Porcine circovirus-2 capsid protein induces cell death in PK15 cells

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ABSTRACT

Studies have shown that Porcine circovirus (PCV)-2 induces apoptosis in PK15 cells. Here we report that cell death is induced in PCV2b-infected PK15 cells that express Capsid (Cap) protein and this effect is enhanced in interferon gamma (IFN-γ)-treated cells. We further show that transient PCV2a and 2b-Cap protein expression induces cell death in PK15 cells at rate similar to PCV2 infection, regardless of Cap protein localization. These data suggest that Cap protein may have the capacity to trigger different signaling pathways involved in cell death. Although further investigation is needed to gain deeper insights into the nature of the pathways involved in Cap-induced cell death, this study provides evidence that PCV2-induced cell death in kidney epithelial PK15 cells can be mapped to the Cap protein and establishes the need for future research regarding the role of Cap-induced cell death in PCV2 pathogenesis.

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Introduction

Porcine circovirus (PCV)-2 has been identified as the primary causative agent of Post-weaning Multisystemic Wasting Syndrome (PMWS), a clinical syndrome of progressive wasting that mainly affects 6- to 12-week-old pigs (Allan et al., 1999; Bolin et al., 2001). PMWS is characterized by an extensive lymphoid cell depletion and granulomatous inflammation (Chianini et al., 2003; Darwich et al., 2002; Sarli et al., 2001) that correlate positively with high PCV2 viral load in affected animals (Ladekjaer-Mikkelsen et al., 2002). PCV2 is the smallest known autonomously replicating non-enveloped virus with a 1.7-kb single-stranded circular DNA genome, which has three well-characterized open reading frames (ORFs). ORF1 encodes two proteins involved in genome replication, Rep and the splice variant Rep', while ORF2 encodes the dominant immunogenic and only structural capsid protein, Cap (Nawagiti et al., 2002). The third ORF encodes a non-structural protein called ORF3 that has been characterized as a pro-apoptotic protein (Liu et al., 2005).

Having a limiting coding capacity implies that PCV2 must encode for multifunctional products to ensure replication within the host. In fact, beside its role as the only structural protein involved in virus assembly, PCV2 Cap protein plays a role in controlling viral replication via its interaction with Rep protein in the nucleoplasm, may be by influencing DNA synthesis (Finsterbusch et al., 2005; Timmusk et al., 2006). Several other cellular proteins involved in different aspects of viral replication such as transcriptional regulation and intracellular transport were also found interacting with Cap protein (Finsterbusch and Mankertz, 2009). Along the same lines, it has been reported that PCV2 manipulates the autophagy machinery to enhance viral replication; and Cap protein was found responsible for that effect, by promoting the formation of autophagosome (Zhu et al., 2012).

Several studies have linked viral replication with cell death and viral dissemination, although the outcome seems to be virus and cell specific (Berens and Tyler, 2011; Levine and Deretic, 2007). PCV2 has been shown to induce apoptosis in porcine kidney epithelial PK15 cells (Liu et al., 2005). On one hand, ORF3 protein was identified as a contributing factor to apoptosis in PK15 cells (Liu et al., 2005); although, its role as the only factor causing lymphoid depletion has been a subject of controversy (Juhan et al., 2010). On the other hand, i) enhanced PCV2 replication was associated with cell death in PCV2 infected cells with a densely localized, perinuclear Cap protein expression (Dvorak et al., 2013), and ii) the ability of PCV2 to replicate and to induce cytopathic effect in the host seems to be compromised by specific mutations occurring in Cap protein (Fenaux et al., 2004; Krakowka et al., 2012). Altogether, these data suggest that PCV2-Cap protein may be involved in inducing cell death late in the replication cycle. Here we have investigated the hypothesis that Cap protein holds the capacity to induce cell death in pig cells, as a result of viral replication. Our objectives were to determine whether i) enhancing PCV2 replication with interferon gamma (IFN-γ) treatment in
PK15 cells is associated with death in cells expressing Cap protein and ii) Cap protein expression alone is capable of promoting the same effect in the absence of the viral genome and other PCV2 products. Here we report that Cap protein expression induces cell death in the PK15 cell line and that effect is enhanced by IFN-γ. A cell death by “bystander” effect has also been observed in cells devoid of any sign of infection. Our results also show that the transient PCV2 Cap protein expression induces cell death at similar rate to PCV2 infection, whether the expression is nuclear or cytoplasmic, suggesting Cap protein’s ability to interact with different cell death signaling pathways. This study provides the basis for future research regarding the role of Cap-induced cell death in PCV2 pathogenesis.

**Materials and methods**

**Generation of recombinant eukaryotic expression vectors**

Coding sequences of ORF2 were PCR-amplified from PCV2a (GenBank accession number: JQ994269) and PCV2b (GenBank accession number: JQ994270) strains using oligonucleotide primers (Table 1). The PCV2a and PCV2b full genomes were cloned in pDNA plasmids that have been kindly provided by Dr. Carl Gagnon (University of Montreal, Quebec, Canada). The anti-sense ORF2 constructs were served as negative control constructs for Cap expression. PCR was performed with iProof™ Hi-Fidelity DNA Polymerase (BioRad, Mississauga, Ontario, Canada) in a GeneAmp® PCR System 9700 (PE Applied Biosystems, Carlsbad, CA). PCR cycle profile consisted of a pre-denaturation step at 98 °C for 30 s followed by 36 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s, extension at 72 °C for 60 s and a final extension step at 72 °C for 10 min. After separation by agarose gel electrophoresis, PCR products of expected size were purified using a QIAquick™ Gel Extraction Kit (QIAGEN). The NotI/Sall fragments of ORF2 were cloned into the corresponding sites of the eukaryotic expression plasmid pCI (Clontech, Mountain View, CA) under the control of a human cytomegalovirus (CMV) promoter (pCMV-PCV2bCap). A pCMV-HA plasmid (Clontech, Mountain View, CA) was used to clone the full length and truncated Cap without the nuclear localization signal (ΔNLS-2bcap) under CMV promoter and HA tag. The first 41 amino acids at the N-terminal of full length Cap (Liu et al., 2001) were removed using suitable primers (Table 1) to generate ΔNLS-2bcap fragment. The NotI/Sall fragments of full length Cap and ΔNLS-2bcap were cloned into the corresponding sites of the pCMV-HA. The resulting clones pCMV-HA-2bcap and pCMV-HA-ΔNLS-2bcap were confirmed by sequencing and the expression was confirmed by western blot using Rabbit anti-PCV2b-Cap. The ORFs cloned in this vector are expressed under CMV promoter in mammalian cells as a tagged protein with a N-terminal HA-tag.

**Table 1**

| Oligonucleotide primers to generate/sequence recombinant Cap constructs. |
|-----------------------------|-----------------------------|
| Constructs                  | Primer Sequence (5’ to 3’)  |
| PCV2a Cap (sense)           | CCAAGGAGG                   |
|                             | R-AGTCCGCGGCGTATTAGGGTTAAGGG |
| PCV2b Cap (sense)           | AAGGAACTGAGCCGCGTATTAGGGTTAAGGG |
|                             | R-AGTCCGCGGCGTATTAGGGTTAAGGG |
| PCV2a Cap (anti-sense)      | AAGGAACTGAGCCGCGTATTAGGGTTAAGGG |
|                             | R-AGTCCGCGGCGTATTAGGGTTAAGGG |
| PCV2b Cap (anti-sense)      | AAGGAACTGAGCCGCGTATTAGGGTTAAGGG |
|                             | R-AGTCCGCGGCGTATTAGGGTTAAGGG |
| ΔNLS-PCV2bCap (Sall)        | AAGGAACTGAGCCGCGTATTAGGGTTAAGGG |
|                             | R-AGTCCGCGGCGTATTAGGGTTAAGGG |
| HA-pCMV (sequencing primer) | CATCCGGTACTAGAGAAGAATCASAAC |

**Cell culture**

PCV-free porcine kidney epithelial PK-15 cell line was maintained in growth media (Eagle’s minimum essential medium (Invitrogen), 10% fetal bovine serum (FBS) (Invitrogen), 2% penicillin-streptomycin solution (Invitrogen), 1% sodium pyruvate (Invitrogen), 1% essential amino acids (Invitrogen). The human embryonic kidney epithelial 293T cell line was grown in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% FBS and 2% penicillin-streptomycin solution.

**Infection and transfection**

9 × 10⁴ TCID₅₀ of PCV2b were used to infect 60–70% confluent PK15 cells in 12-well plates. Cells were incubated for 90 min at 37 °C in 5% CO₂ and fresh culture medium was then added to PCV2b infected cells, which were further incubated for 48 h. For the purpose of increasing viral replication, PK15 were also treated with 500 U/ml of swine recombinant IFN-γ (Gibco) before or after PCV2b infection. Non-infected PK15 cells treated with IFN-γ were used as control. For transfection experiments, 12-well plates (3.8 cm² per well) were coated with poly-D-lysine (Sigma-Aldrich) and cells were seeded one day prior to transfection in growth medium without antibiotics (1 ml per well in a 12-well plate). When the cells were 70% to 80% confluent, 2 µg of DNA was transfected into the cells with Lipofectamine 2000 (Invitrogen, Burlington, Ontario, Canada). The growth media is added 4 h post-transfection and cells were analyzed 48 h later.

**Flow cytometry-based intracellular staining of the cap expression**

Mock and PCV2b-infected or transfected cells were trypsinized and washed with phosphate-buffered saline (PBS). For intracellular staining, cells were fixed in 4% formaldehyde and cell count was determined using Petroff-Hauser Chamber (Hauser Scientific partnership) and phase contrast microscopy (CKX41, Olympus Canada). Cells were diluted to 10⁶ cells/ml with PBS, treated with the permeabilization buffer (PBS, 0.1% saponin, BSA) for 30 min at RT and stained for intracellular Cap expression using a rabbit polyclonal anti-PCV2b Cap as a primary antibody (1:12792) and Goat anti-Rabbit-FITC as a secondary antibody. The samples were analyzed by flow cytometry (Cell Lab quanta TM SC MPL, Beckman Coulter, Canada).

**Cell cytotoxicity analysis**

All cells including both detached and adherent cells were collected, centrifuged at 2000 rpm for 5 min and washed twice with PBS. The concentration was adjusted to 10⁶ cells/ml. For each treatment, 100 µl of cell suspension was stained using molecular probes™ live/dead™ fixable dead cell stain kits (Invitrogen) according to the manufacturer’s protocol. The cells were then fixed with 4% formaldehyde and intracellular staining for Cap was done as described above. The samples were analyzed by flow cytometer and percentage of live/dead cells among the Cap expressing cells were calculated. Etoposide (Sigma-Aldrich), an apoptosis-inducing chemical, was used as a positive control in this assay.

**Immunofluorescence assay (IFA)**

For Cap protein localization study, PK15 and 293T cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature (RT). Cells were washed with PBS and treated with the permeabilization buffer (PBS, 0.1% saponin, BSA) for 30 min at RT. The cells were incubated with a polyclonal rabbit anti-PCV2b Cap primary antibody (1:200-dilution) at 37 °C in dark for 90 min. Cells were washed three times with PBS-T (PBS, 0.1%
Teneur et al. (1999), and were incubated in the dark with Alexa Fluor 568-conjugated goat anti-rabbit IgG ( Molecular probes, Invitrogen) at 37 °C for an hour. Cells were washed three times with PBS-T, counterstain with 4'-6-diamidino-2-phenylindole (DAPI) and visualized with Olympus IX51 fluorescence microscope.

**Statistical analysis**

Statistical analyses were performed using SPSS 17.0 software. Independent samples t-test was performed and differences were considered significant when P-value < 0.05. At least three independent trials were conducted for each experiment.

**Results and discussion**

**IFN-γ enhances PCV2 replication that leads to cell death in PK15 cells**

It has been shown that PCV2 replication induces apoptosis in the PK15 cell line (Liu et al., 2005), although it remains unclear whether cell death is a consequence of direct cytoxicity, a “bystander” effect or both. Since IFN-γ is a pro-inflammatory cytokine that has been found to play a role in increasing PCV2 replication in porcine cell lines (Ramamoorthy et al., 2009; Meerts et al., 2005), PK15 cells were infected with PCV2, treated with IFN-γ for 48 h, subjected to a double staining with fixable live/dead dye and anti-Cap antibody and analyzed by flow cytometry. Our results show an initial rate of infection of 3% in PK15 cells, which increases to 9% in IFN-γ treated cells regardless of whether the cytokine is added before or after infection (Fig. 1). We further show that the 2.5- to 3-fold increase in PCV2-Cap positive cells following IFN-γ treatment was accompanied by a higher rate of cell death when compared to untreated cells (80% vs. 40%, p < 0.05) (Fig. 2). Notwithstanding the limited understanding of how IFN-γ modulates PK15 cells permissiveness to PCV2 (Meerts et al., 2005) and how this leads to cell death, a study has reported IFN-γ's ability to decrease Cyclin A (Cyc A) expression (Sibinga et al., 1999), whose phosphorylation of NLS cargo proteins through nuclear pores (Harreman et al., 2004). The over-expression suppresses PCV2 replication by altering PCV2-Rep nuclear import of Cap protein remains unclear. It was hypothesized that the phosphorylation of the NLS regulates the import of NLS cargo proteins through nuclear pores (Harreman et al., 2004). Whether the phosphorylation of NLS regulates the import of NLS cargo proteins through nuclear pores (Harreman et al., 2004). Whether the phosphorylation of NLS may enhance or decrease the nuclear localization of PCV2 is a frequent event that accompanies the induction of apoptosis- induced cell death (Blachon et al., 2005; Heilman et al., 2006). To determine whether IFN-γ treatment has any effect on Cap, cells were infected with PCV2 and were either pre- or post- treated with IFN-γ and then stained for Cap protein expression. Our results show a clear change in the intracellular distribution of Cap protein 48 h post-infection, with a major shift in the ratio of cytoplasmic-to-nuclear Cap protein localization from 2:1 in untreated cells to approximately 1:11 in IFN-γ pre-treated cells and 1:5.6 in IFN-γ post-treated cells (Fig. 3). Although, PCV2-Cap has an NLS that tags this protein for nuclear translocation (Cheung and Bolin, 2002), the exact mechanism behind the nuclear import of Cap protein remains unclear. It was hypothesized that the phosphorylation of the NLS regulates the import of NLS cargo proteins through nuclear pores (Harreman et al., 2004). Whether the Cap-NLS has a kinase phosphorylation site or whether the phosphorylation of NLS may enhance or decrease the nuclear localization is unknown.

**IFN-γ enhances nuclear localization of the PCV2 cap protein**

Studies have shown that the nucleo-cytoplasmic shuttling of viral proteins is a frequent event that accompanies the induction of apoptosis-induced cell death (Blachon et al., 2005; Heilman et al., 2006). To determine whether IFN-γ treatment has any effect on Cap shuttling, cells were infected with PCV2 and were either pre- or post- treated with IFN-γ and then stained for Cap protein expression. Our results show a clear change in the intracellular distribution of Cap protein 48 h post-infection, with a major shift in the ratio of cytoplasmic-to-nuclear Cap protein localization from 2:1 in untreated cells to approximately 1:11 in IFN-γ pre-treated cells and 1:5.6 in IFN-γ post-treated cells (Fig. 3). Although, PCV2-Cap has an NLS that tags this protein for nuclear translocation (Cheung and Bolin, 2002), the exact mechanism behind the nuclear import of Cap protein remains unclear. It was hypothesized that the phosphorylation of the NLS regulates the import of NLS cargo proteins through nuclear pores (Harreman et al., 2004). Whether the Cap-NLS has a kinase phosphorylation site or whether the phosphorylation of NLS may enhance or decrease the nuclear localization is unknown.

**PCV2-caps protein expression induces cell death**

To rule out the possibility that the PCV2-induced cell death might be due to ORF3 expression, we sought to determine whether the expression of PCV2-Cap protein expression alone is capable of inducing cell death in the absence of viral replication. To mimic the late stage of PCV2 infection, the Cap protein of PCV2a and PCV2b,
the two major pathogenic genetic variants that differ only in the Cap amino acid sequence, was expressed under the control of the CMV promoter and used to transiently transfect PK15 and 293T cells. Cells were collected 48 h post-transfection and analyzed by flow cytometry for cell death and Cap protein expression. Regardless of which PCV2 variant had been used for transfection, cell death was induced in 60% of PK15 cells expressing Cap protein with the same extent as etoposide (a potent inducer of cell death), (Fig. 4). Neither PCV2 Cap transfection nor etoposide treatment were strong inducers of cell death in human 293T cells (Fig. 4), despite a 4- to 5-fold higher rate of expression of Cap protein in 293T cells (20–30%) as compared to PK15 cells (5-6%) (Fig. 5). The difference in cytotoxicity of PCV2-Cap between PK15 and 293T cell lines cannot be explained by the levels of Cap expression, as both PCV2a- and PCV2b-Cap constructs expressed the protein at a very similar level in both cell lines (data not shown). These data suggest that 293T cells may be specifically resistant to the PCV2-Cap protein effect, as they had been previously found to be sensitive to the pro-apoptotic effect of PCV2-ORF3 (Chaiyakul et al., 2010), and which further indicate that the mechanism through which PCV2-ORF3 and PCV2-Cap induce cell death may be distinct. The ability of Cap protein alone to induce cell death suggests that PCV2-induced apoptosis may reflect the need for Cap protein accumulation late in the replication cycle to induce cell death. No difference between PCV2a and PCV2b Cap cytotoxicity in transfected PK15 and 293T cells, although the ability of these two strains to propagate in VR1BL cells seems to be different (Dvorak et al., 2013). It has been suggested that the difference in the replication kinetic of PCV2 strains can be
compromised by a lower efficiency of PCV2 entry or Cap protein susceptibility to serine proteases cleavage that occurs in the endosome-lysosome system upon infection (Misinzo et al., 2008). Although further comparative analysis of Cap protein-induced cell death from different strains is needed, our data provide evidence that cytotoxicity can be mapped to the Cap protein and can be cited as a virulence determinant for PCV2.

Cell death is not dictated by specific cap protein sub-localization

Given that i) PCV2 replicates in PK15 but not in 293T cells (Hattermann et al., 2004), ii) Cap protein is expressed in both PK15 and 293T cells and iii) cell death is induced only in PK15 but not in 293T cells prompted us to investigate whether the difference between PK15 and 293T cells observed with regard to cell death is due to specific sub-cellular localization of Cap protein. The PK15 and 293T transfected cells were collected, assessed for Cap protein expression by IFA and a difference in the Cap protein sub-cellular localization has been found between PK15 and 293T cells. Interestingly enough, Cap protein was localized preferentially in the nucleus of PK15 cells (Fig. 6), whereas its expression was predominantly cytoplasmic in 293T cells (Fig. 6). Next we sought to analyze whether the nuclear localization of Cap protein was associated with cell death in PK15. For this purpose, we generated a construct to express a truncated form of Cap protein devoid of NLS, this deletion will restrict the expression to the cytoplasm (Liu et al., 2001). Both PK15 and 293T were transfected with pCMV-HA-2bcap and pCMV-HA-ΔNLS-2bcap and analyzed for Cap protein expression and cell death. Our data shows that the truncated form of Cap was expressed in the cytoplasm, whereas the full length Cap protein was detected predominantly in the nucleus (Fig. 6). Of note, the expression of Cap protein using pCMV-HA-2bcap and pCMV-HA-ΔNLS-2bcap constructs induce similar cell death rate in transfected PK15 cells (Fig. 8), suggesting that the cell death in our cell model is not dictated by specific Cap protein cell localization. This seems also to be true for PCV2 infected cells pre or post-treated with IFN-γ. Although, a difference was observed in the cytoplasmic-to-nuclear Cap protein localization ratio between IFN-γ pre- or post-treated cells (1:11 vs. 1:5.6), similar induced-cell death rate has been observed. These data suggest that Cap protein is capable of triggering different cell death signaling pathways in PK15 cells. Studies have indeed demonstrated Cap protein localization in the nucleoli, recognized as a site for ribosome biogenesis, which also holds components

![Fig. 6](image) Sub-cellular localization of Cap protein in PK15 and 293T cells. Recombinant construct with PCV2b-Cap under CMV promoter was transfected into PK15 and 293T cells. Cells were collected 48 h and analyzed by IFA. Representative images of three independent experiments are shown. In PK15 cells the Cap is localized predominantly in the nucleus (N*), whereas in 293T (C*) cells it is mostly cytoplasmic.

![Fig. 7](image) Sub-cellular localization of Cap protein in PK15 under HA tag. (a) Full length PCV2b-Cap (HA-2b) and (b) truncated Cap without NLS (HA-ΔNLS-2b). Recombinant constructs with full length PCV2b-Cap and PCV2b-Cap without NLS under HA tag and CMV promoter were transfected into PK15 cells. Cells were collected 48 h post-transfection and analyzed by IFA. The full length Cap protein is observed in different subcellular levels cytoplasm(C*), nucleus (N*) and also in the nucleolus (Nu*) of some cells. In the absence of NLS the Cap is localized in the cytoplasm (C*) alone.
that play a role in executing active cell death (Horký et al., 2002). In addition, virus-like particles have been shown to interact with the inner and outer membranes of mitochondria, the cellular organelles intrinsically involved in the apoptotic pathway (Rodríguez-Cariño and Segalés, 2009; Rodríguez-Cariño et al., 2010).

We conclude that Cap protein individually expressed has the ability to induce cell death in PK15 cells, and that effect may account for nearly all the cell death observed in PCV2 infected cells. The Cap protein’s ability to cause cell death regardless where it is expressed raises the possibility that cell death may be triggered in the nucleus by immature virions and enhanced by mature virions in the cytoplasm to promote the virus release. Although further investigation is needed to gain insights into the nature of the pathways involved in Cap-induced cell death, this study provides the basis for future research regarding the role of Cap-induced cell death in PCV2 pathogenesis.

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Fig. 8. Cell death of cytoplasmic versus nuclear expression of 2cap in PK15. Cells were transfected with full length Cap-ORF (PCV2b) and truncated cap (ANLS-PCV2b) under HA tag. Cells were collected 48 h after transfection and stained using molecular probes live/dead fixed cell stain kits (Invitrogen) followed by a staining for Cap protein expression. Values that are significantly different (P < 0.05) from the values for negative controls, e.g., mock- or empty vector controls (*) are indicated above the bars.

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