Investigating Porcine Circovirus Type 2 Viral Protein 3 Multimerization Capabilities

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ABSTRACT

Porcine Circovirus Type 2 (PCV-2) is a single-stranded non-enveloped DNA virus belonging to the Circoviridae family that produces a novel viral protein, Viral Protein 3 (VP3) that is suspected of selectively killing transformed human cancer cells via an unknown mechanism. To understand this mechanism, PCV-2 VP3 is often compared to the Chicken Anemia Virus (CAV Apoptin), which has been shown to harbor killing capacity towards transformed cells in a p-53 independent manner. Here we investigate by Fluorescence Resonance Energy Transfer (FRET) PCV-2 VP3’s ability to multimerize as part of a mechanism-of-apoptosis.
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INTRODUCTION

CANCER AND ITS TREATMENTS

The term, “cancer” is a broad term used to describe over one hundred diseases that are caused by uncontrolled and uninhibited cellular growth. Cancer can be brought on by a variety of reagents such as radiation, infectious organisms, inherited mutations, and immune conditions (1). Approximately 12,549,000 cases of cancer prevalence have been reported in the United States as of January 1, 2009 (1). If left untreated, cancer can spread throughout the body and result in death. In 2011, the American Cancer Society projected that approximately 577,190 Americans would die of cancer related issues in 2012 – more than 1,500 deaths a day. These numbers provide a clear indication of the seriousness of the disease and justify the need for further research towards the development of a cure. Until a cure is found, it is important to invest in the development of more effective and promising cancer treatments.

Some of the most promising cancer treatments of the modern age involve chemotherapy, radiation, and surgery. Cancer treatment is determined on a case-by-case basis and often depends on the cancer’s location, type, and stage of diagnosis. Due to the inability of many treatments to specifically target transformed cancer cells, they are considered cytotoxic to the body, being just as dangerous as they are beneficial. Hence, the study of targeted therapies is a rapidly growing area of research in the Pharmaceutical and Biotechnology fields. By targeting molecular features that are unique to transformed cells, therapies decrease the risk of damage to normal tissue. Many cancer therapies modulate the human tumor suppressor protein 53 (p-53) to prevent genomic-compromised cells from proliferating. P-53 has been shown to activate DNA damage repair and prevent cell division (2). However, nearly half of all
cancer patients are either missing or have mutations in the p-53 gene, rendering these treatments useless. For this reason, other targeted therapy research aims to distinguish transformed cells from normal cells in a p-53 independent manner. One area of research involves the use of Circoviruses as cancer treatments.

**Circoviruses**

Circoviruses are single-stranded non-enveloped DNA viruses belonging to the genus Circovirus of the Circoviridae family. Circoviruses have been found to include the smallest known autonomously replicating viruses present in eukaryotic host cells (3). The Circoviridae family includes Porcine viruses as well as Avian viruses, of which the Chicken Anemia Virus (CAV) is a member. CAV belongs to the Gyrovirus genus and differs from the Circovirus genus in genomic organization and capsid morphology (3). Circoviruses are typically very small, measuring approximately 17-22nm in diameter. They contain a genome that codes two major open reading frames (ORFs) which allow for viral replication and for the formation of a single capsid protein (4; 3). Circoviruses utilize a host cell’s Polymerases and repair activity to form dsDNA by using the virus’ ssDNA as a template (5).

Porcine Circoviruses are present in swine in Europe, North America, and Asia and were first discovered in 1974 as a contaminant found in cultured swine kidney cells (6). Two known Porcine Circoviruses exist, which are roughly 70% similar in nucleotide homology. (4). These viruses are known as Porcine Circovirus Type 1 (PCV-1), consisting of 1759 nucleotides, and Porcine Circovirus Type 2 (PCV-2), consisting of 1768 nucleotides (3). PCV-1 does not produce clinical infections and is nonpathogenic in swine, while PCV-2 causes Post-Weaning Multisystemic Wasting Disease, also known as Porcine Circovirus Associated Disease
(PMWS/PCVAD), in swine. This disease, along with PCV-2, was first discovered in North America in 1991 and has devastated many of the world’s largest swine-producing nations (3; 6). PCVAD is a disease affecting swine of about six weeks of age. The disease may cause lesions to form on several of the swine’s organ systems and can result in death, respiratory dysfunction, progressive weight loss, anemia, and diarrhea (5; 3; 6).

PCV-2

PCV-2, being the smaller of the two known Porcine Circoviruses, contains a genome that is approximately 1.76 kb and contains two major ORFs. ORF1 is called the rep gene and encodes for two proteins involved in viral replication: Rep and Rep’ (7). ORF2, called the cap gene, contains the genome for a 27.8 kDA protein which forms the major structural protein viral coat and is also used as the main antigenic determinant for the virus (7; 3). The virions of the Porcine Circoviruses form an icosahedral structure created by the self-assembly of sixty capsid protein molecules that arrange themselves in twelve flat, pentamer clusters (8). These two ORFs compromise 93% of the entire Porcine Circovirus genome (9). Several other ORFs have been predicted, but with the exception of ORF3, expression has not been seen (3). The third ORF consists of 315 bases in PCV-2 and encodes a novel viral protein, given the name Viral Protein 3 (VP3). VP3 has been shown to selectively induce apoptosis in transformed cancer cells.

VIRAL APOPTOSIS

Programmed cell death is an important part of the cell life-cycle and is integral for the health and survival of an organism. Apoptosis, in particular, is a, “genetically controlled process involved in the regulation of homeostasis, tissue development, and the immune system by eliminating cells that are no longer useful,” (10). Chromatin condensation, DNA fragmentation,
membrane blebbing, cell shrinkage, and compartmentalization of dead cells into apoptotic bodies are all unique characterizations to distinguishing cell death by apoptosis (10). Some speculations exist as to why a virus would contain a genome encoding for a viral apoptotic protein. Because viruses are dependent on the host cell’s machinery for replication and growth, it would be advantageous for a virus to produce a protein that blocks programmed cell death until sufficient progeny have been produced. Alternatively, the final stages of apoptosis of the host cell can also be advantageous to the virus. During this final stage, the cell becomes vacuolized into cellular apoptotic bodies (10). In the event of viral infection and apoptosis of a host cell, vacuoles would contain and protect viral particles, which may then be absorbed into surrounding cells. In partaking in this pathway, virions evade host immune responses and protect the progeny virus from host enzymes and antibodies (10).

**Viral Protein 3 (VP3)**

As was stated above, PCV-2 VP3 causes apoptosis in transformed cancer cells. In an experiment by Lie et al., expression of a GFP-fused PCV-2 ORF3 variant in swine cells demonstrated that PCV-2 VP3 is not essential for viral replication, but was shown to induce apoptosis in its host via capsase 8 and capsase 3 (7; 3). This leads to the belief that PCV-2 VP3 is an important factor in viral pathogenicity in vivo (7). It is hypothesized that PCV-2 VP3 is responsible for causing PCVAD in swine, and that the genetic permutations of PCV-1 VP3 and PCV-2 VP3 account for the observed difference in virulence in swine (7).
VP3 Isoforms

While PCV-2 may be present in swine, not all swine exhibit symptoms of PCVAD. This supports that death of swine infected with PCVAD is not guaranteed (9). However, in 2004, Canada’s swine industry experienced a dramatic increase in swine deaths due to PCVAD at a percent much higher than normal. While the cause was not readily and immediately understood, a hypothesis was formed that a more virulent strain of PCV-2 had developed. Post-examination of the swine proved this hypothesis to be true, as two forms of PCV-2 were discovered and named PCV-2a and PCV-2b respectively. PCV-2b was the name given to the newly discovered mutation of the Porcine Circovirus, and was shown to be much more virulent than PCV-2a (11). Further research, including the research completed in this lab, investigate the genetic and functional variances of the viral proteins of these two Circoviruses.

The two mutations of PCV-2 have remarkable nucleotide and amino acid homology but account for drastically different pathogenicity, as caused by mutations in their ORF3, resulting in two isoforms of PCV-2 VP3. Nucleotide alignments indicate 98% homology of the two isoforms, with mutations occurring at positions 121, 207, 283, 291, and 304. A difference of three amino acids is observed in the two isoforms post-translation, occurring at positions 41, 102, and 104. It has been speculated that the mutation occurring at the 41st amino acid would result in a functional change of a putative Nuclear Export Sequence (NES), which may account for the increase in pathogenicity. To examine this hypothesis, an experiment was completed that investigated the apoptotic abilities of the NESs and Nuclear Localization Sequences (NLSs) of the two PCV-2 VP3s. By forming chimeras of PCV-2a VP3’s functional NLS and PCV-2b VP3’s putative NES, we have shown that the putative NES of PCV-2b VP3 is likely responsible for induction of apoptosis in transformed cells (12). During this experiment, punctate structures
were observed in the cytoplasm of the host cells, indicating possible multimerization capabilities and defined cytoplasmic localization of PCV-2 VP3. Interestingly, CAV produces a viral protein, much like PCV-2 VP3, that has been shown to require self-multimerization in order to induce apoptosis (13).

**Chicken Anemia Virus (CAV)**

The Chicken Anemia Virus is a small, non-enveloped ssDNA virus with a genome of approximately 2.3kb (14; 8; 13). By replicating in thymocytes and erythrocytes, CAV has been found to produce severe anemia and immunosuppression in young chicks, often leading to death of the host (14). Much like PCV-2, CAV also contains three ORFs, of which the third produces a potent apoptosis protein named Apoptin. CAV Apoptin is a small 13.6 kDa protein that causes G2/M arrest and apoptosis of a host cell. However, apoptosis has been shown to be selective to a wide range of transformed human cancer cell lines (14; 15; 16). Additionally, CAV Apoptin has been shown to induce apoptosis in a p53-independent manner. In conjunction, these properties make CAV Apoptin one of the most intensely studied viral proteins for use in cancer treatment.

Unlike PCV-2 VP3, which has been shown to localize in the cytoplasm of both transformed and primary host cells, CAV Apoptin has been shown to localize in the nucleus of transformed cancer cells. In primary cells, however, CAV Apoptin will localize in the cytoplasm and fail to induce apoptosis (13). DNA damage in primary cells signals the nuclear localization of CAV Apoptin (14). This suggests that nuclear localization is required for induction of apoptosis. However, in an experiment that fused a strong NLS with CAV Apoptin, it was shown that the protein localized in the nucleus but did not demonstrate killing activity (13). This suggests that
another factor is essential for inducing apoptosis in transformed cells. Researchers have noted
distinct punctate structures in transformed cells infected with CAV Apoptin, and have suggested
that the multimerization of CAV Apoptin plays a significant role in the protein’s mechanism-of-
apoptosis (16).

According to Leliveld et. al., “...recombinant CAV Apoptin protein spontaneously forms
non-covalent multimers compromising 30-40 subunits in vitro.” The same study also
hypothesized that, “the multimeric state [of CAV Apoptin] is the functional form of
recombinant MBP-Apoptin.” It is interesting to note that the hypothesized multimerization
domains of CAV Apoptin also overlap with the NES (aa33-46) of the protein. Leliveld et. al.
showed that the NES amino acids of CAV Apoptin might fold as an anti-parallel beta sheet
forming a beta-turn or hairpin structure. In this case, the amino acids would orient in such a
way as to allow hydrophobic amino acids to protrude from one side of the hairpin, and the
hydrophilic amino acids on the other. This structure would account for CAV Apoptin’s
multimerization properties as the hydrophobic NES amino acids couple together and deactivate
the NES (16).

**CAV Apoptin and PCV-2 VP3**

As has already been mentioned in this text, CAV Apoptin and both isomers of PCV-2 VP3
have a multitude of genetic and functional similarities. Both CAV and PCV-2 have a genome that
consists of three ORFs. Each has a novel viral protein that is encoded in their third ORF that has
been shown to selectively induce apoptosis in transformed cells. Figure 3 shows the amino acid
alignment of CAV Apoptin and PCV-2 VP3 for both isomers. It can be seen that all three viral
proteins share some degree of homology at the amino acid level. The highest degree of
homology is seen at the NES sites. Though there is homology, there are many amino acid differences between all three proteins. What is important to note is that the physical properties of the amino acids remain the same for all three proteins, as can be seen in Figure 4.

Though physical properties of the proteins show similarity, the striking difference in subcellular localization raises certain alarms. Because subcellular localization is linked to multimerization in CAV Apoptin, and both are required to induce apoptosis, it is imperative to investigate the multimerization capabilities of PCV-2 VP3. Furthermore, although multimerization activity for PCV-2 VP3 has been previously hypothesized, it has not been scientifically proven. This is an area that must be investigated. If PCV-2 VP3 exhibits cytoplasmic localization and no multimerization capabilities, its mechanism of apoptosis is distinctly different from CAV Apoptin. Where CAV Apoptin has been used as a template for research on PCV-2 VP3, our findings here may shift PCV2 VP3’s candidacy as a novel cancer therapeutic in a different direction.

**FLUORESCENCE (OR FÖRSTER) RESONANCE ENERGY TRANSFER**

In order to expose the multimerization capabilities and dependencies of PCV-2 VP3, a novel Fluorescence Resonance Energy Transfer (FRET) technique will be used. According to Invitrogen, FRET is, “a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon.” This schematic can be observed in Figure 8. While other fluorescent microscopy techniques allow for the viewing of localization, FRET determines intermolecular interactions, making it a well-suited technique for determining multimerization.
GFP and DsRed as Fluorescent Dyes

The current dye standards used in FRET exhibit certain complications. The leading FRET partners (CFP/YFP) entail suboptimal donor excitation that hinders FRET imaging on many confocal microscopes (17). Additionally, CFP/YFP emissions have a large spectral overlap that further hinder and complicate imaging. DsRed, however, features spectra that resolve such issues. The DsRed fluorophore will be used as the acceptor molecule and the GFP fluorophore will be used as the donor molecule. Vectors that code for either GFP or DsRed fluorophore proteins will be used in these experiments.
MATERIALS AND METHODS

CONSTRUCT VERIFICATION AND SYNTHESIS

Restriction Digest

Isolation of PCV-2 VP3 inserts and DsRed vector was accomplished by a double digest of PCV-2a VP3 and PCV-2b VP3 DNA in GFP vector stock solutions and CAV DsRed-Apoptin vector stock solutions with EcoRI and BamH1 restriction enzymes. First, 7µL PCV-2a VP3 and PCV-2b VP3 in GFP constructs were digested with 2µL EcoRI and 2µL BamH1 and 2µL Buffer E. The mixtures were allowed to sit for two hours at 37 degree Celsius. To separate the inserts from the vectors, electrophoresis was run on the restricted samples using a 0.9% agarose gel (1X Tris-Acetate-EDTA (1M Acetate and 0.05M EDTA), 2µL Ethidium Bromide). The plasmid DNA was prepared for electrophoresis by mixing the restricted samples and 2µL of 5X DNA Loading Dye (30% glycerol, 0.25% bromophenol blue, 69.75% ddH2O). The columns were loaded with 20µL of each restriction and the gel was run for 90V for 60 minutes before imaging under a UV lamp to confirm restriction and presence of DNA. Approximately 12µL of a 2-log DNA ladder was used as a size reference.

Insert and Vector Purification

Restricted PCV-2 VP3 inserts and DsRed vector were excised from the gel using a sterile blade and purified using Promega’s Wizard SV Gel and PCR Clean-up System (Cat. # A9281). Gel containing the inserts and vectors were weighed separately in sterile tubes and 10µL of Membrane Binding Solution and 10µL Sodium Iodide (NaI) was added to each tube per 10mg of gel slice. Each tube was then vortexed and incubated at 60 degrees Celsius until the gel was dissolved. The gel mixtures were then transferred to a Minicolumn assembly and centrifuged at 16,000 x g for 1 minute. After discarding the flowthrough, 700µL of Membrane Wash Solution was added to the Minicolumn and pulled through the column by centrifugation at 16,000 x g for
1 minute. The process was repeated with 500µL of Membrane Wash Solution and centrifuged
for 5 minutes at 16,000 x g. The flowthrough was discarded and the column assembly was re-
 centrifuged at 16,000 x g for 1 additional minute. The Minicolumn was then transferred to a
sterile tube and 50µL of Nuclease-Free water was added to the column and allowed to incubate
for 1 minute at room temperature. After incubation, the Nuclease-Free water was pulled
through the Minicolumn by centrifugation at 16,000 x g for 1 minute to remove DNA from the
Minicolumn.

Insert and Vector Ligation
DsRed-PCV-2 VP3 construct synthesis was performed using T4 DNA Ligase and the
purified PCV-2 VP3 inserts and DsRed vector. The procedure was performed on ice. To ligate
PCV-2 inserts and DsRed vectors together, 1µL of T4 DNA Ligase and 1µL Ligase Buffer were
added to three ratios of vector to insert: 2µL vector to 6µL insert to 20µL total, 1µL vector to
10µL insert to 20µL total, 1µL vector to 1µL insert to 20µL total, 2µL vector to 6µL insert to 10µL
total, 1µL vector to 7µL insert to 10µL total, and 1µL vector to 1µL insert to 10µL total. A
negative control was also created that contained 1µL of T4 DNA Ligase and 1µL Ligase Buffer
with 2µL DsRed vector and 16µL ddH2O. Following 60 minutes of incubation at room
temperature, the ligated constructs were transformed into JM109 one-time use chemically
competent E. coli cells.

Construct Transformation Into Competent E. Coli Cells
DsRed-PCV-2 VP3 constructs were transformed into competent E. coli cells for construct
eexpression and amplification. Frozen JM109 cells were thawed on ice. While the cells were
thawing, LB-Kanamycin plates and LB media were warmed to 37 degree Celsius. Once thawed,
the JM109 cells were gently flicked to mix. Ligated constructs were transformed into the
competent cells by pipetting 5µL of the ligation mixture into the thawed cells and flicked to mix. The cells were incubated on ice for 15 minutes to allow complexes to form. Once complexes were formed, the cells were heat-shocked in a 42 degree Celsius water bath for exactly 60 seconds and then immediately returned to ice for 2 minutes. Once the cells had recovered, 450µL of warm recovery media was transferred to each E. coli tube and pipetted to mix. Each mixture was then incubated on at 37 degrees Celsius with rotation for a minimum of 1 hour. To select for transformation, 50µL of the transformed E. coli cells were grown over night at 37 degrees Celsius on the pre-warmed LB-Kanamycin plates.

Additionally, 2µL of stock GFP-PCV-2 VP3 DNA constructs were transformed into JM109 competent E. coli cells according to the procedure detailed above.

**Construct Inoculation**

To amplify DNA and screen for proper uptake of transformed constructs, transformed E. coli cells from construct plates were grown over night in LB-Kanamycin media. Following overnight incubation of the construct plates, colony growth was checked. Four random colonies from GFP-PCV-2 VP3 plates were added to 100mL of warm, sterile LB media containing 50µL 1000X Kanamycin and allowed to grow for 16 hours with shaking at 37 degrees Celsius in a sterile environment.

**Constructs Midiprep**

To extract GFP-PCV-2 VP3 Promega’s PureYield Plasmid Midiprep system (Cat. No. A2492) was used with 100mL of the inoculated transformed E. coli cells. The inoculated cells and media were separated into two 50mL conical tubes and centrifuged at 5,000 x g at room temperature for 10 minutes to pellet the cells. Pelleted cells were then resuspended in 3mL Cell
Resuspension Solution. Following resuspension, 3mL of Cell Lysis Solution was added to each tube and mixed by gently inverting 5 times. Cell lysis mixtures were allowed to incubate at room temperature for 3 minutes. After incubation, 5mL of Neutralization Solution was added to each tube and mixed by gently inverting 10 times. The lysate was then centrifuged at 15,000 x g at room temperature for 15 minutes. A column stack was assembled by placing a blue PureYield Clearing Column on top of a white PureYield Binding Column. The column stack was then placed on a vacuum manifold. Lysated supernatant from tube was poured directly into the column stack and the vacuum was applied until all liquid had passed through both the clearing and binding columns. The vacuum was slowly released from the filtration device and the blue clearing column was removed, leaving behind the white binding column on the vacuum manifold. To wash the lysate, 20mL of Column Wash Solution was added to the binding column and pulled through the column by applying the vacuum. Once all Column Wash Solution had passed through the binding column, the column membrane was dried by applying the vacuum for 60 seconds. The vacuum was then released and the column was removed and tapped on a paper towel to ensure all ethanol was removed. The binding columns were placed into new 50mL conical tubes. To elute the construct DNA, 600µL of Nuclease-Free Water was added to each of the columns and allowed to incubate at room temperature for 1 minute. The columns and conical tubes were then centrifuged in a swinging bucket rotor at 2,000 x g for 5 minutes. Filtrate was collected and stored at -20 degrees Celsius.
**Construct DNA Quantification**

To determine the concentration of GFP-PCV-2 VP3 and DsRed-Apoptin construct DNA, 300-fold dilutions of each purified DNA construct were created and vortexed to mix. Quartz cuvettes were cleaned with ddH2O, and 300uL of each of the diluted DNA plasmid constructs were added to the cuvettes and read on a UV-Vis Spectrophotometer with DNA/RNA setting at 260nm and 280nm.

**DNA Construct Transfection Into H1299 Cells**

To express construct DNA for experimentation, H1200 human lung cancer cells were grown and transfected with each construct in a sterile environment. H1299 human lung cancer cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic formulation PSF (penicillin, streptomycin, and fungizone) at 37 degrees Celsius in 5% CO2 air. Cells analyzed by fluorescence microscopy were grown to 40% confluency in 6-well plates containing square microscope cover slips sterilized in 70% ethanol. GFP-PCV-2 VP3 with DsRed-Apoptin and GFP-Apoptin with DsRed-Apoptin constructs were co-transfected into H1299 cells using the Qiagen Effectene Transfection Kit (Cat. No. 301425). GFP-Apoptin, DsRed-Apoptin, and GFP-PCV-2 VP3 constructs were also transfected into H1299 cells independently to be used for FRET calibration.

In order to achieve transfection, concentrated DNA constructs were diluted with EC buffer to a final concentration of 5µg/µl in 200µL. Once diluted, 6.4µL of Enhancer was added to each tube and vortexed to mix. The mixtures were allowed to incubate at room temperature for 2 minutes before 20µL of Effectene Reagent was added to each tube and complexes were allowed to form for 15 minutes at room temperature. While complexes were allowed to form,
the plates were washed with sterile 1X Phosphate Buffered Saline (PBS) and resuspended in 1.6mL of fresh media. Following the 15 minute incubation, DNA complexes were combined appropriately (DsRed-Apoptin with GFP-PCV-2 VP3 and GFP-Apoptin with DsRed-Apoptin). The mixed DNA complexes were then added to 1.2mL of fresh media and pipetted to mix. Approximately 710µL of the mixture was added to each of 2 wells dropwise. The 6-well plates were then allowed to incubate overnight at standard cellular proliferation conditions.

Following overnight incubation, the plates were washed with 1x PBS and the cells were fixed with 2ml of 1x PBS with 4% Paraformaldehyde before light shaking for 15 minutes. The cells plates were then rewashed in 1X PBS and the cover slips with cells were sterilized with 2mL of 70% Ethanol and allowed to incubate at room temperature for 10 minutes. The cover slips and cells were air dried and mounted onto slides using 20µL of mounting media (1x PBS with 50% glycerol and 2.5% DACBO) before being sealed with nail polish and allowed to dry in a dark box.

**FLUORESCENCE MICROSCOPY**

To observe multimerization, mounted cells will be imaged and FRET efficiencies will be calculated. FRET analysis will begin by imaging the GFP-Apoptin and DsRed-Apoptin transfected cells to give correction constants for eliminating excitation and emission cross-talk. Using lasers, GFP-Apoptin and DsRed-Apoptin constructs will be excited at their peak intensities of 488nm and 557nm respectively. Once fluorescence intensities are analyzed, PMT gain and laser dose factors will be calibrated for ideal donor (GFP) and acceptor (DsRed) controls. Correction images will be taken. The GFP-PCV-2 VP3 and DsRed-Apoptin co-transfected cells will then be
imaged and excited at 488nm. In each cell viewed, 6 regions of interest (ROI) will be selected for precise calculation of FRET efficiencies ($E_A(i)$) using the following formula:

$$E_{A(i)} = \frac{(B - A) \times (b - C) \times c}{C}$$

Where $A =$ Donor emission, $B =$ FRET emission, $b =$ Donor emission + crosstalk ratio, $C =$ Acceptor emission, $c =$ Acceptor emission + crosstalk ratio based on methods described previously (18).
RESULTS
IN-SILICO ANALYSIS OF PCV AND CAV

The Porcine Circovirus (PCV) contains a 1.76kb, single-stranded, circular genome. As seen in Figure 1, the genome contains three Open Reading Frames (ORF). ORF1 codes for the rep gene which produces two replication proteins, Rep and Rep’. ORF2 codes for the cap gene which produces a capsid protein. ORF3 contains a gene that is known as the VP3 gene and produces a novel viral protein (VP3) that has been shown to induce apoptosis in transformed cancer cells selectively. The PCV genetic model is very homologous to the Chicken Anemia Virus (CAV) genetic model. The PCV has been found to exist in two forms, PCV-1 and PCV-2, the latter being the cause of death in swine by Post-Weaning-Multisystemic-Wasting-Disease.

Two isoforms of The Porcine Circovirus Type 2 were found, PCV-2a and PCV-2b. They contain mutations in the third ORF that results in two forms of VP3. The difference in virulence between the isoforms is attributed to these mutations. Figure 2 shows the amino acid alignment of the two PCV-2 VP3 isoforms. Amino acid mutations of the isoforms and can be seen at positions 41 (Glycine to Serine), 102 (Phenylalanine to Leucine), and 104 (Lysine to Glutamine).

Of the three PCV-2 VP3 amino acid mutations, one is found in the putative Nuclear Export Sequence (NES) of VP3 at position 41. In CAV Apoptin, this NES domain is considered to be the domain responsible for self-multimerization, which is necessary to induce viral apoptosis in transformed cells by the protein. Figure 3 shows the amino acid alignment of the PCV-2 VP3 isoforms against CAV Apoptin with the NES domains highlighted in yellow. The highest degree of amino acid homology can be seen in this domain. However, varying amounts of dissimilarity
between the alignments both in and outside of this domain leads to uncertainty of complete NES homology. In Figure 4, however, it can be seen that the NES domains (highlighted in yellow) of both PCV-2 VP3 isoforms are predicted to be neutrally charged and hydrophobic. These properties mirror the properties of CAV Apoptin’s NES. Figure 5 shows the possible multimer motif of NES-multimerized proteins. The hydrophobic NES areas attract one another and are subsequently buried in the motif.

**Formation and Verification of DNA Constructs**

In order that NES similarities and multimerization be investigated, FRET fusion protein constructs were created and verified. GFP-PCV-2 VP3 and DsRed-Apoptin constructs were verified by a double digest with EcoRI and BamH1 followed by gel electrophoresis on a 0.9% agarose gel to separate inserts from vectors. The separated inserts and vectors can be seen as separate bands on the gel image found in Figure 6. The log 2 ladder was used as a size marker and the vector and insert band sizes were verified against known PCV-2 VP3, Apoptin, GFP, and DsRed genome sizes. The GFP and DsRed vectors are approximately 4.4kb and the PCV-2 VP3 inserts are approximately 315b. Apoptin was verified to be 363b. These sizes are consistent with literature reported sizes.

DsRed-PCV-2 VP3 construct formation is ongoing according to the schematic detailed in Figure 7 followed by transformation into chemically competent cells.

**Transformation and FRET**

To image PCV-2 VP3 and Apoptin NES multimerization interactions, GFP-PCV-2 VP3 and DsRed-Apoptin DNA constructs were successfully transformed into human lung cancer H1299
cells. The mounted cells were imaged for epifluorescence in order to verify successful transformation. Appropriate fluorescence was observed; co-transfected cells displayed presence of both GFP and DsRed tagged proteins. Co-localization of the co-transfected cells and presence of punctate structures was observed.

FRET technique is currently underway to investigate cross-multimerization and self-multimerization. GFP-PCV-2 VP3 and DsRed-Apoptin as well as GFP-PCV-2 VP3 and DsRed-PCV-2 VP3 co-transfected cells are being investigated for signs of multimerization.
The characteristic ability of the Porcine Circovirus Type 2 Viral Protein 3 (PCV-2 VP3) and the Chicken Anemia Virus Apoptin protein (CAV Apoptin) to selectively target transformed cells and induce cell death via apoptosis in a manner that is independent of p-53 makes these viral proteins ideal contenders as cancer therapeutics. CAV Apoptin has been intensely researched and has been hypothesized to self-multimerize in the nucleus of transformed cells before interacting with the Anaphase Promoting Complex/Cyclosome (APC/C) and inducing apoptosis via a protein cascade (13). Most importantly, CAV Apoptin has been hypothesized to undergo multimerization at its Nuclear Export Sequence (NES) domain (16). In this case, the consequential multimer motif would display NES hydrophobic coupling inside the multimer (Figure 5). This motif supports the presence of CAV Apoptin multimers specifically in the nucleus, as NES activity would be deactivated as a consequence of the unexposed domain.

In contrast to CAV Apoptin’s nuclear localization in transformed cells, PCV-2 VP3 localizes in the cytoplasm specifically. These two proteins have often been compared because of their genomic structure homology and ability to induce apoptosis in transformed cells. The mechanism-of-apoptosis of the two viral proteins has been considered to be very similar, if not the same. However, the observed difference in subcellular localization raises question to this thought. Under the condition that CAV Apoptin multimerizes at its NES domain, all NES function would be rendered inactive; this is seen. However, if the same multimerization domain exists for PCV-2 VP3, observed cytoplasmic localization cannot be explained. PCV-2 VP3 has been shown to contain a functional Nuclear Localization Sequence (NLS), in which case, deactivation of its NES would result in nuclear localization. For these reasons, amino acid alignments (Figure
3) were performed for both isoforms of PCV-2 VP3 and CAV Apoptin to investigate NES homology.

The highest degree of homology is seen in the NES domain on the amino acid alignment. The degree of homology, however, is not enough to scientifically prove that the NES domains are similar enough to demonstrate identical multimerization. Therefore, the amino acid sequences of both isoforms of PCV-2 VP3 were entered into a program to predict secondary structure, specifically average charge, surface areas, and hydrophobicity/hydrophilicity (Figure 4). The results of the prediction algorithm pertaining to the NES domain mirror the secondary structure properties of CAV Apoptin’s NES domain. It is likely, then, that the domains are homologous in function. To investigate this further, FRET was attempted to verify multimerization.

While it is important to identify self-multimerization of PCV-2 VP3, it is of equal importance to examine cross-multimerization between PCV-2 VP3 and CAV Apoptin to identify NES similarity and multimerization capabilities. H1299 lung cancer cells were transfected with fluorophore fusion proteins (GFP-PCV-2 VP3 and DsRed-Apoptin) to examine cross-multimerization by utilizing FRET. Transformation was successful, and upon imaging the cells for epifluorescence, the presence of both fluorophores in co-transfected cells was confirmed. Before attempting FRET, the localization of the fusion proteins was noted. The fluorophore tagged PCV-2 VP3 and CAV Apoptin proteins appeared to co-localize in the nucleus. This data, however, is not enough to verify multimerization, but may be a precursor to the discovery of NES homology.
Though FRET is currently underway, certain speculations may be considered before results are attained. Due to the potential of co-localization, it may be likely that NES homology is great enough as to allow PCV-2 VP3 to multimerize with CAV Apoptin at the NES domain and initiate FRET, as is illustrated in Figure 9A. However, under this condition, self-multimerization is brought into question. NES domain multimerization for PCV-2 VP3 would most likely allow self-multimerization. The resulting motif would result in NES burying and inactivation, which is not consistent with the cytoplasmic localization of punctate PCV-2 VP3 structures. It may be possible, that the NES of CAV Apoptin is overwhelming PCV-2 VP3 and dragging it into the nucleus.

Figure 9B illustrates the second of three possible FRET results. The inability of PCV-2 VP3 to self or cross-multimerize would not initiate FRET. This observation would be consistent with the cytoplasmic localization of PCV-2 VP3, but would also most likely mean that the two viral proteins interact with transformed cells in different manners and cause apoptosis via different mechanisms. In this case, it would be unwise to continue to compare PCV-2 VP3 and CAV Apoptin in further research. CAV Apoptin has been shown to localize in the nucleoli (DNA-rich areas) of transformed cells. It is there that CAV Apoptin has been hypothesized to interact with APC/C and damaged DNA ends (19). It would be of best interest to investigate PCV-2 VP3 interactions with damaged DNA ends by exposing the protein to single stranded DNA. If the protein interacts with the ssDNA, it may allude to a similar mechanism-of-apoptosis as CAV Apoptin, but may indicate interactions taking place either earlier or later down the mechanistic cascade.
The last of the three possible FRET results is illustrated in Figure 9C. It is possible that PCV-2 VP3 is dissimilar enough to CAV Apoptin at the NES domain as to disallow multimerization with CAV Apoptin and not initiate FRET. However, because the presence of punctate structures of PCV-2 VP3 has been observed, self-multimerization of PCV-2 VP3 may be allowed. For this reason, FRET should be completed for GFP and DsRed tagged PCV-2 VP3 to examine self-multimerization. Self-multimerization at the NES domain conflicts with the cytoplasmic localization of PCV-2 VP3. However, self-multimerization of PCV-2 VP3 may occur at a different domain that is dissimilar to CAV Apoptin. It may also be possible that a non-traditional NES is present in PCV-2 VP3 that is not inactivated during multimerization. To investigate this phenomenon, mutagenic probing of other possible NES domains of PCV-2 VP3 should be completed.

The results presented in this study suggest nuclear co-localization of PCV-2 VP3 and CAV Apoptin by multimerization, but does not prove any such behavior. It is vital that FRET be completed for PCV-2 VP3 to examine cross-multimerization and self-multimerization in order to compare or elucidate mechanisms-of-apoptosis. Depending on the results retrieved from these experiments, PCV-2 VP3’s candidacy as a novel viral protein cancer therapeutic may be supplemented or hindered. Under the condition that PCV-2 VP3 undergoes a different mechanism-of-apoptosis than CAV Apoptin, a slew of other possible cancer therapeutics may be uncovered and researched further.
FIGURES

Figure 1: PCV-2 Genome. The PCV-2 genome (~1.76 kb) consists of three open reading frames (ORF). ORF1 encodes for viral replication proteins (Rep and Rep'). ORF2 encodes for the capsid protein. The third ORF encodes for a viral protein that is similar to CAV Apoptin. It is suspected of harboring killing capacity towards transformed cancer cells.

Figure 2: PCV-2 VP3 Amino Acid Alignment. PCV-2a VP3 and PCV-2b VP3 Amino Acid sequences are 97% homologous and differ by 3 amino acids located at positions 41 (Glycine to Serine), 102 (Phenylalanine to Leucine), and 104 (Lysine to Glutamine). Mutation 41 occurs in the putative NES of PCV-2 VP3.
Figure 3: PCV-2 VP3 Isoforms and CAV Apoptin Amino Acid Alignment. Highly conserved areas are indicated in red, moderately conserved areas are indicated in pink, and non-conserved areas and gaps are indicated in blue. The most homology is seen at CAV Apoptin’s NES (position 37 to 46) and PCV-2 VP3’s putative NES (position 40 to 47) which are highlighted in yellow. Still, much dissimilarity exists.

Figure 4: PCV-2 VP3 Amino Acid Physical Properties. PCV-2 VP3’s putative NES (highlighted in yellow) is predicted to be hydrophobic and neutrally charged. This software does not predict NES as being a surface region. National Center for Biotechnology Information (NCBI) secondary structure prediction software was used to determine PCV-2 VP3 properties. These properties exactly mirror CAV Apoptin NES properties, alluding to similar NES structure and behavior.
Figure 5: Possible NES Multimerization Motif. NES multimerization due to hydrophobic interactions result in “buried” NES regions, rendering the NES inactive.

Figure 6: Construct Verification Gels. 0.9 % Agarose Gel Electrophoresis of EcoRI and BamH1 restricted GFP-PCV-2 VP3 and DsRed-Apoptin constructs verified the presence of appropriate inserts and vectors. A Log 2 DNA ladder was used to verify insert and vector sizes.
Figure 7: DsRed-PCV-2 VP3 Construct Synthesis Schematic. Double digest restriction of GFP-PCV-2 VP3 and DsRed-Apoptin constructs will yield separated PCV-2 VP3 inserts and DsRed vector. Ligation of these inserts and vectors will allow for the formation of DsRed tagged PCV-2 VP3 proteins.

Figure 8: FRET Technique Diagram. Excitation of the GFP-PCV-2 VP3 fusion protein by at 488nm will excite the DsRed-Apoptin fusion protein if the proteins are within 10nm of each other (indicating multimerization). DsRed Direct Excitation and GFP Direct Excitation bleedthrough will be accounted for using independent fluorophore FRET controls.
Figure 9: Possible PCV-2 VP3 and CAV Apoptin Multimerization Outcomes. FRET using GFP-PCV-2 VP3 and DsRed-Apoptin will result in three possible outcomes. PCV-2 VP3 and CAV Apoptin will multimerize and induce FRET (A), PCV-2 VP3 and CAV Apoptin will not multimerize and fail to induce FRET and no PCV-2 VP3 self-multimerization will be observed (B), and/or PCV-2 VP3 and CAV Apoptin will not multimerize and fail to induce FRET and PCV-2 VP3 self-multimerization will be observed (C).
REFERENCES


