are crucial for maintaining the survival function of the death suppressor VP5. The BH1 and BH2 domains are found in all death antagonists of the Bcl-2 family (Kelekar and Thompson, 1998). Residues in the BH1 and BH2 domains are essential for the survival function of the death suppressors Bcl-2 and Bcl-xL and for interactions with death agonists such as Bax (Oltavai et al., 1993) and Bak (Chittenden et al., 1995). Furthermore, VP5 is absent in the C-terminal signal-anchor sequence (TM), unlike other Bcl-2 family members that predominantly target the outer mitochondrial membrane, endoplasmic reticular membrane, and outer nuclear envelope (Krajewski et al., 1993). We propose that VP5 might act through a different mechanism involved in regulating cell death by IPNV infection, although further experiments are required to clarify the interaction of other proteins with viral protein in the lower vertebrate (fish) system.

Many viruses have evolved genes encoding proteins that effectively suppress or delay apoptosis and consequently still maintain host survival for the production of sufficient quantities of progeny. For example, the adenoviral E1B gene (19-kDa protein) (Boyd et al., 1994), the BHRF1 gene of EBV (Henderson et al., 1993), and the LMW5-HL gene of ASFV (Chacon et al., 1995) encode homologues of cellular Bcl-2, an inhibitor of apoptosis. To the best of our knowledge, to date, no member of the viral Bcl-2 family has yet been reported from any double-stranded RNA virus or fish virus. The IPNV virus replication process is localized in the cytoplasm and one replication cycle takes about 16–20 h at 22°C, resulting in a characteristic cytopathic effect (CPE) (Monney et al., 1996). In our system, we found that viral protein synthesis or virus replication is associated with the down-regulation of the expression of the survival factor Mcl-1, which induces host cells to undergo apoptotic cell death at a relatively low m.o.i. We propose that viral protein expression between 6 and 8 h subsequent to IPNV infection may be involved in either the direct or the indirect down-regulation of the Mcl-1 pathway (Hong et al., 1999b). Our data show (Figs. 4 to 6) that VP5 can also delay the induction of DNA internucleosomal cleavage to rescue or prevent cells from undergoing apoptotic cell death either by maintaining the Mcl-1 protein level or by limiting specific viral protein expression during IPNV infection. VP5 is the early expression gene as shown in Fig. 1 (Magyar and Dobos, 1994). We found that VP5 may play a novel function that both blocks the host defense system from triggering apoptosis (Tschopp et al., 1998; Roulston et al., 1999) and regulates viral-death gene expression in an early replication stage, thus enhancing the virus production. On the other hand, we examined the anti-apoptotic role that VP5 plays following UV irradiation, the results of which appear to be consistent with those presented in Fig. 4 that demonstrate the general anti-death function that VP5 displays. Recently, we reported that the death effector Bad gene could be induced, its expression occurring during the early replication cycle (Hong and Wu, 2002), and that other effectors such as caspase(s) (Boulakia et al., 1996; Monney et al., 1996) may be activated in order to drive the apoptotic death pathway. Finally, however, how VP5 induces the down-regulation of Mcl-1 and disrupts VP5 function for inducing postapoptotic necrosis at the pre-late apoptotic stage (Hong et al., 1998) warrants further investigation.

**MATERIALS AND METHODS**

**Cells and viruses**

Chinook salmon embryo cells (CHSE-214) were obtained from the American Type Culture Collection (ATCC). CHSE-214 cells were grown at 18°C in plastic tissue-culture flasks (Nunc) using Eagle's minimum essential medium (MEM) supplemented with 10% (v/v) fetal calf serum and 25 μg/ml of gentamicin.

The isolated virus, E1-S, a member of the Ab strain of IPNV, was obtained from Japanese eels in Taiwan (Wu et al., 1987) and was propagated in CHSE-214 monolayer cells at a m.o.i. of 0.01 per cell. Virus-infected cultures were incubated at 18°C until an extensive CPE was observed (Dobos, 1977). Virus plaque assays (Dobos, 1977) and TCID₅₀ were performed on confluent monolayers of CHSE-214 cells (Nicholson and Dunn, 1974).

**Radioactive labeling and analysis of viral protein from IPNV-infected cells**

Confluent CHSE-214 monolayer cells in plastic tissue culture plates (60 mm in diameter, Nunc) were infected...
with IPNV (for the virus-infected group) at 18°C at a m.o.i. of 10, the cells being allowed 1 h for adsorption. Following this, the medium was removed from the virus-infected group and the normal control group culture plates, the monolayers were rinsed three times with phosphate-buffered saline (PBS), and the uncovered plate was irradiated with UV light as previously described by Wu et al. (1998). Methionine-free medium (Gibco) that contained actinomycin D (1 μg/ml) was used for the virus-infected group and the normal control group, and starvation proceeded for 2 h at 18°C. At 2 h postinfection, growth medium was added containing 50 μCi/ml of [35S]methionine. Subsequent to the completion of the labeling period, and at various time intervals thereafter, including 1, 2, 4, 6, 8, and 22 h, respectively, the cell layer was washed with PBS, and the monolayer was lysed in 0.4 ml of electrophoresis sample buffer and then heated for 2 min in a boiling water bath. Radioactively labeled proteins were analyzed in the slab gels, using a discontinuous SDS-gel system as previously described by Laemmli (1970). The 12% separating gel and 5% stacking gel were prepared with an acrylamide to bisacylamide ratio of 30:0.8. Fifty to 60 μl of labeled cell lysate placed into a total volume of 400 μl (and exhibiting a 20,000- to 40,000-cpm intensity) was loaded into wells. Electrophoresis continued for 14 to 16 h at 60 V. The slab gel was dried under vacuum, autoradiographed, and then exposed to undeveloped film.

Cloning of VP5

VP5 was amplified by one-step RT-PCR from total RNA derived from cells 6 h p.i. (Chirgwin, 1979) with VP5 mRNA as a template. Two oligonucleotide primers were used including VP5p1 (5′-ATGGCGAAAG CCTTCTCTAC-3′) and VP5p2 (5′-ACAGACTTCCCTCGAAGTTG-3′). The VP5 mRNA was reverse-transcribed in 50 μl of reaction mixture containing 1 μg of total RNA, 20 ng each of the oligonucleotide primers, the dNTPs at a final concentration of 10 μM each, 2 units each of Superscript II reverse transcriptase and T7q DNA polymerase (RT/Taq mixture), and 25 μl of reaction buffer at 42°C for 30 min. The first-strand cDNA of VP5 was followed by pre-denaturation at 94°C for 2 min. The second-strand cDNA of VP5 was amplified using a 35-cycle PCR process, with each cycle consisting of a denaturation step of 15 s at 94°C, an annealing step of 30 s at 42°C, an extension step at 72°C for 1 min/kb, and one cycle of a final extension at 72°C for 10 min. The PCR products were fractionated using gel electrophoresis, and DNA fragments of the expected size (0.45 kb) were extracted from the gel by an oligonucleotide purification kit and cloned into pcDNA3.1/V5/His-Eukaryotic TOPO TA cloning vector (Shuman, 1994). The resulting clones were sequenced using a single base reaction and using the ABI Prism 377 DNA sequencer according to the manufacturer’s protocol. Complete sequence analysis was then performed on a representative clone. The VP5 gene accession number is AF160258.

VP5 and VP5 BH domain-deleted transfectants

VP5-producing cells were obtained by transfecting CHSE-214 cells with a TOPO vector containing the VP5 gene and by selecting with G418 for 10–12 weeks (Hong et al., 1999a). The VP5 BH1 and BH2 domain-deleted constructs were used as the specific primers to amplify the VP5dBH1-2 (249 nt) and VP5dBH2 (373 nt) by PCR and to then ligate the inserts to the TOPO vector. The plasmids were transfected with Lipofectamine Plus (Gibco BRL) to CHSE-214 cells and selected with 800 μg/ml G418 for 10–12 weeks.

Quantification of cell viability

To test whether VP5 functions in an anti-apoptotic capacity during IPNV infection, the transfectants that we used contained the CHSE-Neo and two VP5 stable cell lines. The CHSE-Neo, CHSE-VP5-1, and CHSE-VP5-2 cell lines were seeded in plastic tissue plates (60 mm in diameter, Nunc) and were either infected with m.o.i. 1 of IPNV E1-S for 0, 4, 8, 12, and 24 h or with m.o.i. 10 for 12 and 24 h at 18°C. At the end of the various culture periods, both virus-infected CHSE-Neo cells and virus-infected VP5 stable cells were examined by light microscopy using phase-contrast optics (Nikon), following which the cells were washed with PBS and the monolayers treated with 0.5 ml of 0.1% trypsin–EDTA (Gibco) for 1 to 2 min. Cell viability was determined using a trypan blue dye-exclusion assay (Alnemri and Litwack, 1990), the results of which were expressed as means ± SEM. Data were analyzed using either a paired or unpaired Student's t test as appropriate. A value of P < 0.05 was taken to represent a statistically significant difference between mean values of groups.

DNA preparation and gel electrophoresis

Approximately 10⁶ of CHSE-Neo and CHSE-VP5-1 cells per milliliter were seeded on a 60-mm petri dish and allowed to remain in culture conditions for more than 20 h. The CHSE-Neo and CHSE-VP5-1 cell monolayers were treated with virus at m.o.i. 1 and were incubated for various time periods including 0, 6, 8, and 12 h. Uninfected control cells were also incubated for 0 and 12 h. At the completion of incubation, cells were lysed with lysis buffer (10 mM Tris–HCl, 0.25% Triton X-100, 1 mM EDTA, pH 7.4). After treatment with phenol:chloroform:isoamyl alcohol (25:24-1), genomic DNA was precipitated in the presence of 0.3 M sodium acetate and cold absolute ethanol at −70°C for 2 h and then resuspended in 10 mM Tris–HCl (pH 7.4) and 1 mM EDTA. Aliquots of 20 μl containing approximately 5 to 10 μg of genomic DNA were then electrophoresed in 1.2% agarose gels for 2 h at
40 V. Gels were stained with ethidium bromide and photographed under UV transillumination.

Immunoblotting

Approximately $10^5$ of CHSE-Neo and CHSE-VP5-1 cells per milliliter were seeded on a 60-mm petri dish and allowed to remain in culture for more than 20 h. The cell monolayers were rinsed twice with PBS, infected with m.o.i. 1 of IPNV, and incubated for various time periods including 0, 6, 12, and 24 h. Uninfected control cells were also incubated for the same periods of time. At the completion of each incubation period, the culture medium was aspirated, and cells were washed with PBS. The culture monolayers were rinsed twice with PBS, infected with m.o.i. 1 of IPNV, and incubated for various time periods including 0, 6, 12, and 24 h. Uninfected control cellswere also incubated for the same periods of time. At the completion of each incubation period, the culture medium was aspirated, and cells were washed with PBS and then lysed in 0.3 ml lysis buffer (Kelekar and Thompson, 1998) (10 mM Tris-base, 20% glycerol, 10 mM SDS, 2% β-ME, pH 6.8).

Proteins were separated by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), electroblotted, and subjected to immunodetection as described (Kain et al., 1994). Blots were incubated with a 1:1500 dilution of immunoglobulin fraction (Clontech) and a 1:7500 dilution of peroxidase-labeled goat anti-rabbit conjugate (Amer sham). Chemiluminescence detection was performed according to the instructions provided with the Western Exposure Chemiluminescent Detection System (Amer sham). Chemiluminescent signals were imaged by exposure to Kodak XAR-5 film (Eastman Kodak, Rochester, NY). Stripping of the primary and secondary antibodies from blots was achieved by incubation in stripping buffer containing 62.5 mM Tris–HCl (pH 6.8), 3.0% (wt/vol) SDS, and 50 mM 1,4-dithiothreitol for 30 min at 55°C with gentle shaking. The blots were washed three times in PBS containing 0.1% (vol/vol) Tween 20 for 10 min each and reprobed with antibodies beginning at the membrane blocking step (Kain et al., 1994).

Prevention of cell death from CHSE-VP5 cell by UV irradiation

To assay the cell viability of the CHSE-Neo and CHSE-VP5-1 cells subsequent to their having been UV-irradiated, confluent control cells including CHSE-Neo and the CHSE-VP5-1 stable cell line in plastic tissue plates (60 mm in diameter, Nunc) were exposed to 0.5–3.0 Gy of UV irradiation (for time intervals of between 30 s and 3 min) and subsequently incubated for 16 h at 18°C. At the end of the culture periods, the cells of both CHSE-Neo and CHSE-VP5-1 stable cells were examined by light microscopy using phase-contrast optics (Nikon). Following this, the cells were washed with PBS and the monolayers were treated with 0.5 ml of 0.1% trypsin–EDTA (Gibco) for between 1 and 2 min. Cell viability was determined using a trypan blue dye exclusion assay (Alnemri and Litwack, 1990). Results were expressed as means ± SEM. Data were analyzed using either a paired or an unpaired Student’s t test as appropriate. A value of $P < 0.05$ was taken to represent a statistically significant difference between mean values of groups.

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