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Fusion of Viral Proteins Apoptin and PCV-1 VP3 C-Terminus: A Study of Localization and Induced Specific Apoptosis of Carcinogenic Cells

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Major Qualifying Project

Fusion of Viral Proteins Apoptin and PCV-1 VP3 C-Terminus: A Study of Localization and Induced Specific Apoptosis of Carcinogenic Cells.

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Abstract

Apoptin and Porcine Circovirus Type1 (PCV-1) VP3 are two viral proteins from chicken anemia virus and porcine circovirus type I respectively. Both proteins have been shown to selectively cause apoptosis in transformed (cancerous) cells. PCV-1 VP3, 621 bp, is roughly double the size of apoptin, 366 bp. The first 315 bps of PCV-1 VP3 share homology with apoptin, while the second 306 bps, known as the "Tail" region, are non-homologous to apoptin. Apoptin is characterized by nuclear localization in transformed cells, whereas PCV-1 VP3 stays cytoplasmically localized, due to a strong nuclear export sequence (NES) in the 'Tail". To determine the necessity of nuclear localization of apoptin to induce apoptosis, the "Tail" region of the PCV-1 VP3 was fused to the 3' end of the apoptin gene. The fused protein was transfected into H1299, non-small cell lung carcinoma, cells. Localization and apoptosis studies were performed and it was discovered that the fused protein localized to the cytoplasm but did not induce apoptosis, therefore implying that nuclear localization may indeed be a critical step in the mechanism by which apoptin induces apoptosis.

Acknowledgments

We would first and foremost like to thank Professor Destin Heilman. His guidance and input throughout this project was a key factor to its success. We would also like to thank the lab manager Edward Partlow III for all of his contributions to our project, including his overlap extension PCR method and his continued help and support throughout the entirety of the project.

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Introduction

In 2014, the United States population is expected to endure over 1.6 million new cases of cancer and over half a million cancer related deaths[1]. To put this number further into perspective, about 1600 people are expected to die per day from cancer alone. This makes cancer the second most common cause of death in the United States[1]. There are many different types of cancer, but they all originate from cells that have lost their ability to regulate the cell cycle. This loss of cell cycle control results in the proliferation of cells that have damaged DNA. These cells deteriorate the body through tumor growth or attack of other somatic tissues[1].

The body has many different mechanisms by which it regulates cancer; however, one of the most important "protectors" from cancer formation is the TP53 gene. This tumor suppressor gene codes for the protein p53. Within the cell, the binding of the p53 protein to DNA stimulates the production of p21, a protein which interacts with a cell division-stimulating protein, cdk2. When this cdk2/p21 complex is present, the cell cannot continue to the next stage of the cell cycle. Aside from causing cell cycle arrest, p53 can activate DNA repair mechanisms, and also the induction of apoptosis, or programmed cell death. Although p53 is critical in protecting an organism from cancer, the simplest mutation can cause a nonfunctional p53, which can lead to the development of cancer[2]. Mutations in the p53 gene are very common among cancer patients. In fact, over half of the patients suffering from cancer lack functional p53[3].

Cancer treatment today is predominantly comprised of chemotherapeutic agents and radiation. These treatments of cancer cells are regarded as the best strategies that we have at the moment for anti-cancer treatment. The problem with these treatments is that they do not selectively localize to oncogenic cells. This non–selective localization causes stress in the

somatic tissue of the patients undergoing those treatments. Also, most chemotherapeutics and radiation treatments rely on a functional p53 pathway to induce the apoptosis of cancerous cells[4]. Therefore their effectiveness at combating cancer is diminished when there is non-functional p53.

In recent years however, it has been discovered that a family of viruses, the *Circoviridae* family, produce a protein which selectively kills cancerous cells regardless of whether or not there is a functional version of P53. This family contains viruses that are non-enveloped, icosahedral, single stranded, circular DNA viruses. These viruses are very similar when it comes to structure and function.

One virus of the *Circoviridae* family, the chicken anemia virus (CAV), produces a protein that has been found to be involved in an apoptotic pathway that is independent of p53. Chicken anemia virus is a virus that causes anemia, bone marrow atrophy, and severe immunosuppression in poultry or what is commonly known as Blue Wing Disease[5]. The virus produces three viral proteins, of which the third viral protein (VP3), also known as apoptin, has been investigated for its ability to induce apoptosis. Apoptin's ability to induce apoptosis is selective to cancer cells, having been shown to only induce apoptosis in transformed cells but not in normal cells[6].

The mechanisms by which apoptin induces apoptosis in transformed cells remain unclear. However there are domains of the protein which have been determined to be crucial in the successful induction of apoptosis. Apoptin has been shown to contain an N- terminal nuclear export sequence (NES) and a bipartite C-terminal nuclear localization signal (NLS)[7]. The inclusion of both the NES and the NLS are critical to the selective localization between cell types, as fragments containing either the NES or the NLS do not exhibit selective localization[7]. It

has also been shown that apoptosis induction is dependent on the formation of apoptin multimers[8]. Furthermore, apoptin has been shown to interact with the anaphase promoting complex / cyclosome (APC/C), causing G2/M phase arrest and leading to apoptosis[7]. As with many viruses that have compact genomes, certain domains of the protein overlap. This is true with apoptin, as the multimerization domain overlaps the NES and the APC/C binding domain overlaps the NLS[7]. Although apoptin's protein characteristics and some interactions are known, the precise mechanisms that lead to the induction of apoptosis after the cells have gone into G2/M arrest are unclear.

Another subset within the Circoviridae family, which has been shown to have similar physiologic effects as CAV, is the porcine circovirus or PCV. There are two subtypes of PCV, PCV-1 and PCV-2. PCV-2 is known to cause postweaning, multisystemic, wasting syndrome (PMWS) in pigs[9]. PCV-1 on the other hand is non-pathogenic[9]. Both PCV-1 and PCV-2 encode a protein, viral protein 3, which shares homology with apoptin, and also have been shown to share apoptotic ability[10]. PCV-2 VP3 is similar in size to apoptin and has been shown to have both a functional NES and NLS and is believed to have the ability to multimerize with itself[11,12]. PCV-1 VP3 has also been shown to have a putative NES and NLS sequence in a region of the protein which is homologous to apoptin[13]. However, the PCV-1 VP3 protein is roughly double the length of apoptin, containing an extended C-terminus known as the "Tail" region of the protein. Both PCV-1 VP3 and PCV-2 VP3 have been observed to selectively cause apoptosis from the cytoplasm of cancerous cells, having no apparent selective localization, unlike apoptin[11,14].

The "Tail" region of PCV-1 VP3 is of particular intrigue, as it is not found in the other circovirus proteins to which it is homologous. It comprises about half of the protein, and makes it

double the length of its homologs. It is composed of roughly 60% hydrophobic amino acid residues. Within the "Tail" region there is a strong putative NES sequence which is believed to be the driving force behind its cytoplasmic localization, as shown by C-terminus fragments of PCV-1 VP3 localizing to the cytoplasm[13].

To further elucidate the pathway by which apoptin causes apoptosis specifically in cancer cells, this study will fuse the "Tail" of the PCV-1 VP3 protein to the end of apoptin. This will be done in an effort to pull apoptin into the cytoplasm utilizing the strong NES of the PCV-1 VP3 "Tail". There have been other attempts to keep apoptin cytoplasmically localized by mutagenesis of the NES and NLS domains, however since these domains overlap other critical domains such as the APC/C binding domain and the multimerization domain, site specific mutagenesis of these areas may also have unintended consequences on the apoptotic ability of the protein. The addition of the "Tail" domain with the strong NES will hopefully have no undesired consequences on the protein, as there is no mutagenesis of the actual apoptin protein.

Subsequent transfection of the human non-small cell lung carcinoma cell line, H1299, will allow for localization and apoptosis studies. By adding the strong NES of the "Tail" portion of PCV-1 VP3, it is expected that the fused protein will remain cytoplasmically localized. If this occurs, then the apoptotic ability of the fused protein can be analyzed, and the importance of the nucleo-cytoplasmic shuttling of apoptin in the induction of onco-specific apoptosis can be analyzed. This will further elucidate the mechanisms by which apoptin causes selective apoptosis in transformed cells, and determine if nuclear shuttling is a key step in the apoptotic pathway.

Methods and Materials

Fusion of Apoptin and PCV-1 VP3 "Tail"

Two methods were used simultaneously to fuse the apoptin and PCV-1 VP3 "Tail" genes together. Only the plasmid generated from the overlap extension (OE) PCR method was successful in transforming an *E. coli* culture, therefore this was the method utilized to fuse the gene together.

Method 1: Traditional PCR, Restriction, and Ligation

The "Tail" vector had been previously generated using EcoRI and BamHI sites to insert the "Tail" gene into a pEGFP-C1 vector, as shown in Figure 1. Forward and reverse primers were designed in order to amplify the apoptin gene with restriction sites for SacI and EcoRI, such that it could be inserted into the MCS of the "Tail" vector in frame, as shown in Figure 2. A PCR master mix was created using nuclease-free water, reverse primer (5 pmol/µL), forward primer (5 pmol/µL), pEGFP-C1 apoptin vector, and GoTaq buffer. The reaction was run in the thermal cycler under the following conditions; an initial dsDNA denaturing (95 °C for 2 minutes), then 25 cycles of; dsDNA denaturing (95°C for 30 seconds), primer annealing (55 °C for 30 seconds), and extension (72 °C for 30 seconds). A final extension time (72 °C for 2 minutes) finished the reaction and it was kept at 10 °C for storage purposes. The PCR results were verified using gel electrophoresis with a 0.9% agarose gel containing ethidium bromide. After verification the PCR was purified using the Wizard® SV Gel and PCR Clean-Up System using the published protocol[15]. The purified apoptin and the pEGFP-C1 PCV-1 VP3 "Tail" vector were restricted, separately, with EcoRI (12 u/µL) and SacI (10 u/µL) in 10x Buffer E. After 1 hour incubating in a 37 °C water bath, the restricted products were isolated using gel

electrophoresis with a 0.9% agarose gel. The DNA was purified using the Wizard® SV Gel and PCR Clean-Up System protocol. The purified products were combined with 10x T4 DNA Ligase buffer and T4 DNA ligase (3u/µL) and incubated at room temperature for 1 hour.

Method 2: Overlap Extension (OE) PCR

This process consists of 2 sequential PCRs, where the product from the first reaction is used as the primer in the second reaction; see Figure 3 for a schematic of the procedure. Forward and reverse primers for the first PCR were designed to amplify the PCV-1 VP3 "Tail" gene with floppy ends that were complimentary to the apoptin pEGFP vector, such that the product of the reaction would be the "Tail" insert flanked on both sides by sequences complimentary to the pEGFP-C1 apoptin vector, see Figure 4. The first PCR master mix was created using nuclease-free water, reverse primer (5 pmol/µL), forward primer (5 pmol/µL), pEGFP-C1 PCV-1 "Tail" vector, and GoTaq buffer. The reaction was run in the thermal cycler under the following conditions; an initial dsDNA denaturing (95 °C for 2 minutes), then 25 cycles of; dsDNA denaturing (95°C for 30 seconds), primer annealing (55 °C for 30 seconds), and extension (72 °C for 30 seconds). A final extension time (72 °C for 2 minutes) finished the reaction and it was kept at 10 °C for storage purposes. The product was electrophoresed on a 0.9% agarose gel, and the DNA was purified from the gel using the Wizard® SV Gel and PCR Clean-Up System using the published protocol[15]. This purified PCR product was then used as the primer in the second PCR reaction. The second PCR reaction was set up using purified PCR product from the first reaction, pEGFP-C1 apoptin vector, $MgSO_4$, dNTPs, nuclease free water, Phusion® polymerase, and Phusion buffer. The reaction was run in the thermal cycler under the following conditions; dsDNA denaturing (95°C for 30 seconds), primer annealing (55 °C for 30 seconds), and extension (72 °C for 5 minutes and 40 seconds), and this was repeated for 17 cycles. The

products of this second PCR reaction were then treated with DpnI and incubated at 37 °C for 3.5 hours to destroy the methylated, supercoiled original apoptin pEGFP-C1 plasmid.

Transformation of Competent *E. coli*

The DpnI OE PCR product (3µL) was added to thawed JM109 competent *E. coli* (50µL, L2005 Promega). These were incubated for 15 minutes on ice to allow DNA complexes to form, then heat shocked for 60 seconds at 42 °C and returned to ice for 2 minutes. LB media (450 µL) was added, and the cells recovered at 37 °C for 1 hour. The transformed *E. coli* were plated on an agar plate containing kanamycin and grown overnight (18-24 hours).

Small Scale Plasmid Purification

Four colonies were randomly selected and transferred to separate pre-warmed LB media (100 mL) containing 1000x kanamycin for inoculation. These were incubated with shaking (220rpm) at 37 °C overnight (18-24 hours). Plasmid DNA was then isolated by Wizard® Plus Minipreps DNA Purification System using the published protocol[16]. The resulting plasmids were verified for fused protein through a restriction digest.

Restriction Digest

Restriction digests of each of the eluted plasmid DNA were performed using 1 µL of EcoRI (12 u/µL), 1 µL of BamHI (10 u/µL), 10x Buffer E, ddH_2O, and the eluted DNA. These four master mixes were incubated at 37 °C for 1 hour. The restrictions were then analyzed using gel electrophoresis with a 0.9% agarose gel containing ethidium bromide, to confirm the presence of the fused apoptin – PCV-1 VP3 “Tail” gene.

DNA Quantification and Sequencing

The eluted plasmid DNA was first diluted 100X in ddH_20. The approximate concentration was then determined by measuring the absorbance at 260nm of this dilution. The concentration was determined to be 0.64 µg/µL. DNA products were diluted to a concentration of 200 ng/µL, then sent to Macrogen USA to be sequenced and sequences were analyzed using MacVector.

Cell Culture Maintenance

H1299s, non-small human lung carcinoma cells, lacking endogenous P53 were maintained with Dulbecco's Modified Eagle Medium (DMEM)/High Glucose with 10% Fetal Bovine Serum and PSF (100 units/mL Pen G sodium; 100 mg/mL streptomycin sulfate; 0.25 mg/mL amphotericin B). The cells were incubated in 37°C and humidified by 5% CO2 and confluence of the cells was maintained at or below 75% by frequent passage.

Transfection in H1299 Cell Lines

H1299 cells were transfected with the fused apoptin – PCV-1 VP3 "Tail" protein, wild-type apoptin, wild-type PCV-1 VP3, and GFP. The cells were transfected using the Effectene Transfection Reagent Kit (Qiagen) with the published protocol. H1299 cells were passed into a 6-well plate with square coverslips at 40-50% confluence. The H1299 cell line was transfected after approximately 24-36 hours of incubation, at a confluence of 70-80%. At approximately 24 hours post transfection, the media was aspirated off the cells, which were washed with 1x sterile PBS, and subsequently treated with the apoptosis detection kit.

Apoptosis Detection in Transfected H1299 Cell Lines

The transfected cells were treated with the Image-it^{TM} LIVE Red Caspase-3 and -7 Detection Kit using the published protocol. The cells were incubated for 1 hour with the caspase-3 and -7 detection red fluorophore. The kit detects apoptosis through the use of a fluorophore

covalently bound to a quencher by a caspase recognition sequence. If the cells are non-apoptotic, there is no caspase activity, and the excited photons emitted from the fluorophore are absorbed by the quencher. If the cells are apoptotic, there are active caspases, and the caspases will cleave the quencher, which is cell permeable and will therefore diffuse out of the cell, from the fluorophore, allowing the fluorophore to excite photons that can be viewed as red fluorescence in the cell. After 1 hour of incubation with the fluorophore, the media was aspirated from the wells. The cells were subsequently washed twice with a 1:10 dilution of the 10X Apoptosis wash buffer. The cells were then fixed and prepared for fluorescence microscopy.

Fixing Cells and Fluorescence Microscopy

The cells were fixed using 4% paraformaldehyde in PBS with slow agitation for 15 minutes. The cells were washed with 1x PBS and mounted on slides with mounting media (50% glycerol; 100 mM Tris (pH 7.5); 2% DABCO, 10µg/mL DAPI). The slides were imaged by epifluorescence and confocal microscopy using the Leica SP5 confocal microscope.

Results

The Fusion of PCV-1 VP3 C-Terminus "Tail" with Apoptin

It has been well characterized that apoptin, the third viral protein produced by the chicken anemia virus (CAV), induces apoptosis selectively in cancerous cells. It has also been shown that this selectivity seems to be connected to a differential localization of apoptin in transformed cells, to the nucleus, as compared to normal, non-cancerous cells, in which it localizes to the cytoplasm. This nuclear localization is thought to be a key event in the pathway by which apoptin selectively causes apoptosis in transformed cells. Interestingly however, a homologous protein created by PCV-1, its own third viral protein, VP3, has also been shown to induce cancer specific apoptosis, but without nuclear localization. The key difference between apoptin and PCV-1 VP3 is an area on the PCV-1 VP3 known as the "Tail" region. This region is highly hydrophobic, roughly 60%, and also contains a strong NES, which keeps the protein cytoplasmically localized. Because the proteins share homology and selectivity, but not localization, it raises the question as to whether the nuclear localization of apoptin is indeed a critical step for the induction of apoptosis in transformed cells.

Studies have been performed in which the NLS of apoptin was mutagenized in order to pull it into the cytoplasm in transformed cells, and these studies have been successful. However, there were undoubtedly unintended consequences of this mutagenesis, because of apoptin's small size, and overlapping functional domains. It is known that this mutagenesis does indeed effect at least one of the other functions of apoptin, which is its multimerization. Therefore, it was proposed that in order to keep all of apoptin's functional domains intact, but still pull it into the

cytoplasm, that the "Tail" of PCV-1 VP3, with its strong NES, be fused to apoptin. A successful fusion would allow for the further study of the localization and apoptotic abilities of apoptin.

Overlap extension PCR (OE PCR) was used to construct the fused protein tagged to GFP in a pEGFP-C1 vector, as shown in Figure 3. The product from the OE PCR was used to transform *E. coli* cells. Positive transformation was screened for using agar plates containing kanamycin, as the pEGFP-C1 vector contains a kanamycin resistance gene. Four transformed colonies were then mini-prepped and tested to confirm the successful fusion of the two genes. First the plasmids from the mini preps were restricted using EcoRI and BamHI, which flanked the fused gene, and then electrophoresed on an agarose gel. It can be seen that colony number 4 contained a product roughly 600 base pairs in length which was indicative of a successful fusion of the two genes, as seen in Figure 5 lane 4. The mini prepped plasmid that tested positive for the 600 base pair product was then sequenced and compared to an in-silico fused gene sequence, as shown in Figure 6. There can be seen complete homology between the translations of the sequence from the mini prepped plasmid and the in-silico fusion of apoptin and PCV-1 VP3 "Tail". This confirmed that PCV-1 VP3 "Tail" was successfully fused with apoptin.

The Apoptin PCV-1 VP3 "Tail" Fused Protein Localizes to the Cytoplasm But Does Not Induce Apoptosis

Once the fusion was confirmed, the next step was to define the localization of the fused protein, and whether or not apoptosis still occurred. To determine the localization and apoptotic ability of this protein, transfections of the fused protein, apoptin, PCV-1 VP3, and GFP were performed on non-small-cell-lung-carcinoma, H1299, cells. Apoptosis assays were run on the cells 24 hours post transfection. The assay used employed a red fluorophore attached to a

quencher by a caspase-3 and caspase-7 recognition sequence. In the absence of caspase activity, the quencher remains attached to the fluorophore and the quencher absorbs any excited photons that are emitted by the fluorophore. When the caspases are activated, by the induction of apoptosis, the quencher is cut from the fluorophore and due to the permeability of the quencher it diffuses out of the cell allowing the fluorophore to emit photons that make the cells fluoresce red. A kit for the detection of caspase-3 and -7 was chosen because it has been characterized that apoptin induces an apoptotic pathway, which activates both of these caspases.

Wild type PCV-1 VP3 was used as a positive control and showed that apoptosis of the transformed cells was induced by the protein from the cytoplasm, as shown in Figure 7 bottom row. Apoptin, another positive control, showed strong induction of apoptosis from inside the nucleus, as seen in Figure 7 third row. GFP was used as a negative control, and showed that transfection alone did not induce apoptosis involving caspase-3 or -7, as seen in Figure 7 second row. The fused protein of interest can be shown with strong cytoplasmic localization, but no apoptosis, Figure 7 top row. To further demonstrate the discrete localization patterns of the fused protein, PCV-1 VP3, and apoptin, ImageJ was used to generate surface plots of selected representative cells from each transfection, as shown in Figure 8. It can be seen that GFP does not have a specific localization in the cells, as expected. It can also be seen that both the fused protein and PCV-1 VP3 localized almost exclusively to the cytoplasm. Although it appears that apoptin does not localize to a specific location in the cell, based on the surface plot, upon careful examination of the cell, it is apparent that it is in late stages of apoptosis, and that the nuclear envelope has been degraded, spilling the contents of the nucleus, along with apoptin into the remainder of the cell, as seen in Figure 7.

Discussion

Cancer is the second leading cause of death in the United States, and there is expected to be an additional 1.6 million cases in 2014 alone[1]. Of these new cases over half will be characterized by a non-functional p53 tumor suppressor protein[2]. Given the fact that most cancer therapeutics rely on a functional p53, it would be ideal to create a cancer therapy that is p53 independent. In this regards, it has been shown that members of the *Circoviridae* family create a protein, which selectively causes apoptosis in cancer cells in a p53 independent manner.

Apoptin, the third viral protein of CAV, and PCV-1 VP3 are two such proteins, which share homology as well as selectivity of apoptosis. However, whereas apoptin has differential localization in transformed cells, in which it is transported into the nucleus, PCV-1 VP3 is consistently cytoplasmic. This is intriguing, as it is thought that the nuclear localization of apoptin in transformed cells is a critical step in the apoptotic pathway. However, PCV-1 VP3 which is homologous to apoptin and has similar effects does not need to be in the nucleus to induce apoptosis. This leads to questions as to whether the nuclear localization of apoptin is indeed a necessary step in the apoptosis pathway. If it were found that it was not a necessary step, then apoptin could be a target for novel therapeutics against cancer, as its relatively small size, cancer selectivity, and p53 independent apoptotic ability would make it a universal cancer treatment.

The apoptin and PCV-1 VP3 "Tail" fused protein was observed to have localized to the cytoplasm. It was believed that this localization was due to the strong nuclear export ability of the "Tail". However, it was intriguing that no apoptosis was observed in the transfected cells. There could have been many possible explanations for this observation. One explanation may

have been that the addition of the "Tail" interfered with multimerization of the protein, which has been shown to be important in the apoptotic mechanism of apoptin[8]. Another possible reason could be that the addition of the "Tail" region, which is around the same size as apoptin alone, could have blocked a region of the protein, as yet undefined, which is critical for interactions that lead to the induction of apoptosis. A third possibility is that nuclear localization is indeed a critical step in the apoptotic pathway of apoptin, and that without the localization there can be no apoptosis.

We have shown here, for the first time, cytoplasmic localization of apoptin without mutagenesis is feasible. The results shown suggest that nuclear localization of apoptin may be necessary for the induction of apoptosis in transformed cells. Studies have previously shown that nuclear localization is dependent on activation of the DNA damage pathway[17]. This further suggests that nuclear localization is in fact critical for apoptosis. The nuclear localization of apoptin could present a problem in the development of novel cancer therapeutics because small molecules are not able to differentially localize within a cell. This would take away the onco-specificity that is characteristic of apoptin. The results presented show promising initial findings towards the larger goal of deducing the mechanism by which apoptin localizes to the nucleus and induces apoptosis.

It is also interesting to note that even though apoptin and PCV-1 VP3 share homology, their localization within a transformed cell differs. PCV-1 VP3 induces apoptosis in the cytoplasm as shown in Figure 7. This indicates that the pathways by which these two proteins induce apoptosis may be different. However, the differences may arise from changes in conformation of the proteins and the pathways may in fact be similar.

It was noticed that in a few of the observed cells there was nuclear contraction, which is indicative of a degrading genome, as seen in the top left panel of Figure 7. This is usually telling of early stages of apoptosis. It cannot be asserted whether the fused protein is the cause of this degradation, and indeed the contracted nucleus may have been caused by the transfection itself or by other apoptotic factors. However, it does lead to interesting possibilities. It may be that different transfection time points are needed to observe apoptosis with the fused protein. The introduction of the "Tail" region may have reduced the kinetics of the apoptin protein in regards to its interaction with other cellular factors that are also involved in the apoptin induced apoptotic pathway. Therefore, the protein may need more time to begin its induction of apoptosis in the transfected cells. Because of time constraints, the transfections were only allowed for a 24-hour time period. Therefore, longer transfections of the cells before apoptosis assays are performed may bring about different results than what was observed.

Another interesting observation was the cell death that was seen on the fused protein slide. After 24 hours, some cells had completely exploded releasing their cellular content into the surrounding areas (data not shown). Therefore, it may also be advantageous to look at earlier time points during the transfection, as the protein may be inducing apoptosis earlier than 24 hours, and the cells that were observed in Figure 7 were cells that were transfected with smaller amounts of the plasmid. Cells do not receive equal amounts of plasmid during transfections;

therefore the cells which ruptured earlier may have taken up more plasmid and therefore been able to express the fused protein for a longer time period. This may also explain the contracted nucleus, as this cell may have only taken up one or a few plasmids, and was just reaching an expression level of the fused protein at which apoptosis would begin to occur.

Figures

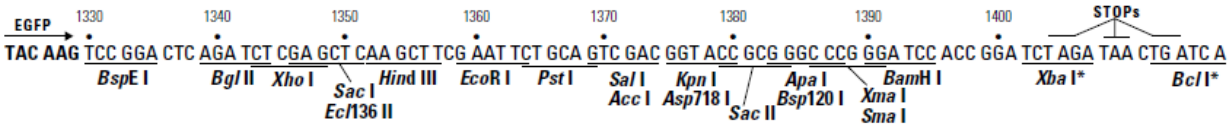

Figure 1 Multiple Cloning Site of pEGFP-C1. Pictured above is the multiple cloning site of the pEGFP-C1 vector into which the PCV-1 "Tail" gene had previously been inserted. The "Tail" gene was inserted using a restriction digest with EcoRI and BamHI restriction enzymes. Therefore the PCV-1 "Tail" insert was located between these two cut sites. The apoptin gene was inserted using a restriction digest with SacI and EcoRI such that it was inserted in frame and spliced to the PCV-1 "Tail" insert.

Forward Primer:

5' ACGTGATCGTGAGCTCGTATGAACGCTCTCCAAGAAGA 3'

Reverse Primer:

5' CCTACGTGACGAATTCGTCAGTCTTATACGCCTTTTTG 3'

Figure 2 Primers Used in Method 1 PCR. The primers are color coded by what each section was utilized for. The black sequences are the sections that are complimentary to apoptin. The green sequences are base pairs added to keep the sequence in frame with GFP and PCV-1 VP3 "Tail". The blue sequence in the forward primer is the restriction sequence for Sac I. The purple sequence in the reverse primer is the restriction sequence for EcoRI. And the red sequences are 10 random base pairs inserted upstream of the cut sites to make the restriction more efficient and to balance out the G:C content of the primers to influence the melting points.

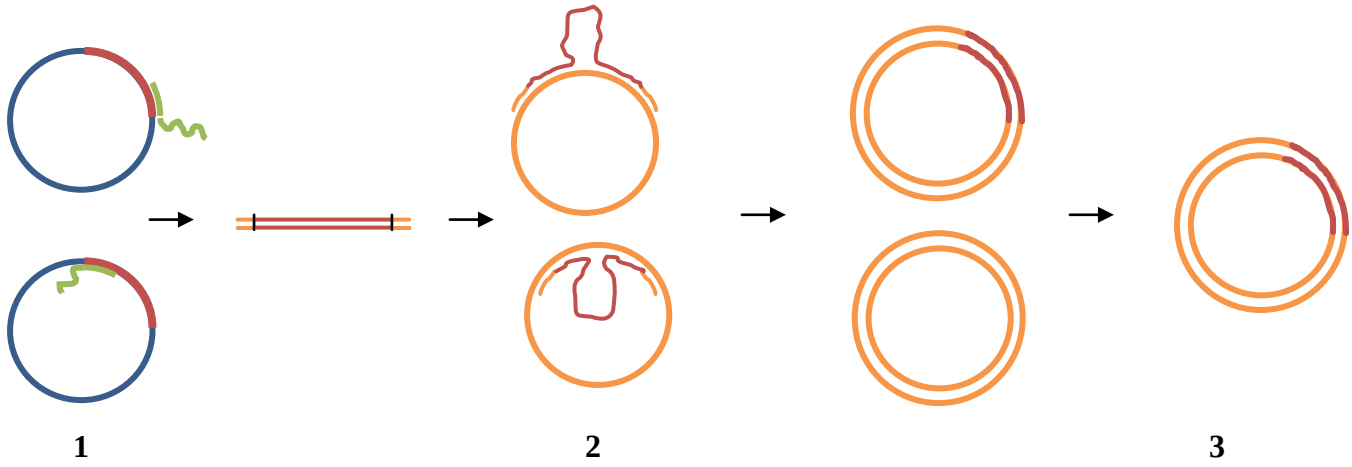

Figure 3 Mechanism of Overlap Extension Polymerase Chain Reaction. Pictured above is the mechanism by which apoptin and PCV-1 VP3 "Tail" were fused. First, a PCR with apoptin (insert) was done using primers with floppy ends that contained complementary sequences to our target vector. Second, the product obtained from the first PCR is used in a second PCR to fuse PCV-1 VP3 "Tail" (insert) into apoptin (target vector). Third, the products that were generated in step 2 (fused vector and methylated supercoiled original vector) were treated with DPN1, which destroys the original methylated vector and leaves only the fused protein of interest.

Forward Primer

5’ AGC CGA CCC CGA ACC GCA AGA AGG CGT ATA AGA CTG GTG GCC TTC TTT ACT GCA GTA TTC 3’

Reverse Primer

5’ GCT GAT TAT GAT CAGTTATCT AGA TCC GGT GGATCC TCA GTG AAA ATG CCA AGC AAG AAA 3’

Figure 4 Primers Used in Method 2 OE PCR. The primers are color coded by what each section was utilized for. The blue sequence in the forward primer is 36 base pairs (bp) complimentary to the end of the apoptin gene. The orange sequence in the forward primer is 24 bp complimentary to the beginning sequence of the PCV-1 VP3 “Tail”. The blue sequence in the reverse primer is 36 bp complimentary to the pEGFP-C1 vector at the end of the apoptin gene. And the orange sequence in the reverse primer is 24 bp reverse complimentary to the end of the PCV-1 “Tail” gene.

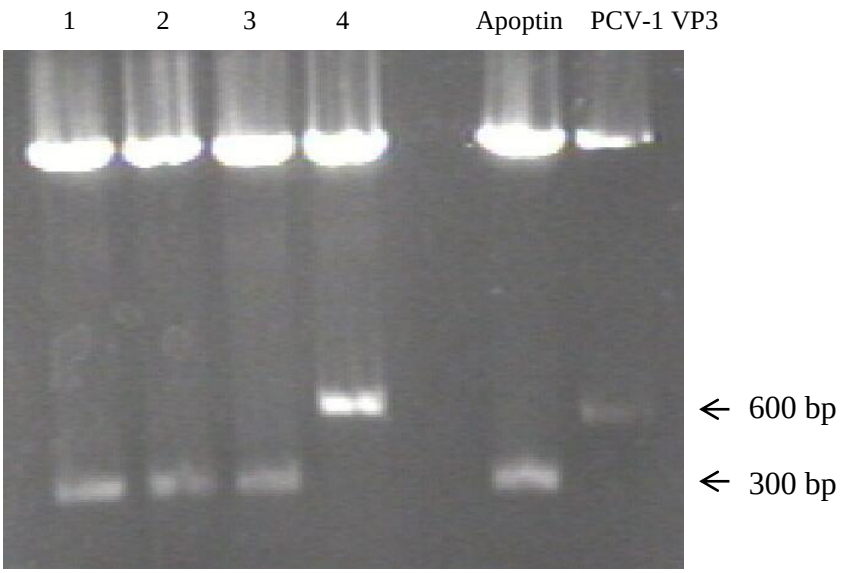

Figure 5 Gel Electrophoresis Results from Restricted EcoRI and BamHI Restriction Digest of Mid-Prepped Transformed Colonies. To verify that the transformed colonies carried plasmids with the fused gene, midi-preps from 4 different colonies on the same plate were restricted with EcoRI and BamHI, as these sites flanked the fused gene. As a comparison, an apoptin vector and a PCV-1 VP3 vector were also digested using EcoRI and BamHI. Colony 4 was the only colony that showed positive results for the fused gene, having a digest band at roughly 600 bp, similar to that of the PCV-1 digest band.

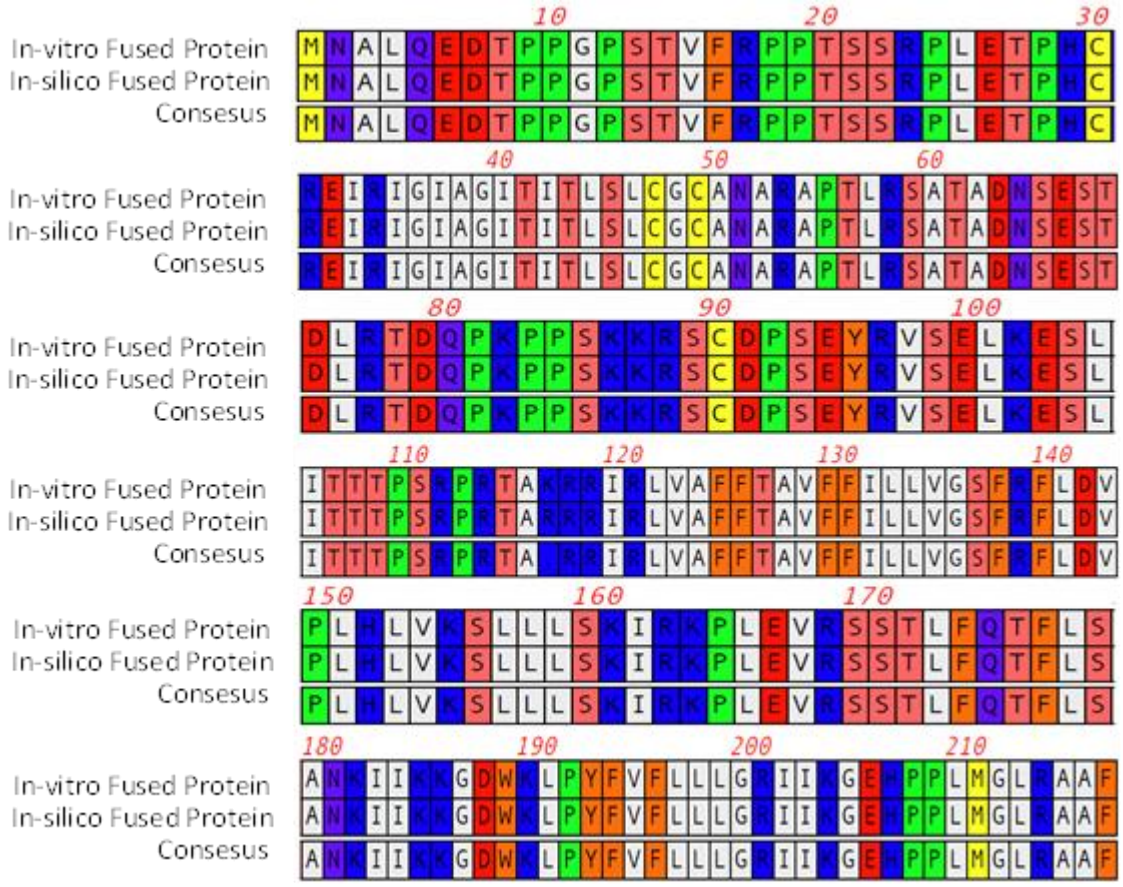

Figure 6 Translation of Sequencing Results Compared to In-Silico Fusion of Apoptin and PCV-1 "Tail" Genes. The sequenced gene from colony 4 was translated using MacVector (In-vitro fused protein). This was compared to a translation of apoptin and PCV-1 "Tail" fused in-silico (In-silico fused protein). The consensus sequence shows that the translation of the sequenced gene is a 100% match to the translation of the in-silico fused gene.

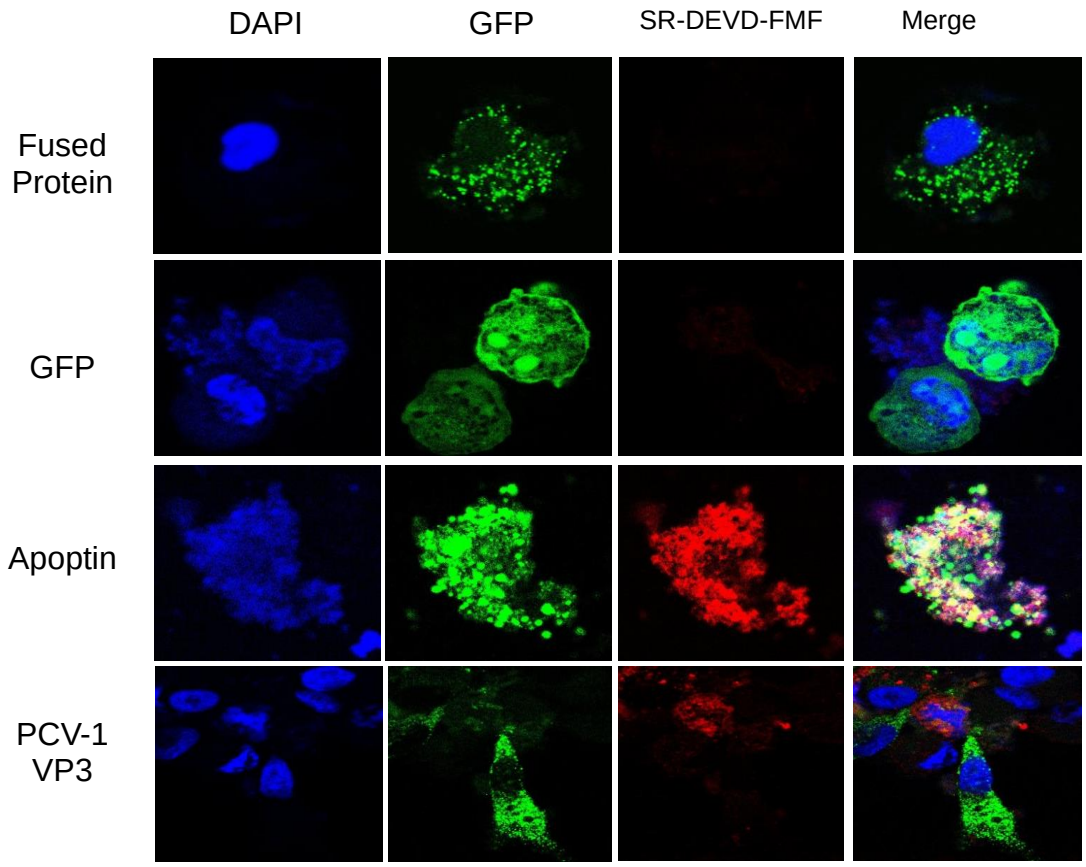

Figure 7 Localization and Apoptosis Assays in H1299 Cells. The gene that was transfected into the H1299 human non-small cell lung carcinoma cells is labeled to the left of the pictures and the stain is labeled above. DAPI stains for the nucleus, GFP stains for the transfected protein, and SR-DEVD-FMF stains for caspase 3 & 7 activity indicative of apoptosis.

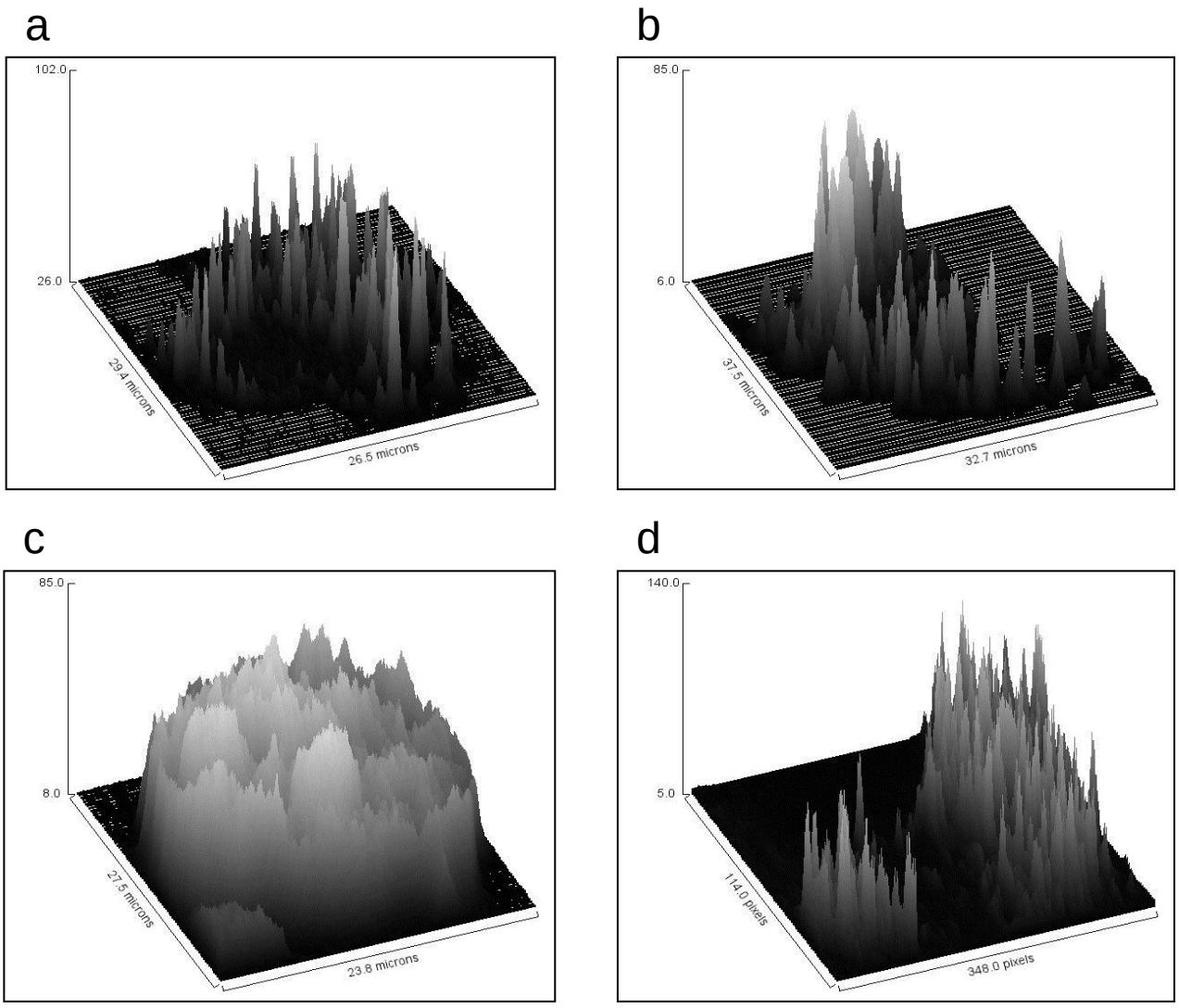

Figure 8 Surface Plots of GFP Expression. GFP expression profiles in transfected cells were generated using surface plots in ImageJ. Peaks indicate high GFP expression. Panel a) is an H1299 cell transfected with the fused protein. Panel b) is an H1299 cell transfected with apoptin. Panel c) is an H1299 cell transfected with GFP. Panel d) is an H1299 cell transfected with PCV-1 VP3.

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