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Uncoupling the Multimerization and Nuclear Export Activities of CAV VP3 via Site-Directed Mutagenesis

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Uncoupling the Multimerization and Nuclear Export Activities of CAV VP3 via Site-Directed Mutagenesis

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1. Apoptin
2. cancer
3. nuclear export

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Abstract

The VP3 (Apoptin) protein from the Chicken Anemia Virus has been shown to selectively kill transformed cells; an activity that is dependent on subcellular localization. Studies note that Apoptin forms large multimers during this process and the role of this aggregation in cell type-specific apoptosis is unclear. Previous studies indicate that the nuclear export and multimerization activities occur via the same domain, however all current mutations affecting export also interfere with multimerization. Here we show that introduction of a point mutant (I37A) in the nuclear export sequence of Apoptin uncouples multimerization and nuclear export activities. Separation of these activities should elucidate the role of multimerization in cell type-specific apoptosis.

Introduction

Viral Replication

Many viruses manipulate the host cell cycle to replicate in the cell and propogate infection. The cell cycle is controlled by a series of proteins that regulate each stage of cell division. The major regulators are the cyclins, a family of proteins named for their oscillatory concentrations during cell cycle progression. Cyclins complex with and activate the protein kinase function of cyclin-dependent kinases (Cdk). These complexes are regulated via Cdk-activating kinases and Cdk inhibitors. Combinations of different cyclin and Cdk proteins lead to the activation of different stages in the cell cycle.[20] Manipulation of the cell cycle is possible through a variety of viral regulatory proteins that interact and interface with cyclins and/or Cdks (directly or indirectly). For example, small DNA viruses have been found to drive the cell into S phase by inactivating pRb. The retinoblastoma family of proteins regulates the cell cycle at S phase by binding and inhibiting transcription factors in the E2F family, namely E2F-1, which activate genes involved in S-phase such as *cdt1+* (an initiation factor) and *hsk1+* (a protein kinase). This allows the virus to make use of the host's DNA replication processes for the proliferation of the viral genome. Another example is the Epstein-Barr virus (EBV), which causes multiple forms of lymphoma (cancer in lymphocytes) and carcinoma (cancer in epithelial cells), through expression of a protein, EBNA-3C, that also binds and inhibits Rb. [21] [4]

Host cell cycle manipulation by viral proteins can also occur through repression of p53. p53 is responsible for two major cellular functions, initiation of apoptosis and/or cessation of the cell cycle via G_1 arrest. In this process p53 (which serves as a transcription factor) upregulates transcription of the p21 protein, which inhibits the kinase complexes required for G_1/S transition,

terminating the cell cycle. Over half of all human cancers have a mutated p53 gene and many repress p53 activity. Inactivation of p53 allows the virus to continue proliferation because cell function cannot be halted through p53-mediated arrest or apoptosis. [4] Thus, many viruses attempt to either inhibit p53 production or p53 activity to allow for unregulated growth and increased S-phase induction. Several instances of such viral interference with p53 have been studied. For example, the Epstein-Barr virus (EBV) activates the A20 gene which inhibits the mediation of apoptosis by p53.[2] Several instances of viral interaction with p53 have been studied, such as that of Simian virus 40 (SV40). At the onset of infection, SV40 transforms the host cell by expressing the large and small T antigens, which bind to p53 and inactivate it. The cell then enters a cycle of unmonitored proliferation for SV40.[1] Some viruses encode proteins that interfere with the activity of p53 and Rb, while others encode proteins that stimulate apoptosis.[1][8]

Virus-induced apoptosis

Animal viruses can also encode proteins that stimulate apoptosis, or programmed cell death. Apoptosis is typified through cell shrinkage, partitioning of the nucleus, cleavage and degradation of DNA, and phagocytosis by surrounding cells.[12] Apoptotic activity that results from these proteins may allow the virus to spread throughout the host, however this phenomenon remains unclear.

Two major pathways for the activation of caspases, a family of cysteine proteases which are used in cell dismantling, have been discerned. The intrinsic pathway occurs in mitochondria in a process by which cytochrome c within the mitochondrial membrane is released into the

cytosol. Cytochrome c then binds Apf1, a caspase-activating protein, which subsequently forms a caspase-activating complex (which includes cytochrome c, Apf1, and initiator caspases) that triggers the initiation of the caspase activation cascade.[2][23] Intrinsic apoptosis is hindered by Bcl-2 and some related family members, which are located on the outer mitochondrial membrane and downstream from caspase 3. Bcl-2 is the mammalian correspondent of regulatory genes initially discovered in *C. elegans* and is part of a complex family of proteins that have both pro- and anti-apoptotic functionality. This protein was found to avert apoptosis by preventing the release of cytochrome c within the mitochondria. These experiments also showed that cytochrome c release precedes other early apoptotic events such as mitochondrial membrane depolarization.[27]

Extrinsic apoptosis is dependent on the tumor necrosis factor (TNF) superfamily of receptors. Upon ligand binding, the receptor's intracellular death domain clusters and proceeds to form the death-inducing signaling complex (DISC). DISC formation is followed closely by initiator caspase activation and ultimately apoptosis. It has been argued that intrinsic and extrinsic pathways are capable of interaction. Caspase-8, an initiator activated in the extrinsic pathway, has been shown to cleave a pro-apoptotic member of the Bcl-2 family, Bid. Entry of cleaved Bid into mitochondria results in cytochrome-c release into the cytosol, triggering activation of the intrinsic pathway.[19][26]

Caspases are activated through these pathways and serve major roles in apoptosis. Enzymatic degradation is an irreversible process that results in cell death, thus few approaches exist for controlling caspase activity. All identified caspases have a similar structure: an N-terminal prodomain (which is cleaved for activation), a large 20 kDa subunit and a small 10 kDa

subunit. The caspases also exhibit substrate specificity.[2][23] Caspase-mediated apoptosis includes inactivation of proteins, through degradation, that safeguard the cell from apoptotic changes. Caspases activate through a cascade that is initiated by acute specificity for aspartic acid. Each caspase has an aspartic acid cleavage site that allows for activation of caspases further along the cascade. These are broadly divided into two groups: initiation and effector caspases. Effector caspases then inactivate the aforementioned regulatory proteins.[2][23]

Some viruses are apoptotic and more specifically, others are cancer-killing viruses. A select number of viruses have been found to induce apoptosis, a subset of which induces apoptosis in a transformation-specific manner such as adenovirus type 5. Additionally, some viruses encode a single protein capable of selectively inducing apoptosis in cancer cells. An example is viral protein 3 of the chicken anemia virus.

The Chicken Anemia Virus (CAV)

CAV, a virus of the family *Circoviridae* and the genus *Gyroviridae*, was discovered during an investigation of a Marek's disease outbreak. Marek's disease is a highly contagious virus in chickens. The isolates from this study were originally designated a chicken anemia agent (CAA) found in commercially produced chickens [28] however further studies indicated that CAA biochemically resembled the porcine circovirus, and CAA was therein denoted CAV. The virus itself is small in size (the genome is approximately 2,300 nucleotides) and is composed of circular single-stranded DNA.[25]

CAV infects chickens shortly after hatching. Within 14 days of infection the virus induces anemia (deficiency in red blood cells) and leads to secondary infections.[7] Physical

symptoms observed 16 days after inoculation with CAV include degradation of bone marrow (yellow color), bone marrow lesions, shrunken thymus, swollen and discolored liver, hemorrhaged skeletal muscle, and hemorrhaged mucosal membrane. Depression and anorexia are also associated symptoms. Chickens exhibiting the worst symptoms die between two and four weeks of age.[28]

The CAV genome encodes three viral proteins (VP1, VP2, and VP3) in three overlapping open reading frames, all of which are required for viral replication. Few discoveries have been made about the functions of VP1 of ORF3 (52kDa; the only CAV capsid protein) and VP2 of ORF1 (24 kDa; significant in CAV replication and influences virulence) to date.[25] Koch *et. al.* [8] have proven that VP2 is necessary for CAV assembly and Peters *et. al*. [17] have shown that VP2 is a protein-tyrosine phosphatase (PTPase) after discovering a remarkable homology between the sequence of VP2 and other dual-specificity phosphatases.[16] Dual specificity phosphatases are characterized by their ability to remove phosphate groups from phosphotyrosine and phosphothreonine residues via cysteine and arginine residues in the catalytic motif (which are highly conserved among PTPases), both of which were observed in kinetic studies of VP2. VP2 is expressed at nearly undetectable levels at the onset of infection, an attribute of regulatory proteins necessary for viral replication that serve a non-structural role.[17]

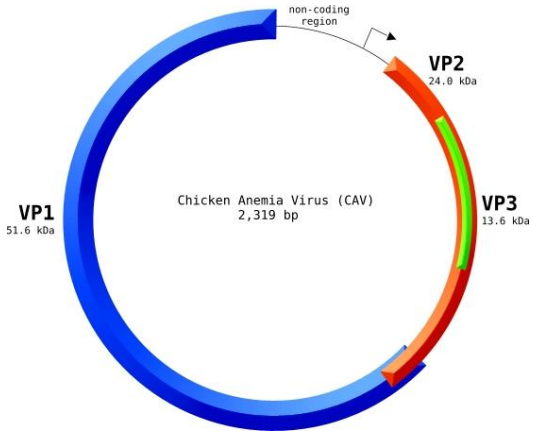


Fig. 1 CAV genome. A schematic representation of the CAV genome. The CAV genome transcribes a single mRNA with multiple translational start sites for the three CAV proteins: VP1, VP2 and VP3.

CAV VP3: Apoptin

VP3, herein referred to as Apoptin, consists of 121 amino acids with two proline-packed stretches and two basic regions.[29] Much like VP2, Apoptin is a non-structural protein that shortly after infection is distributed in a dispersed granular pattern within the cell however Apoptin later accumulates in proximity to isolated apoptotic structures. Bcl-2, which was found to generally inhibit intrinsic apoptosis via pathways such as p53, can inhibit Apoptin-mediated apoptosis, indicating that Apoptin-mediated apoptosis follows a mitochondrial intrinsic pathway.[11] In order to effectively induce apoptosis, Apoptin must have proper subcellular localization to the nucleus. Once nuclear localization occurs, Apoptin can bind and inhibit the target of apoptotic interaction.

This target is the Anaphase Promoting Complex (APC/C). The APC/C is a vital protein for cell cycle regulation. The APC/C is a complex protein with thirteen core subunits and two

associated subunits, Cdc20 and Cdh1.[24] The two associated subunits are responsible for the two assemblies of APC/C: APC/C^{Cdc20} and APC/C^{Cdh1}.[15] APC/C^{Cdc20} is important for mitotic regulation. APC/C is the enzyme responsible for the regulation of the cyclins, proteins that are responsible to initiating the various stages of mitosis, through ubiquitin-dependent proteolysis.[15] In addition to APC/C's role in cyclin proteolysis, the complex is responsible for activating separase. This cleavage is the initiation of anaphase.[16] Interference with this essential regulatory protein can severely affect the cell cycle.

Apoptin has been found to inhibit the anaphase promoting complex/cyclosome, leading to G2/M arrest and eventually apoptosis in transformed cells.[6] Apoptin binds to APC1. APC1 is the largest subunit in the complex and performs a scaffolding function. It has been proposed that the APC/C is destabilized when Apoptin binds to this subunit.[5] It was shown that Apoptin binding produces complexes that were significantly smaller in molecular weight than a full APC/C complex and some were the size of a single subunit, indicating destabilization of the APC/C. [22]

This destabilization of a crucial cell cycle regulatory complex leads to mitochondrial apoptosis through the Nur77 pathway, a p53-independent pathway.[10][5] The p53 pathway serves as a primary avenue for apoptosis of cancer cells by chemotherapy. However, this pathway is mutated in nearly half of naturally occurring tumors. Therefore, a p53-independent pathway for induced cell death is an important finding for future cancer research.

Studies have shown that in transformed cells, Apoptin is located primarily in the nucleus, while for normal cells Apoptin is located primarily in the cytoplasm.[13][6] In primary cells, Apoptin formed a pattern similar to cytoskeletal structures, suggesting that Apoptin may be

aggregating and complexing with the cytoskeleton or forming a filament itself. [22] In the nucleus of transformed cells, Apoptin binds to APC/C, however Apoptin does not bind to the APC/C in primary cells. It has been concluded that the presence of Apoptin in the nucleus of transformed cells is necessary for the selective apoptotic effect. [21]

Nuclear Import and Export of Apoptin

Major transport into and out of the nucleus is mediated by proteins called karyopherins and membrane proteins called nucleoporins. Karyopherins tag proteins in the cytoplasm and nucleoplasm for import and export. An adapter protein is often used to bind the protein to be transported, which in turn is bound to the karyopherins. Either the karyopherin or adapter protein binds to key amino acid residues on the protein. Subsequent protein conformation of the karyopherin allows it to be recognized by the nucleoporin and bound. Once bound, the entire complex is transported across the membrane through the nucleoporin. For import, the importin-α family of karyopherins is often used as an adapter protein while the importin-β family of karyopherins can bind importin-α or act independently. Nuclear export is usually handled by the binding of the karyopherin Crm1.[14]

The nuclear localization sequences (NLS) of proteins have been difficult to define. There has yet to be a conserved sequence to identify the NLS.[13] In contrast, nuclear export sequences (NES) have a loosely conserved sequence of hydrophobic residues, mostly leucine and isoleucine.[3] The karyopherin Crm1 is responsible for interaction with the NES.[8][38] This karyopherin makes use of the Ran-GTP cycle. Ran complexes with the protein to be

exported by recognizing and binding the nuclear export sequence found on the protein.[14] Crm1 recognizes Ran-GTP and exports the cargo across the nuclear membrane. Once exported, Crm1 dephosphorylates Ran-GTP to Ran-GDP and the substrate to be transported is dissociated.

Apoptin undergoes nucleocytoplasmic shuttling through the functions of its nuclear export and nuclear import sequences.[6] It has been shown to be exported from the nucleus by Crm1 and imported by importin-β1.[18][6] This indicates that Apoptin uses existing cellular mechanisms to move between the nucleus and cytoplasm. To prove that Apoptin is transported out of the nucleus, nuclear export was blocked by leptomycin B. This resulted in the nuclear accumulation of Apoptin in primary cells.[6] To prove that Apoptin is transported into the nucleus, Ran dependent nuclear import was blocked with a dominant-negative Ran GTPase mutant. This resulted in the ctyoplasmic accumulation of Apoptin.[6] In addition, both import/export sequences are active at the same time, resulting in an equilibrium in protein shuttling. This shuttling has been shown to be critical to the cell-type specific apoptosis induced by Apoptin, however the exact role is unknown. Removal of either the nuclear export sequence or the nuclear import sequence results in a loss of cell type-specific localization.[6]

Studies have shown that introducing a generic nuclear localization sequence does not affect Apoptin's ability to selectively induce apoptosis. Apoptin's NLS can be replaced with a heterologous sequence, SV40 large T-antigen with no detected change in the cell-type specific localization of Apoptin.[6] This indicates that it may be the nuclear export sequence that is the key to the cell type selectivity. The NES of Apoptin, a leucine-rich region between amino acids 33 and 46 (IRIGIAGITITLSL), was replaced with an established NES, such as that of the Rev protein, results in a similar localization pattern for both transformed and primary cell types.

Interestingly, in all cases where export is lost, the same cytoplasmic filamentous pattern was not produced.[6]

Apoptin: Multimerization

Studies indicate that an unspecified domain is responsible for Apoptin multimerizing with itself.[6][10] Data indicates that Apoptin multimerizes with itself in both transformed and primary cells. However, the extent of multimerization differs between cell types. In normal cells Apoptin forms mostly large, insoluble complexes in the cytosol. In transformed cells Apoptin is mostly soluble (unpublished data, D.W. Heilman). When it does multimerize in transformed cells, it interacts with Promyelocytic Leukemia (PML) bodies in the nucleus, which contain APC/C.[9] Multimerization may be critical for this localization and for the induction of apoptosis. [29]

Studies in which N-terminal residues were mutated in Apoptin resulted in elimination of aggregate formation [9], the ability to induce apoptosis, and loss of cell type selectivity [6]. The marked reduction in multimerization from the replacement of the NES indicates that Apoptin's NES contains important amino acid residues required for effective multimerization.[6] In an effort to determine the role of multimerization and nuclear export activities in cell-type specific apoptosis, these two activities must first be separated. All previous attempts at uncoupling nuclear export and multimerization have been unsuccessful. Therefore, it is the aim of this study to uncouple the nuclear export and multimerization activities of Apoptin through executing a novel point mutation to a conserved isoleucine residue, I37, in the nuclear export sequence.

Materials and Methods

Creation of mutants. PCR based site-directed mutagenesis was used to create an I37A point mutation within the NES in Apoptin. To ligate the gene into the correct reading frame and ensure propoer orientation in the expression vector, an EcoR I site was introduced onto the N-terminal primer and a BamHI site was introduced onto the C-terminal primer. (ApI37A forward primer: 5' TGCAGAGAGATCCGGATTGGTGCCGCTGGAATTACAATCACTCTA 3'; ApI37A reverse primer: 5' TAGAGTGATTGTAATTCCAGCGGCACCAATCCGGATCTCTCT GCA 3'. Ap forward primer: 5'GCGAATTCATGAACGCTCTCCAAGAAGATACTC 3'; Ap reverse primer: 5; GCGGATCCTTACAGTCTTATACGCCTTTTTGCGG 3') The following PCR protocol was used: 95 °C, 4 min, 1x | [95 °C, 30 s] [55 °C, 30 s] [72 °C, 1.5 min], 30x | 72 °C, 5 min, 1x | 10 °C, ∞. PCR products were agarose gel electrophoresed using a TAE (0.04M Tris-HCl, 1 mM EDTA, 57.2µL glacial acetic acid) and 9% agarose gel. The products were purified from the agarose gel using a Promega Wizard PCR Preps DNA Purification System (cat. #A7170).

Mutant cloning. PCR products were subcloned into Promega pGEM-T Vector (cat. #A3600). DH5α *E. coli* cells were chemically transformed with the ligated T vector. The cell cultures were screened via x-gal and IPTG selection. Colonies were grown on ampicillin plates and select colonies were alkaline lysed (0.2N NaOH, 1% SDS). Presence of the gene insert in the T-vector was verified by colony PCR. The gene insert was isolated by restriction digest with EcoR I and BamH I and purified by Promega Wizard PCR Preps DNA Purification System (cat. #A7170). The gene insert was ligated into the Clontech pGBKT7 vector (cat #K1612-1) with a Promega

T4 DNA ligase (cat #M180A). The Clontech pACT2 vector (cat #K1604-A) with wild type Apoptin insert was provided by D.W. Heilman. The positive colonies were inoculated in either ampicillin or kanamycin. Select positive colonies were alkaline lysed (resuspension solution: 50 mM glucose, 25 mM Tris·HCl, 10 mM EDTA at pH 8.0; lysis solution: 0.2 N NaOH, 1% SDS; neutralization solution: 3 M K^+, 5 M OAc^-) and purified for plasmid DNA by a Promega Wizard® Plus Midipreps DNA Purification System (cat #A7640). Dideoxy DNA sequencing of the purified product was performed by MacrogenUSA with T7 primers for both strands of the T-vector to confirm the I37A mutation.

Yeast transformation and selection. Y187 yeast were cultured in Clontech YPD Medium (cat. #630410). The Y187 cells were cultured to a cell density with absorbance of 0.6. 50 mL of this culture was centrifuged and washed, after which the cells were resuspended in 100 mM LiAc. The Y187 were transformed with 240 µL PEG-4000, 36 µL 1.0M LiAc, 25 µL single-stranded carrier DNA (2.0 mg/mL), 50 µL of H2O and 2 µL of plasmid DNA: pGBKT7/pACT2, pGBKT7-p53/pACT2-T (provided by D.W. Heilman), pGBKT7-ApWT/pACT2-ApWT, or pGBKT7-ApI37A/pACT2-ApWT. The yeast were transformed for 30 minutes at 30˚C and then heat shocked at 42˚C for 20 minutes. The transformed Y187 were plated onto selective media (Clontech Minimal SD Agar Base (cat. # 630412) and Clontech -Leu/-Trp DO Supplement (cat. # 630417)).

Beta-galactosidase assay. After three days of growth at 30 °C, colonies were lifted onto 3 mm Whatman filter paper and flash frozen in liquid nitrogen for 10 seconds. The filter paper was

pressed to another Whatman filter paper that had been soaked in a 3.28 mg/mL *x*-gal, 0.0379M β-mercaptoethanol, 15.79 g/L $Na_2HPO_4 \cdot 7H2O$, 5.40 g/L $NaH_2PO_4 \cdot H2O$, 0.75 g/L KCl, and 0.264 g/L $MgSO_4 \cdot 7H2O$ solution and incubated for 1.5 hours.

Results

ApI37A site-directed mutagenesis. In an effort to uncouple nuclear export from multimerization, a canonical isoleucine within the NES was mutated to an alanine (Figure 2A). I37 is among the conserved residues necessary to bind to Crm1, a major exportin. Alanine is a small, inert amino acid which is expected to have no interaction with Crm1, which would likely eliminate nuclear export. Previous studies have demonstrated that the N-terminal leucine residues in the NES were likely necessary for multimerization, as mutation of these resulted in elimination of both nuclear export and multimerization. A bipartite nuclear localization sequence is located on the N-terminal side of Apoptin, however this sequence will not be examined in this study.

To execute the I37A mutation, PCR-based site-directed mutagenesis using the "megaprimer" technique was performed to construct half mutants. Due to the location of NES, the mutant primers would create PCR products of different lengths. As the PCR would only elongate to the primer, truncated PCR products would be formed. Screening the PCR products via agarose gel electrophoresis of Tax positive control demonstrated effective PCR and the negative control, which contained no DNA, ultimately showed no PCR product, indicating that there was likely no contamination of wild type Apoptin. The first round of site-directed mutagenesis confirmed the creation of 0.1 kb and 0.2 kb half mutants (Figure 2B, lanes 3 and 4). Full-length ApI37A was assembled during the second round of site-directed mutagenesis. Due to the fact that truncated PCR products have overlapping regions, they act as full-length templates for one another. This would produce a PCR product of a full-sized Apoptin gene with the point mutation. Gel electrophoresis confirmed the size of the full-length ApI37A (Figure 2C, lane 3).

This full-length product was purified in preparation for ligation into the expression vector.

Cloning of ApI37A. To determine protein-protein interaction the ApI37A mutant was inserted into pGBKT7, a component of a yeast 2-hybrid system. Subcloning into T-vector was performed to increase the efficiency of restriction digest by EcoR I and BamH I, as using restriction enzymes on a PCR product are highly inefficient due to the terminal location of restriction sites. Ligation into the T vector was possible because the Taq polymerase used in PCR non-specifically adds adenosine bases to the end of a DNA sequence. These overhanging adenosine bases base pair with the overhanging thymidine (T) residues in the vector, allowing for ligation. To confirm that ApI37A was present in the T-vector a restriction digest of the ligated T-vector was performed and confirmed the presence of full-length Apoptin (Figure 2D). To insure that the site-directed mutagenesis was successful in introducing the necessary point mutation, DNA sequencing was performed. Sequencing in both directions on the ligated T-vector was necessary to ensure accuracy of the DNA sequence. DNA sequencing confirmed the presence of the point mutation of I37A and retained homology with the rest of the gene (Figure 2E).

A yeast 2-hybrid system was implemented to assess the degree of multimerization between ApWT and ApI37A. The ApI37A that was excised from the T-vector by restriction enzymes EcoR I and BamH I was ligated into the pGBKT7 expression vector. This vector also contains the TRP1 gene and the gene for the DNA-binding domain, half of a necessary transcription factor for the Gal4 promoter. The primers used for mutagenesis were designed so that ApI37A was inserted in the correct reading frame for fusion to GAL DNA-binding domain. After cloning ApI37A into pGBKT7, a restriction digest of the ligated pGBK vector was

performed to ensure ApI37A was present in the positive clone. The digest produced an excised fragment of approximately 0.4 kb, confirming the presence of ApI37A (Figure 2F).

Transformation and expression of pGBKT7-ApI37A in Y187 yeast. The second vector used in the yeast 2-hybrid system was a previously-constructed pACT2 into which ApWT was ligated. pACT2 contains the LEU2 gene as well as the gene for the transactivating domain, the second half of the Gal4 transcription factor, fused to Apoptin. The transactivating domain binds to RNA polymerase II for transcription initiation. 50 mL of 0.6 A of yeast were LiOAc transformed with pGBKT7 and pACT2 vectors. To confirm that the Y187 yeast, which are deficient for Leu and Trp metabolism, were transformed with both plasmids, the yeast were plated onto -Leu/-Trp media for selection. Yeast that were transformed in the 2-hybrid system would express the LEU2 and TRP1 genes, introduced by pGBKT7 and pACT2, and grow in -Leu/-Trp media (Fig. 3, panels 1-5). Untransformed controls did not grow on the -Leu/-Trp media, as expected (Fig. 3, -C panel). Yeast that had been transformed with both the pGBKT7 and pACT2 vectors in this assay were selected for the colony lift β-galactosidase assay because both halves of the Gal4 transcription factor are required to assess the retention of multimerization.

β-galactosidase assay. When protein-protein interaction occurs, the DNA-binding domain from the pGBKT7 vector and the transactivating domain from the pACT2 are brought in proximity. The DNA-binding domain binds the Gal4 promoter and the transactivating domain recruits RNA polymerase II to the lacZ gene, which results in transcription of lacZ. Transcription of the gene results in β -galactosidase production, which cleaves x-gal into galactose and 5-bromo-4-chloro-

3-hydroxyindole (which stains the colonies blue). To assess the degree of multimerization, a colony lift β-galactosidase assay was performed (Figure 4A). Colonies were lifted to 3 mm Whatman paper, fractured in liquid nitrogen, and assayed for β-galactosidase activity. The two positive controls, pGBKT7-p53/pACT2-T and pGBKT7-ApWT/pACT2-ApWT showed β-galactosidase activity (Figure 4B, upper right and lower left), indicating protein-protein interaction. These controls were selected because p53 and the SV40 Large-T antigen are known to interact with one another. Similarly, wild-type Apoptin is well known to multimerize.In addition, pGBKT7-ApI37A/pACT2-ApWT also showed β-galactosidase activity (Figure 4B, lower right). pGBKT7/pACT2 vectors demonstrated no β-galactosidase activity, as the two halves of the Gal4 promoter were not brought in proximity since Apoptin was not present in the vector and protein-protein interaction could not take place. (Figure 4B, upper left)

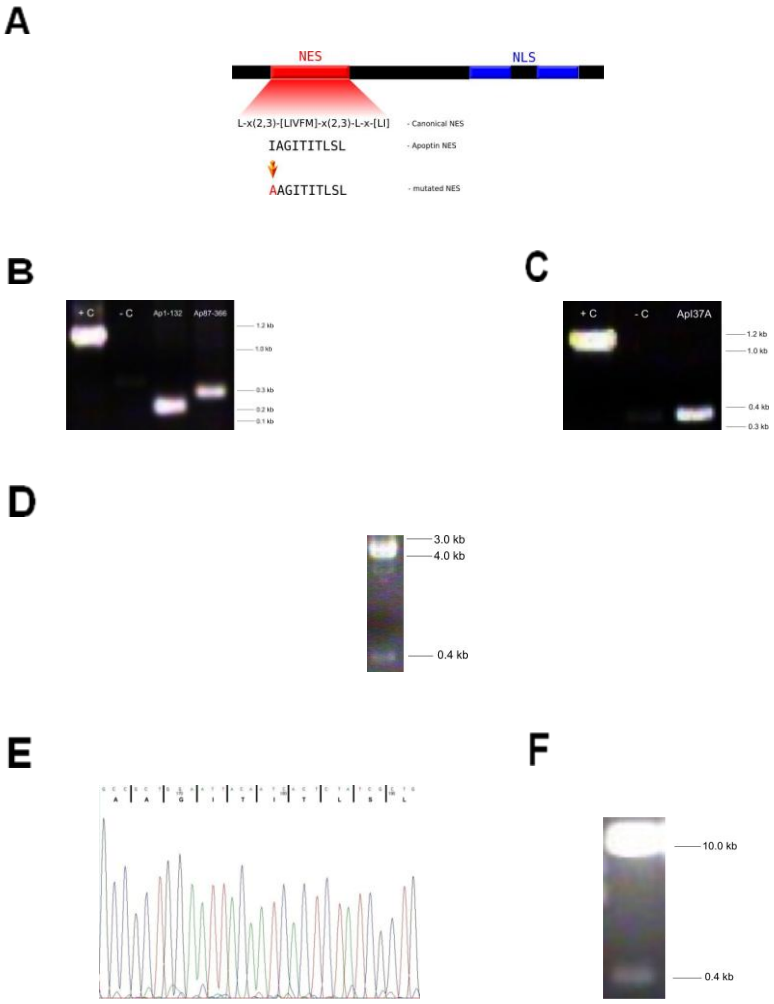


Fig. 2 ApI37A site-directed mutagenesis and cloning into pGBKT7 vector. ApI37A mutation was generated using the "megaprimer" method of PCR-based site-directed mutagenesis. Apoptin mutants were cloned into the pGBKT7 2-hybrid vector (a) A graphic representation of the Apoptin localization sequences indicating the canonical nuclear export sequence (NES) of Apoptin with the ApI37A mutant. (b) Electrophoretic results of the first step of site-directed mutagenesis. Shown are Tax positive control (lane 1), negative control (lane 2), and ApI37A half mutants at 0.1 kb (lane 3) and 0.2 kb (lane 4). (c) Electrophoretic results of the second step of site-directed mutagenesis. Shown are Tax positive control (lane 1), negative control (lane 2), and ApI37A at 0.4 kb (lane 3). (d) Electrophoretic results of restriction digest of T-vector-ApI37A. One positive clone produced a band of approximately 0.4 kb, indicating the existence of ApI37A. (e) A portion of the chromatographic sequence analysis of the T-vector-ApI37A showing the successful I37A point mutation. (f) Electrophoretic results of the restriction digest of pGBKT7-ApI37A. Both positive clones produced a band of approximately 0.4 kb, indicating the existence of ApI37A.

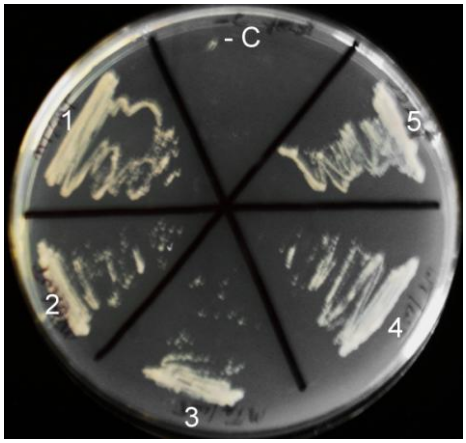
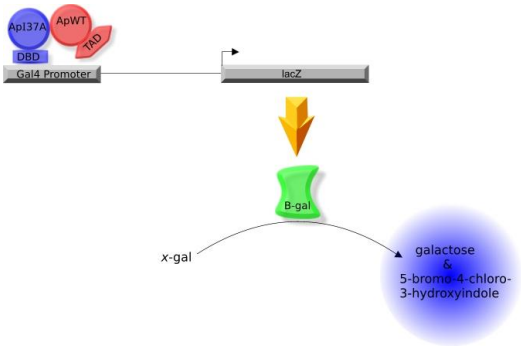


Fig. 3 Transformation into Y187 S. cerevisiae and selection. pGBKT7-ApI37A mutants were cotransformed with pACT2-ApWT and positive clones were selected on -Leu/-Trp. ApWT/ApWT and p53/T pairs were also contransformed for use as controls. [1] pGBKT7/pACT2, [2] pGBKT7-ApI37Aa/pACT2-ApWT, [3] pGBKT7-ApI37Ab/pACT2-ApWT, [4] pGBKT7-ApWT/pACT2-ApWT and [5] pGBKT7-p53/pACT2-T.

A



B

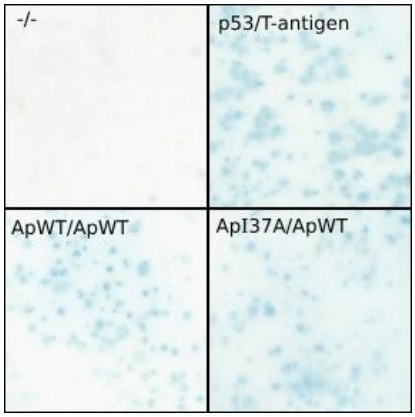


Fig. 4 2-hybrid β-galactosidase assay for protein-protein interaction. Positive yeast clones were cracked and stained with x-gal for 1 hour at 30°C. (a) A graphic representation of the β-galactosidase assay. Multimerization of ApI37A with ApWT will bring two halves of the required Gal4 transcription factor in proximity. This induces activation of lacZ, the product of which is β-galactosidase which will cleave x-gal into galactose and 5-bromo-4-chloro-3-hydroxyindole. (b) Negative control (upper left), positive controls (upper right & lower left), and ApI37A/ApWT (lower right) are shown.

Discussion

Previous studies have shown that Apoptin multimerization and nuclear export are contained in overlapping domains. This study has demonstrated, however, that uncoupling of these activities may be possible. The nuclear export sequence of Apoptin follows a canonical nuclear export sequence. Within the conserved NES, it is established that hydrophobic residues, leucine and isoleucine in particular, are essential for nuclear export. These residues are responsible for recognition by Crm1, which in turn is responsible for nuclear export. This study mutated a hydrophobic residue based on this canonical sequence. Isoleucine 37 (I37) was determined to be one of these important amino acid residues for nuclear export as it was a conserved hydrophobic residue within the NES. It was assumed that mutation of this residue would eliminate nuclear export. As previous mutations of essential hydrophic residues L44 and L46 and the replacement of the NES with an analogous one resulted in concurrent loss of multimerization, it was not certain whether this point mutation would affect multimerization activity. A yeast 2-hybrid system was used to determine if protein-protein interaction was still present. This yeast 2-hybrid system involved fusion of the two halves of the GAL4 transcription factor to our mutant Apoptin and wild type Apoptin. Multimerization of the ApI37A and ApWT brought the necessary transcription factor together, which resulted in transcription of the lacZ gene. This resulted in β-galactosidase activity to visually demonstrate the presence of protein-protein interaction.

The ApI37A mutation, which is at the C-terminal side of the NES, retains multimerization activity. This allows for a few theories as to how the multimerization and nuclear export activities share domains. Previous simultaneous L44A and L46A mutations at the N-terminal

side of the NES resulted in loss of multimerization activity, as well as export.[9] These results indicate that the multimerization domain may share residues only on the N-terminal end of the nuclear export sequence. Alternatively, residues contributing to multimerization may be interspersed within the nuclear export sequence, including those residues not essential for nuclear export. Point mutations, similar to the one performed in this study, of the entire nuclear export sequence will need to be performed to map which residues are shared by the two domains and which are exclusive to nuclear export. If mutations to only N-terminal residues affect multimerization, the former theory will be proven. However, if mutations to amino acids throughout the nuclear export affect multimerization, then the latter theory will be corroborated.

As the two activities were previously coupled, loss of one activity resulted in loss of the other. This made it difficult to determine which function was responsible for apoptosis or cell-type specific selectivity. Uncoupling the multimerization domain and nuclear export is important in determining what roles the two activities have in the induction of apoptosis and cell-type selectivity. Previous data suggests that Apoptin is heavily multimerized in primary cells, but becomes more soluble in transformed cells.[unpublished data, D.W. Heilman] This is theorized to affect cell-type specific subcellular localization of Apoptin. Thus a differential cytoplasmic concentration of Apoptin is observed in primary and transformed cells. If Apoptin is heavily multimerized in primary cells, there would be a small pool of Apoptin that can undergo nuclear localization. If for transformed cells Apoptin is more soluble, then there is a larger mobile pool of Apoptin for nuclear localization. This increased solubility results in greater nuclear localization. It can be theorized that this change in multimerization is the regulatory mechanism responsible for the cell type selectivity in localization. Furthermore, multimerization could

affect the activity of nuclear export simply in its location relative to the NES. As multimerization activity and nuclear export activity share domains, multimerization of Apoptin could prevent Crm1 access to the nuclear export sequence. If the nuclear export sequence is responsible for apoptotic interactions, the differential multimerization of Apoptin between cell types could explain cell type-selective apoptosis.

The isolation of multimerization is important for determining what role differential multimierization has in cell-type specific apoptosis, assuming differential multimerization is not affected by eliminating nuclear export. This would confirm the theory that transformed cells alter Apoptin multimerization activity. The next theory to address is whether multimerization is responsible for apoptotic interaction. If multimerization is responsible for apoptotic interaction, then cell-type specific apoptosis is dependent on multimerization alone, and that nuclear export activity is not required.

Assuming differential multimerization is affected by the elimination of the NES, the theory that nuclear export activity is required for cell-type specific localization is confirmed. Next it must be determined whether nuclear export is also responsible for apoptotic interaction. If this theory is corroborated, then our Apoptin mutant would not be able to induce apoptosis. There is also the possibility that both domains are responsible for only one of the activities, differential aggregation and apoptosis. In this case our mutant may retain differential multimerization but fail to induce apoptosis, or vice versa. Additionally, there is the more complex scenario of both functions being required cell-type specific apoptosis. For example, multimerization may be responsible for differential multimerization, and the NES is responsible

for induction of apoptosis. However, it is possible that differential multimerization is a prerequisite to apoptotic interaction.

Once nuclear export deficiency has been verified, the role of multimerization in cell type-specific apoptosis can be elucidated. The role of multimerization in cell specific apoptosis is vital to the design of a small synthetic analog. If multimerization is required for apoptotic interaction, then Apoptin is interacting with its apoptotic target via a large protein area. The synthetic drug would have to be designed to simulate this large protein interaction. Essentially, this makes the drug extremely difficult to design. On the other hand, if multimerization is not required for apoptotic interaction, then a single Apoptin molecule is responsible for apoptosis. This translates into small protein-protein interaction surface area, where only a few residues are required. The drug would then only have to simulate a small interaction area. This would simplify the design and manufacturing costs of this drug. Therefore, the role of multimerization in the apoptotic interaction is important for the feasibility of developing a synthetic analog to mimic the mechanism of Apoptin cell-type specific apoptosis.

Though it can be reasonably assumed that the point mutation of the canonical amino acid residue within the NES will eliminate nuclear export activity, this will need to be verified. To verify that these activities are uncoupled, fluorescence analysis to determine localization of ApI37A is required to determine if nucleocytoplasmic shuttling is preserved. To insure that nuclear localization can only be affected by the nuclear export sequence, the NLS of Apoptin can be removed or mutated. Apoptin can then be fused to a fluorescent tag such as GFP or HA, though because GFP is hypothesized to have abnormal effects on the subcellular localization of Apoptin, another fluorescent tag such as HA would be prefereable. The data should show diffuse

localization of Apoptin if nuclear export activity was eliminated. Otherwise, the data would show localization of Apoptin within the cytoplasm exclusively if nuclear export activity was retained.

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