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Dissecting the Mechanism for the Selective Induction of Apoptosis in Transformed Cells by CAV Apoptin: a Dissertation

Destin W. Heilman
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A Dissertation Presented

By

Destin Heilman

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

March 1, 2006
DISSECTING THE MECHANISM FOR THE SELECTIVE INDUCTION OF APOPTOSIS IN TRANSFORMED CELLS BY CAV APOPTIN

A Dissertation Presented

By

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University of Massachusetts Medical School Interdisciplinary Graduate Program

March 1, 2006
DEDICATION

Qualities of unwavering love and support, gentle kindness, and absolute strength of character come so rarely together in a single person. I would like to dedicate this work to my grandparents, Charles and Eileen Gee, two people with such virtues, who are a rare and cherished gift for which I am ever grateful. Their support and encouragement of my pursuits in the sciences, as well as in the arts, has given me confidence and direction; their generosity and tender compassion has given me hope; their fortitude and integrity has given me the will to embrace with courage those things which I find adverse and demanding. Though they are no longer with me, I will not forget the unspoken lessons they have taught me, nor will I forget to share them with those in my life.
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Second, I would like to thank Dr. Jose Teodoro. As a postdoctoral mentor, Dr. Teodoro imparted a tremendous amount of hands on guidance and support to me in the laboratory. His knowledge and expertise in science, as well as in many other disciplines, is extraordinary and has been an inspiration and a delight. I will be forever grateful for his friendship and for the examples he has set for me in science and otherwise.

I would also like to extend my thanks to those Green lab members whom I have closely befriended over the last five years. Together, they have enriched my performance in science and have been supportive and thoughtful friends.

I would like to thank my Advisory Committee for their ever-present support and advice during my Ph.D. studies. Your true devotion to the welfare of the students in the graduate program is, and has been, greatly appreciated.

Lastly, I would like to thank my loving wife Susan, whose genuine support and encouragement throughout this Ph.D. has been invaluable. She has shown the utmost resilience to adversity and has demonstrated strength of character in the graduate program. She continues to be an inspiration for myself and other students alike.
ABSTRACT

Most existing chemotherapeutics lack adequate specificity for transformed cells and therefore have high rates of collateral damage to normal tissue. Moreover, such therapies often depend on p53 to induce cell death and are ineffective on the large number of human cancers that have lost p53 function. The discovery of novel p53-independent cancer therapies is therefore of significant interest. The Chicken Anemia Virus protein Apoptin selectively induces apoptosis in transformed cells in a p53-independent manner while leaving normal primary cells unaffected. This selectivity is thought to be largely due to cell type-specific localization: in primary cells Apoptin is cytoplasmic, whereas in transformed cells the protein localizes to the nucleus. The basis for this cell type-specific localization remains to be determined. In this study, Apoptin is revealed to be a nucleo-cytoplasmic shuttling protein whose localization is mediated by an N-terminal nuclear export signal (NES) and a C-terminal nuclear localization signal (NLS). Both signals are required for cell type-specific localization, as Apoptin fragments containing either the NES or NLS fail to localize differently between transformed and primary cells. Significantly, cell type-specific localization can be rescued in trans by co-expression of the two separate fragments, which are able to interact through an Apoptin multimerization domain. Interestingly, this multimerization domain overlaps with the NES suggesting that these two activities may be functionally coupled in cytoplasmic retention in primary cell types. Factors present in transformed cells induce localization of
Apoptin to the nucleus where a biochemically distinct, more soluble form of the protein exists.

Using affinity-purification and mass spectroscopy it was found that, specifically in transformed cells, Apoptin is associated with APC1, a subunit of the anaphase-promoting complex/cyclosome (APC/C). The APC/C is required to establish a mitotic cell-cycle checkpoint, and its inhibition results in G2/M arrest and apoptosis. Expression of wild type Apoptin in transformed cells inhibits APC/C function and induces G2/M arrest and apoptosis, whereas Apoptin mutants that are unable to associate with APC1 have no effect. In p53 null cells, ablation of APC1 by RNA interference induces a G2/M arrest and apoptosis analogous to that observed following Apoptin expression. Furthermore, Apoptin was found to induce the formation of PML bodies and to recruit APC/C subunits to these nuclear structures suggesting a mechanism involving sequestration and subsequent inhibition of the APC/C.

Thus, the results of this study clarify Apoptin cell type-specific localization behavior and explain the ability of Apoptin to induce apoptosis in transformed cells in the absence of p53. This study advances a newly emerging field of viral mechanisms of apoptosis involving G2/M arrest and APC/C modulation. The resultant p53-independent apoptosis suggests that the APC/C may be an attractive target for the development of anti-cancer drugs.
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PREFACE

The work contained within this thesis is represented in the following publications:


Figure panels 2.1a-b and 2.2b were contributed by J.G. Teodoro. Experiments in figures 3.7 and 3.8 were performed with the aid of technicians A.E. Parker and J. Zhu, respectively.

Additional unrelated studies performed in fulfillment of the Ph.D. degree will not be presented in this thesis and are represented in the following publication:

CHAPTER I

GENERAL INTRODUCTION
1.1 Cancer. Cancer is a disease of autonomous and uncontrolled cell proliferation generally spawned from stepwise somatic mutations that first deregulate cell division and/or block programmed cell death resulting in perilous increases in cell number. These cells, having a significant growth advantage over their normal surrounding counterparts, will usually expand in number to form a clonal benign neoplasm that will not yet have the capacity to spread. Selection of additional somatic mutations, however, will impart these cells with qualities necessary for further transformation and eventual progression to a fully malignant cancer with invasive capability (see Figure 1.1 and (68) for review).

The development of effective cancer therapies depends upon the discovery of agents that selectively destroy tumor cells with a low degree of collateral damage to their normal counterparts. This requires that an agent be responsive to properties or activities that discriminate between normal and transformed cells. In this regard, early in vitro studies were performed to characterize those features of transformed cells that are altered from normal cells. The general phenotypic qualities elucidated from these efforts are characteristic hallmarks of all cancer cells and a more detailed understanding of these properties will be important for the development of tumor-selective treatments (31, 157).

One hallmark that occurs temporally early in transformation involves cells gaining the capacity for unlimited proliferation (121). Unlike normal cells, which will senesce and/or die after a finite number of divisions, tumor cells become immortalized, in general, through mutations that abolish replicative senescence and abrogate cellular crisis. As mentioned before, this often occurs during the initial steps of transformation
and usually involves prevention of programmed cell death (PCD or apoptosis) and release of cell cycle control (68).

Another feature common to transformed cells is the loss of growth inhibition upon contact with neighboring cells (1, 78). Part of normal organ function depends on the establishment of tight boundaries between tissues and cell layers through the shutdown of cell duplication upon contact with other cells. The outgrowth of a tumor beyond normal cell and tissue boundaries involves mutations that abolish this contact-based signaling that would normally lead to growth inhibition.

Transformed cells also show loss or alteration of growth factor dependence (34). Normally cells require a certain level of growth signals from the environment to advance past the G₀/G₁ restriction point and enter the cell cycle. Additionally, cells from different tissue types will differ in their growth factor requirements, restricting their proliferation to particular microenvironments within the body. Selective pressure will drive the transformed cells to mutate and survive in environments lacking the normal supply of growth factors (54). Metastasis, or separation from the primary tumor site and establishment of residence elsewhere in the body, involves gross changes in growth factor responsiveness for transformed cells.

Malignant cancers also possess the ability to grow in the absence of anchorage (146). Detachment from a growth surface normally results in anoikis, a specialized form of apoptosis resulting from the lack of anchorage-related signaling (66). Fully transformed cells lack this signaling cascade and can readily grow in suspension. Tumor cells that break through tissue barriers are usually shed to the bloodstream by the millions
per day. Most of these cells will die by anoikis, however the rare cells that fail to respond to death signaling will survive the journey to distal tissues and may form metastatic lesions (10). Additional alterations in transformed cells include abnormal or defective differentiation, increased cell motility and gross morphological alterations in cell shape and size (see (31) for review).

Advances made in genetic methodology have allowed for the identification and characterization of the specific genetic aberrations that underlie many of the above mentioned phenotypes in transformed cells. These mutations affect one of two major classes of cancer-related genes: oncogenes and tumor suppressors, which are grouped according to how they functionally contribute to transformation. Oncogenes contribute to neoplastic disease through premature, aberrant or constitutive activation. Such a situation can occur in various ways including increase in copy number via gene duplication, alteration of regulation following chromosomal translocation, or mutation of various regulatory areas (e.g. promoter or silencer elements) that affect protein activation (see (175) for review).

The erbB proto-oncogene (oncogene precursor prior to mutation) for example, which encodes the cell surface receptor for epidermal growth factor (EGF), is normally involved in extracellular signaling responses to mitogenic stimulus (56). Receptor ligation and activation by EGF results in phosphorylation-based signaling cascades within the cell and activation of events including progression of the cell cycle and metabolic stimulation (168). Various mutations of erbB can result in constitutive activation of receptor-tyrosine kinase activity and subsequent intracellular signaling
independent of EGF ligand binding (24). The resultant constancy in proliferative
signaling has been shown to contribute directly to several phenotypic hallmarks including
cellular immortalization (via recovery from crisis) as well as the obvious growth factor
independence of these cancers. Similar oncogenic effects have been observed for other
closely related ErbB family members and many additional proteins of the greater
receptor-tyrosine kinase family (94).

The second class of cancer-related genes is the tumor suppressors. Unlike
oncogenes, it is the mutational ablation of these genes that contributes to cell
transformation. Normally these genes function in various situations to prevent
transformation and tumorigenesis and the loss of such genes results in the lack of this
protective effect. Moreover, unlike oncogenes the dysfunction of tumor suppressors is
functionally recessive and both maternal and paternal copies of the gene must be mutated
to fully contribute to transformation. In most cases one allele is lost through gross
mutation (such as deletion of large chromosomal regions) and only in extremely rare
instances are both alleles inactivated by point mutation (see (185) for review). The
discovery and functional characterization of tumor suppressors has proven more difficult
than for oncogenes due mainly to their recessive nature as well as difficulties inherent to
the isolation of genes based on loss of function. The first characterized, and most well
understood tumor supressors to date, are the pRb and p53 proteins. Initially identified by
inherited susceptibility to retinoblastoma, the pRb protein functions as a veritable
gatekeeper for the cell cycle and blocks S phase induction by binding the E2F family of
transcription factors. In its hypo-phosphorylated state, pRb remains tightly bound to E2F
preventing gene transactivation. Upon phosphorylation by cyclin E/Cdk2 complex in late G1, pRb releases E2F resulting in nuclear translocation and subsequent activation of genes critical for the induction and maintenance of S phase (Figure 1.2). These include a number of genes necessary for DNA replication including those encoding DNA polymerase, dihydrofolate reductase and thymidine kinase (70).

Key to the commitment to begin the cell cycle is the ability to actively monitor the fidelity of the genomic DNA throughout this process. If mistakes occur, pRb must again sequester E2F to block further entries into the cell cycle until the problem can be addressed by repair machinery, thereby avoiding propagation of harmful mutations to the daughter cells. Cellular response to stress and DNA damage in S phase and throughout the cell cycle relies on activation of p53. The p53 protein functions as a transcription factor and serves to activate a great many genes involved in regulation of the cell cycle and induction of apoptosis (63). If the integrity of the DNA is compromised, cellular levels of p53 rise through separation from MDM2, a negative regulator that constitutively binds p53 and induces its degradation. One well-characterized target of p53 transactivation is p21Waf1/Cip1, which actively silences the Cdk2-dependent phosphorylation of pRb resulting in E2F sequestration and halt of S phase gene expression (Figure 1.2). The p21Waf1/Cip1 protein falls into a special class of cell cycle regulators known as cyclin-dependent kinase inhibitors (CKI), a recently recognized class of tumor suppressor proteins often found to be mutated in human cancers (100, 144). If damaging conditions persist or repair efforts fail, p53 can activate the transcription of
various pro-apoptotic proteins (e.g. PUMA and NOXA) and sacrifice the cell entirely; a choice that mechanistically remains poorly understood.

Together, pRb and p53 provide a nodal connection between those processes that govern the entry and progression of the cell cycle and those that control apoptosis; two related and interconnected pathways effecting the rate and multiplicity of cell duplication. It is not surprising then to find that greater than half of all human cancers harbor mutations in these genes (64, 155). Moreover, it is theorized that those cancers that do not present p53 and pRb mutations have likely still lost function of these proteins via disruption of upstream regulators including CKIs (175). Specifically, these proteins regulate two major cell cycle checkpoint mechanisms that monitor the G1/S phase transition and the global cellular response to DNA damage and stress throughout the cell cycle (12, 77). The loss of cell cycle control and apoptosis resulting from the dysfunction of these tumor suppressors often occurs early and constitutes the source of initial proliferative phenotypes observed during transformation.

1.2 Treatment of cancer. Current therapeutic strategies for cancer remain dependent on relatively crude methods of treatment usually involving surgery to remove cancerous tissue, followed by radiation and/or chemotherapy aimed toward destruction of remaining cancer cells. Late stage, highly metastatic cancers with poor prognosis are usually treated without surgery and rely on large doses of chemotherapeutics that lack sufficient specificity for transformed cells. Most broad-spectrum chemotherapeutic agents act to inhibit features of cellular function and division common to all cells but are used at
particular doses where transformed cells are more susceptible. For example, some cytotoxic agents routinely used for chemotherapy are more deadly to transformed cells due to inherent higher rates of division. This narrow therapeutic index results in collateral effects on normal cells that also divide rapidly, such as loss of stomach and intestinal epithelium, hair loss, and immunosuppression (149). Additionally, alkylating agents, antimetabolites, and antibiotics including Adriamycin are among the most commonly used drugs, all of which rely on p53 function to commit cells to apoptosis (162). For this reason, the large number of human cancers that have lost p53 function are refractory to treatment with any of these agents. Clearly there is a need for more transformed cell-specific treatments that do not rely on p53 function to induce programmed cell death.

Certain studies have achieved therapeutic successes by developing drugs that will specifically inactivate well-characterized targets present only in transformed cells. A recent example of this target-based approach involved the design of a small molecule inhibitor of the oncogenic fusion protein BCR-ABL, the causative agent of chronic myeloid leukemia (CML). This oncogene results from a reciprocal chromosomal translocation (t(9;Ph22)) and fusion of the coding regions of the bcr and abl genes (see Figure 1.3a and b). The oncogenicity of the resultant fusion protein involves constitutive activation of the Abl tyrosine kinase conferred by Bcr dimerization activity (see (11) for review). In the early 1990s, a project was started to synthesize a small molecule inhibitor that would act as a highly specific competitive inhibitor of ATP occupancy for the Abl kinase. The most promising compound to emerge from the screen, imatinib mesylate
(STI571 Gleevec), showed exceptional specificity and potency in inactivating the Abl kinase (Figure 1.3c-d) (120). With negligible toxicity, this compound was able to induce a complete hematological remission in all CML patients receiving over 300 mg/day (21, 52). Not surprisingly, the drug entered clinical trials and was quickly approved for use in treating human CML.

Several factors contributed to success in the development of Gleevec, the most prominent being the identification of a causative oncoprotein that was only present in CML transformed cells. This situation is relatively rare as most transformed cell oncoproteins are present in normal cells as well, albeit at different levels and activities. Thus, the fruitfulness of this approach for most cancers will depend on the elucidation of novel transformed cell-specific activities that are sufficiently different or absent in normal cells. This, coupled with the specificity and low collateral toxicity inherent in targeting particular molecular determinants, may produce promising agents with better therapeutic efficacy in the treatment of a broad range of cancers. In this regard, current studies are using diverse strategies to identify novel factors and activities that might discriminate between normal and transformed cells at the molecular level.

1.3 Utility of viruses in cancer research. An increasing number of studies employ the use of viruses or virus-encoded proteins to help unravel the complexities of cancer. In general, viruses have evolved elegant and diverse strategies to manipulate many host cell systems to facilitate their genome replication and protein expression, to abrogate host defense mechanisms, and to facilitate viral egress. Many of these strategies involve viral
perturbation of the host cell genome and/or expression of viral proteins that affect the cell cycle, cell signaling and apoptosis. Thus, it is not surprising to find that many of these viruses and viral proteins are also inherently oncogenic. Over 15% of worldwide cases of human cancer have been etiologically linked to viral infection accounting for nearly 1.5 million cases per year (23). A large number of studies are now focused on deciphering the mechanics of these viruses and viral proteins and how they might help to unravel the complexities of virus- and non-virus-related human neoplasia.

One pivotal example of viral influence on cancer research is the surprising number of proto-oncogenes that were discovered through studies involving retroviruses (23, 31, 186). These RNA viruses integrate their genetic material into the host genome during their life cycle and, as a latent pro-virus, are replicated as part of the normal host cell genome. During RNA genome production, retroviruses often extract host genomic sequences via an imprecise transcriptional process that can result in packaging of incomplete or mutated forms of cellular genes (Figure 1.4). The insertion of these sequences during subsequent infections can result in the expression of a viral oncogene, usually under the control of relatively powerful LTR promoter elements. Retroviruses are also known to induce proto-oncogene activation by integrating directly into host open reading frames (ORF) or through positional effects by integrating near a gene resulting in aberrant LTR promoter transactivation.

Characterization of the first retroviral oncogene began in 1970 with pilot work by Peter Duesberg and Peter Vogt during comparison studies of Avian Leukosis Virus
(ALV) and its derivative Rous Sarcoma Virus\(^1\) (RSV) (53). RSV was understood to be an acutely transforming virus in chickens and was found to carry approximately 1.5kb of additional genomic sequence beyond that of its parent ALV genome. The oncogenicity of RSV was attributed to this extra sequence as mutation or loss of this region ablated its transforming capabilities (with no effect on virus replication) (98, 111). The sequence was eventually identified as a mutant form of the cellular src gene, the first characterized protein-tyrosine kinase, which is involved in intracellular signaling in response to cell surface receptor stimulation (20, 30). The virally encoded src oncogene (v-src) is truncated (as a result of imprecise retroviral gene capture) at the C-terminus where a normally inhibitory phosphorylation takes place (Figure 1.5a) (25). The lack of this site results in a constitutively active Src protein and downstream effects including anchorage-independent cell growth and mitogenic effects similar to that of ErbB dysfunction previously discussed. Thus, the study of RSV revealed the v-src gene, as well as its cellular proto-oncogene counterpart, as a potent oncogene which is now known to be relevant in human disease.

Continued focus on viral oncogenesis has elucidated many other viral and cellular oncogenes. For example, the oncogenic potential of the aforementioned EGF receptor was first identified through studies involving avian erythroblastosis virus (AEV), which carries an oncogenic form of erbB (57). It was not until 1984, when the sequence of the cellular EGF receptor gene was analyzed, that the true identity of v-erbB was revealed (50, 154). Interestingly, the v-erbB mutant from AEV lacks the extracellular ligand

---
\(^1\) RSV, the first clearly characterized and accepted tumor virus, was named for Peyton Rous, who isolated and characterized the virus from a chicken tumor in 1911.
binding domain of the receptor (Figure 1.5b), a truncation later shown to be the most relevant mutation of this gene leading to constitutive activation in human cancers (31). Studies of the Ableson Leukemia Virus in mice led to understanding of the oncogenic potential of the Abelson kinase (v-\textit{abl}) years before the role of the Bcr-Abl fusion in CML was revealed (74, 108, 179). Currently, over two-dozen oncogenes have been characterized by study of retroviruses, all of which are relevant in human disease and are potent inducers of neoplastic transformation (see Table 1.1).

Many endogenous viral proteins have been useful in the understanding of tumor-specific processes as well. The compact nature of viral genomes demands that the limited number of proteins they produce exert a maximal effect on host cell function with a minimal amount of protein interactions. It is then understandable to find that many viral proteins operate at nodal points of cellular protein interaction networks where a single interaction can have vast downstream effects on cellular function. These nodes exist at critical regulatory areas where multiple cellular systems converge, many of which are involved in the control and/or prevention of human malignancy. Thus, study of these proteins has proven fruitful for elucidation and characterization of tumor suppressor proteins as well, primarily because many of the same nodal regulators that oncogenic viral products affect have been found to have functional relevance in non-virus related human cancers.

One of the greatest advances in the understanding of tumor suppressors came in the 1990s with the realization that virtually all DNA tumor viruses have convergently evolved proteins that bind to and inactivate both pRB and p53 (Figure 1.6). This curious
observation seeded the understanding that both of these cellular proteins function as seminal tumor suppressors whose dysfunction may be important, and possibly even required, for cell transformation (175). Examples of such viral proteins include Adenovirus E1A and human papilloma virus (HPV) E7 which bind to pRb and inhibit interaction with E2F. Additional proteins from these same viruses target p53, namely Adenovirus E1B55k and HPV E6. The Simian Virus 40 (SV40) Large T protein is uniquely able to bind to and inactivate both p53 and pRb regulators and is a potent viral transforming agent (see (59) for review). Many other DNA virus proteins have been found to affect other tumor suppressors or have functions that indirectly effect p53 and pRb (see Table 1.2).

In addition to elucidating proto-oncogenes and tumor suppressors, other molecular aspects of oncogenesis have been clarified through viral study. One such area involves the viral suppression of apoptosis, a common and required event for cell transformation and viral infection. One class of these viral products has evolved to specifically counteract host defense mechanisms that induce apoptosis in response to infection. Many of these involve the host innate immune responses centered on cytokine production and chemo-attraction of macrophages, neutrophils and NK cells that will induce apoptosis in infected cells and clear resulting debris (152). Many viruses produce proteins that block signaling pathways involved in cytokine production and death receptor activation by use of molecular mimicry. Cowpox virus, for example, produces two protein products, CrmB and CrmC, which traffic to the plasma membrane and competitively inhibit TNF ligand association by mimicry of the TNF receptor extracellular domain (79). Similarly,
proteins such as Epstein-Barr Virus LMP-1 counteract death signaling by artificial activation of NF-κB pro-survival pathways using a TRAF receptor mimic (48).

Other viruses produce proteins that interfere more directly with downstream and intrinsic apoptotic machinery. Many of these involve mimicry of a class of cellular proteins called cIAPs (cellular inhibitor of apoptosis) which normally function to either bind and block activated caspases or to prevent activation of procaspases in response to survival signaling (152). Many baculoviruses encode vIAPs, viral mimics of cIAPs, which block the action of particular subsets of caspases. Notably, the discovery of cIAPs and their role in apoptosis was elucidated through study of their viral counterparts (72, 105).

Interestingly, just as many early viral life cycle events require inhibition of apoptosis, certain late events are propagated by the timed induction of apoptosis. For example, viruses may trigger cell suicide towards the end of the life cycle as a method of viral egress. Such a mechanism has certain advantages including the virions being packaged into apoptotic bodies that are not only protected from inactivation by host antibody responses but are also taken up by phagocytic cells allowing efficient spread of infection. This strategy is most useful to those viruses that have not evolved complex mechanisms to evade host cell responses or anti-apoptotic factors (152). Some viruses will achieve successful infection cycles through both inhibition and induction of apoptosis by using combinations of viral proteins, the effectiveness of which requires precise timing and expression levels of these protein products. Numerous proteins including Adenovirus E1A and Hepatitis-B Virus HBx induce apoptosis in host cells and
are critical for viral replication competency (107, 163). Of particular note are those viral products that have been found to induce apoptosis independently of p53 activation through actions at the mitotic checkpoint (73, 194). Such viral proteins including Adenovirus E4orf4, HIV-1 Vpr, and HTLV-1 Tax are currently being studied to clarify as yet unclear apoptosis mechanisms from this checkpoint.

The mitotic checkpoint system exists to ensure proper segregation of genetic material to each daughter cell during the late stages of mitosis (reviewed in (134)). The timing of sister chromatid separation is tightly controlled to prevent onset of anaphase until all chromosomes have achieved bipolar spindle attachment and possess adequate spindle tension to align at the metaphase plate. The sensory mechanism involved provides a negative signal from kinetochores that lack spindle attachment to the aptly named anaphase-promoting complex/cyclosome (APC/C), one of the major cellular E3 ubiquitin ligases involved in cyclin turnover (15). The Mad and Bub family of proteins actively associate with and inhibit the mitotic APC/C activator protein cdc20 resulting in delay of onset of anaphase ((165) and reviewed in (134)). Once all sister chromatids have achieved spindle attachment, Mad/Bub signaling ceases and cdc20 can activate APC/C (APC/C\textsuperscript{cdc20}) resulting in the ubiquitination and subsequent destruction of mitotic cyclins as well as securin, the inhibitory subunit of the separase enzyme. Upon activation, separase catalyzes the destruction of cohesin complexes that bind sister chromatids together allowing for separation and migration of chromatin toward opposite spindle poles (Figure 1.7). A single unattached kinetochore can sufficiently activate the spindle checkpoint and inhibit all APC/C function, stabilizing securin and preventing sister
chromatid separation (147). Interestingly, it is now becoming evident that viral products produced by many previously mentioned viruses (e.g. HTLV-1 and Adenovirus) modulate various aspects of APC/C function (194). This emerging field is beginning to describe the mechanics of a novel pathway of virus-mediated G2/M cell cycle arrest and programmed cell death that will be discussed later and may be of great interest in cancer research.

1.4 Viral oncotropism and tumor cell-selective capacities. Observations made as early as the 1920s indicated that some viruses might have specific replicative capacities in transformed cells. Subsequent studies of viral oncotropism has revealed that a surprising number of naturally occurring viruses possess tumor-selective capabilities (19, 148). Examples include parvoviruses, human reovirus and vesicular stomatitis virus (VSV), all of which have been shown to replicate more efficiently in transformed cells (9, 60, 71, 190). The underlying molecular mechanisms for such cancer cell selectivity are only now being elucidated. For example, Parvoviruses, which are excluded from infecting normal quiescent cells because they lack the ability to forcibly induce S phase, seem to take advantage of dysregulated S phase entry in transformed cells and replicate vigorously (148). Human reovirus adopts a more specific strategy relying on common transformed cell dysregulation of the Ras pathway, which studies suggest relieves a block in viral gene translation allowing replication and lysis to proceed (122). Additionally, some viruses with natural oncolytic ability have been either directly genetically altered or adapted by serial passage through transformed cell culture to become more oncotropic.
Adaptation of Newcastle disease virus (NDV) is a classic example of this approach. Serial passage through various tumor cell cultures including human melanoma cell lines has resulted in NDV strains with greater selectivity and replicative vigor in transformed cells that may be of use in the direct treatment of cancer through solid tumor injection (26, 27).

In addition to those viruses that have oncotropic and oncolytic activity, other viral protein products have been found to have dramatic and selective transformed cell activities apart from their parent viruses. Molecular dissection and separate expression of these proteins has only recently yielded interesting insights into tumor cell-specific physiology that may prove useful for the development of future targeted drug therapies. One such protein, chicken anemia virus (CAV) VP3, has such activity and will be the focus of the research and discussion presented herein.

1.5 Chicken Anemia Virus (CAV). CAV was originally isolated and characterized in 1979 from an unusual subpopulation of chickens infected with Marek’s disease virus (MDV). MDV, a transforming herpesvirus, normally progresses with low mortality in chickens mostly due to widespread vaccination, however a subpopulation of chickens displayed rapid mortality and lack of effective immune response to MDV. Upon close inspection it was determined that these chickens were doubly infected with MDV and another uncharacterized virus, CAV. Subsequent inoculation of CAV alone in young chickens resulted in severe anemia due to loss of erythroblastoid cells in the bone marrow, subcutaneous and intramuscular hemorrhaging, and depletion of lymphoid
organs (including thymocytes), which might explain the loss of protective immunity observed with vaccinated adult chickens (2, 192). Cytological analysis of CAV infected tissues in these chickens indicated that losses of erythroblastoid cells and thymocytes was due to large-scale induction of apoptosis in these populations (51). CAV was later isolated and characterized as being non-enveloped and icosahedral, with a rare circular single-stranded DNA genome of approximately 2.3 Kbp (112, 113, 124). These properties placed CAV in a novel taxonomic class as the only member of the family Circoviridae, genus Gyroviridae.

The CAV genome contains only 3 functional open reading frames (Figure 1.8a), all of which are translated from a single polycistronic and polyadenylated transcript controlled by promoter and enhancer elements upstream of the transcription start site (89, 124, 125, 137). This promoter region contains at least 4 near-perfect 12bp repeat elements that are able to recruit cellular SP1 and SP1-like transcription factors, a situation not uncommon with other viral promoter elements (129). Mutational studies indicate that VP1 (51.6kDa), VP2 (24.0kDa) and VP3 (13.6kDa) proteins encoded by CAV are required for the virus infectious life cycle (127, 128). Limited study of VP1 has demonstrated this protein to be largely structural and comprise the major protein component of the virus capsule (127). The VP2 and VP3 proteins are non-structural and have been studied in greater depth in recent years. Detailed studies involving VP2 have demonstrated a dual-specificity phosphatase activity for this protein, a relatively rare activity for viruses of this type. Mutagenesis of the VP2 coding sequence reveals that catalytic cysteines (C95 and C97) are able to coordinate the removal of phosphate groups
from both tyrosine and serine/threonine residues and that these activities are necessary for high titer replication of the virus (135, 136). Authors of these studies speculate that VP2 activity may interfere with signaling pathways involved in host cell defense responses, however little information is available to make any substantial conclusions.

1.6 CAV VP3 (Apoptin). The vast majority of research studies on CAV have focused on the product of the third ORF for several reasons. Immunohistochemical staining of CAV infected tissues revealed that VP3 protein was found in the nucleus and, at later timepoints, showed this protein to aggregate in large clusters concurrent with onset of apoptosis. Cloning and recombinant expression of VP3 in chicken lymphoblastoid T-cells revealed that the activity of this protein alone results in apoptosis similar to that induced by CAV (123, 126). Intriguing results from subsequent studies reported that expression of VP3 in numerous primary and transformed cell lines resulted in apoptosis only in transformed cell types. By contrast VP3 was functionally inert following transient or long-term expression in various primary, normal cell types (37).

Remarkably, induction of apoptosis occurred in transformed cell types lacking functional p53 suggesting a novel pathway to apoptosis of nuclear origin (37, 38, 198). This reproducible transformed cell-specific apoptosis makes VP3 an ideal candidate for the study of cancer cell-specific physiology and the potential discovery of novel and p53-independent cancer treatments.

The mechanism of transformed cell selectivity by VP3, henceforth deemed “Apoptin” (126), is unknown. Analysis of the 121 amino acid primary structure of
Apoptin indicates no structural motifs or homology to any known proteins. Sequence analysis reveals a single putative nuclear export signal (NES) in a hydrophobic patch near the N-terminus (a.a. 37 to 46) and basic residues at the C-terminus that may function in nuclear import (Figure 1.8b). Interestingly, Apoptin activity appears to be dependent on sub-cellular localization; in primary normal cell types Apoptin is cytoplasmic whereas in transformed cells it is nuclear (37, 119, 126, 196-198). Preliminary mutagenesis experiments have shown that abrogation of Apoptin nuclear localization either by truncation of C-terminal sequences or by targeted mislocalization to alternate cellular compartments results in a loss of killing capacity in transformed cell types suggesting that nuclear localization is required for induction of apoptosis (42, 67, 126, 196). Surprisingly however, forced expression of Apoptin to the nucleus of primary cell types does not result in apoptosis indicating this is not sufficient for the Apoptin killing activity and that additional activities are necessary for Apoptin activation (67). Co-expression of Apoptin with SV40-LT has been shown to confer nuclear localization and killing capacity to Apoptin in primary cell types suggesting that particular elements in the transformation process may be linked to Apoptin trafficking and activation (37, 130).

Analysis of recombinant in vitro purified Apoptin by circular dichroism reveals no extensive α-helix or β-sheet motifs yet spectroscopic and electron microscopic analysis suggests that Apoptin exists in large globular clusters that appear to be stable and repeated in conformation. Such clusters of an estimated 30-40 Apoptin monomers will associate into larger, hydrophobic aggregates by virtue of sequences at the N-terminus of the protein. Gross truncations of the N-terminus of Apoptin results in a loss of all
aggregation activity using recombinant protein (101, 102). Aggregation could play a role in regulating sub-cellular localization patterns between primary and transformed cells as Apoptin appears to form punctate structures in the cytoplasm of primary cells as opposed to the nucleus of transformed cells where at similar time points it is diffuse (37). Contributing factors to Apoptin cell type-specific localization are intriguing as such activities are likely to be specific to transformed cells and worthy of future study. Previously, Apoptin was reported to be phosphorylated by an as yet unidentified kinase activity present in transformed cells. Threonine 108 in the C-terminus of Apoptin was shown to be phosphorylated and it is suggested that this event may serve to activate nuclear entry and induction of apoptosis in transformed cells (151). These findings have proven to be controversial as several subsequent studies have either not successfully reproduced phosphorylation of this residue or found no consequence in mutation of it (67, 176). Despite research focused on elucidating such transformed cell-specific kinase activity, the consequences of Apoptin phosphorylation and the nature of its activation are quite unclear (see Appendix).

It is evident that cell death induced by Apoptin in transformed cell types occurs by apoptosis. Early studies reported that expression of Apoptin resulted in changes in nuclear morphology including chromatin condensation, DNA fragmentation and nuclear blebbing as well as other general features of apoptosis including presence of phosphatidyl serine in the outer leaflet of the plasma membrane (37, 126, 138). More recent analyses of Apoptin-induced cell death have focused on clarifying the involvement of specific apoptotic pathways. Stimulation of apoptosis can be divided into two major types:
intrinsic and extrinsic signaling. The extrinsic pathway is activated by stimuli originating outside the cell, usually in the form of cell surface receptor engagement. Specialized death receptors at the plasma membrane (e.g. TNF and Fas receptors) are engaged by cognate ligands which are either in solution or membrane bound (e.g. cytotoxic T-lymphocytes (CTL)). Death receptor activation results in a “suicide signal” and the recruitment of adaptor proteins that initiate caspase activation (reviewed in (85)).

Various studies involving either co-localization or yeast 2-hybrid screening have shown Apoptin association with FADD (Fas Associating protein with Death Domain), Bcl-10, DEDAF (death effector domain-associated factor) and Hippi (Huntingtin-interacting protein-1 protein interactor), all of which are primary or secondary effectors of the extrinsic apoptosis pathway (29, 40, 67). However, functional support for such associations is lacking and subsequent analysis of Apoptin derived cell death has revealed signaling independent of any death receptor pathways. Specifically, Apoptin induced cell death occurs regardless of the status of FADD and caspase 8, key components of the death-inducing signaling complex (DISC) involved in death receptor ligation and activation (109).

More convincingly, Apoptin has been shown to stimulate intrinsic pathways to apoptosis. This type of programmed cell death involves interplay between various members of the BCL-2-related family of proteins, which constitute the bulk of intrinsic apoptotic circuitry for both pro- and anti-apoptotic signaling. When expressed, BCL-2 itself is a potent inhibitor of many resident pro-apoptotic proteins of the same family and generally functions to preserve the integrity of the mitochondria, the hub for modulation
of intrinsic apoptosis (199). Activation from internal stimulus induces various BCL-2-related pro-apoptotic proteins such as BID, BIM, NOXA and PUMA to translocate to the mitochondria and counter the action of BCL-2 (and similar anti-apoptotic proteins), causing a dramatic loss of membrane integrity. Cytochrome-c liberated from the mitochondrial inner membrane will associate with and activate the adaptor protein Apaf-1, which in turn activates procaspase 9. These factors in complex with each other (a structure called the *apoptosome*) cleave and activate various downstream effector caspases (reviewed in (28, 65)). Consistent with intrinsic activation, over-expression of pro-survival factors such as BCL-2 and BCL-X<sub>L</sub> has been shown to block Apoptin induced cell death (22), contrary to earlier less convincing evidence which suggested otherwise (38, 41). Additionally, downstream events following Apoptin expression include the loss of mitochondrial membrane integrity and release of cytochrome-c, followed by apoptosome formation, and effector caspase activation (22).

Cell death brought upon by Apoptin has at least been shown to be dependent on the downstream activation of effector caspase 3. Apoptin expression has been shown to induce the processing of pro-caspase 3 and broad-spectrum inhibition of caspases using the peptide inhibitor zVAD-fmk, which blocks activation of caspase 3, negatively affects Apoptin-induced programmed cell death (PCD) (22, 39). Thus, Apoptin expression in transformed cell types results in death by virtue of intrinsic apoptosis components, however the specific nuclear mechanisms of initiation remain unclear.

Preliminary experiments have been conducted to evaluate the therapeutic potential of direct Apoptin gene transfer into human tumor cells *in vitro* as well as solid
human tumors *in vivo* via different viral vector systems. Success has been achieved in
the selective killing of tumor cells in both cases by using adenoviral vectors for Apoptin
transgenesis. Apoptin treatment of xenografted human hepatomas in nude mice by direct
tumor injection has been shown to decrease mean tumor volumes between 75 and 100
percent with little collateral damage to surrounding tissues (139, 169). Other studies
have had similar successes by coupling Apoptin expression to delivery through
autonomous oncotropic and oncolytic viral vectors, such as rodent parvovirus, thereby
increasing tumor cell selectivity at even higher viral titers conducive to in vivo therapy
(32, 117, 131, 158).

Apoptin may have greater value beyond its uses for direct tumor therapy. Studies
focusing on the mechanics of Apoptin tumor selectivity are likely to produce novel
transformed cell-specific protein activities or molecular targets worthy of further study.
Activities coupled to Apoptin cell-specific localization and/or apoptosis could be
exploited for targeted drug therapies similar to STI571, but useful for a broad spectrum of
cancers. Of particular significance is the apparent lack of dependence on p53
functionality for Apoptin-induced PCD. As mentioned before, the p53 tumor suppressor
regulates the major nuclear pathway to apoptosis in response to many cellular stresses
and would ordinarily be a likely candidate for Apoptin death signaling. However,
apoptosis occurs in those transformed cell types that lack p53 function equally as well as
those that do not suggesting an alternate or downstream mechanism of initiation. Clearly
the study of Apoptin and other novel pathways to apoptosis that do not rely on p53 are of
great therapeutic interest.
The following studies focus on clarification of both the tumor cell selectivity of Apoptin as well as identification of novel targets and pathways involved in the induction of apoptosis by Apoptin in transformed cell types in the absence of functional p53. Many advances have been gained in the understanding of Apoptin structure and function through the research that follows. Of note are also the insights that have been gained spanning to areas involving the cell cycle and cancer biology.
Figure 1.1. Stepwise mutations leading to cancer. The process of normal cell transformation involves the stepwise accumulation of somatic mutations, each affecting a particular gene. Initiating mutations usually involve deregulation of the cell cycle and/or suppression of apoptosis leading to abnormally excessive cell growth, or hyperplasia. Subsequent mutations in other genes result in the acquisition of common cancer cell phenotypes (hallmark abilities) that together will contribute to invasive metastatic disease.
Initiating mutation (hyperplasia)

Secondary mutation (early premalignant)

Tertiary mutation (late premalignant)

Quaternary mutation (invasive metastatic)
Figure 1.2. pRb and p53 tumor suppressor functions. Together, pRb and p53 regulate major mechanisms controlling the rate and multiplicity of cell duplication. The pRb protein, which normally inhibits the function of the E2F family of transcription factors, is phosphorylated following increases in G1 cyclin-Cdk activity. Cyclin D/E-Cdk complexes hyperphosphorylate pRb resulting in a release of E2F, which transactivates many genes involved in the progression and maintenance of S-phase. During DNA synthesis and throughout the cell cycle, if damage or mistakes occur, p53 protein levels rise and initiate a host of responses. One of these responses involves transactivation and expression of p21, a cyclin-dependent kinase inhibitor (CKI) that blocks the activity of various cyclin-Cdk complexes including Cdk1 and Cdk2. This blocks advance of the cell cycle at several points including S-phase initiation. Cdk2 phosphorylation of pRb is inhibited causing E2F sequestration and S-phase gene shutdown. Together, these tumor suppressors provide a plexus where the initiation and progression of the cell cycle is intimately connected to mechanisms that preserve the fidelity of these processes.
Figure 1.2

Cdk1-cyclin B
Cdk1-cyclin A
Cdk2-cyclin A
Cdk2-cyclin E
Cdk4/6-cyclin D
G0
G1
G2
M
S

pRb
E2F

p53
p21

Adapted from Lodish et al. Molecular Cell Biology, 2000.
Figure 1.3. Targeted inhibition of the Bcr-Abl oncoprotein. (A) Schematic diagram of reciprocal translocation event (t(9;Philadelphia 22)) leading to fusion of the Bcr and Abl coding regions. (B) Detail of the Bcr and Abl protein domain architecture. Translocation between coding regions for these proteins results in truncation and fusion of the N-terminal oligomerization region of the Bcr protein with the C-terminal tyrosine kinase domain of Abl. Oligomerization of this fusion protein via Bcr sequences results in autophosphorylation and constitutive activation of the Ableson kinase. (C) Crystal structure of the Abl kinase (green) bound to the STI571 (Gleevec) inhibitor. Gleevec (blue) associates directly with the catalytic binding pocket of the kinase and blocks entry of ATP. (D) Chemical structure of Gleevec illustrating bonding patterns observed in the Abl active site. (Panels C and D from Nagar, B et al. 2002. Cancer Research. (120))
Figure 1.3

A

9 22 9q+ 22q- Ph

Abl-Bcr

Bcr-Abl

B

Abl

TK

Bcr

Bcr-Abl

C

D

SH2

SH3
Figure 1.4. Retroviral oncogene capture.

A hypothesized scheme depicting the steps involved in retroviral cellular gene capture and oncogene production are shown. Proviral integration occurs in the host cell genome frequently in proximity to a cellular genetic locus. Failure in termination during transcription of the viral genome results in partial readthrough transcription of the cellular locus and generation of hybrid virus/host pre-mRNA. Abnormal splicing events can lead to fusion of the viral and host cell exons in the mature mRNA, which is then packaged into virions with another normal copy of the viral genome. Recombination events take place during reverse transcription in subsequent infectious cycles resulting in the stable integration and expression of a hybrid provirus expressing mutated cellular genes under the control of relatively powerful LTR promoter elements.
Figure 1.4

Proviruses and Proto-oncogene Loci

-readthrough transcription

-abnormal splicing

-heterozygous packaging

-adapted from Cooper, G.M. 1995
Figure 1.5. Example retroviral oncogenes. (A) Comparison of the normal cellular Src (c-Src) and viral oncogenic Src (v-Src) proteins. The Src protein has similar domain architecture to that of the Abl protein. In this case, a normally inhibitory phosphorylation site (Y-527) is deleted from v-Src version via truncation of C-terminal sequences as a result of retroviral gene capture. This results in constitutive activation of Src kinase activity and downstream effects that can contribute to transformation. (B) Comparison of the normal cellular erbB (c-erbB/EGF receptor) and viral oncogenic erbB (v-erbB) proteins. In the normal situation, erbB receptor activation occurs in response to EGF ligand docking at the extracellular protein domain. The v-erbB protein lacks this domain and is rendered ligand insensitive. Therefore, activation of this EGF receptor occurs constitutively regardless of the presence or absence of EGF.
Figure 1.5

A

\[ \text{SH3} \quad \text{SH2} \quad \text{TK} \quad \text{SH3} \quad \text{SH2} \quad \text{TK} \]

\[ \text{c-Src} \quad \text{v-Src} \]

B

\[ \text{c-erbB} \quad \text{v-erbB} \]

adapted from Cooper, G.M. 1995
Table 1.1. Retroviral Oncogenes. The study of retroviruses has elucidated over two-dozen oncogenes. Each of these oncogenes originated from a cellular proto-oncogene and as a result of retroviral gene capture, exists in a mutated form that has relevance in transformation. The viral oncoprotein product is indicated as well as the hybrid protein generated upon fusion with viral sequences. Also indicated is the species from which the oncogene was originally liberated (owing to the tropism of the particular retrovirus).
## Table 1.1 Retroviral Oncogenes

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<th>Species</th>
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Cooper, G.M. 1995 *Oncogenes.*
Figure 1.6. Convergently evolved viral inhibitors of p53 and pRb.

The central role of p53 and pRb in cell cycle control and tumor suppression is illustrated by the many convergently evolved DNA tumor virus proteins that bind and inhibit these proteins.

Table 1.2. DNA tumor virus proteins and their activities. The study of DNA virus proteins with transforming capability has resulted in the discovery of the many cellular targets of these proteins that are important in cell cycle regulation and tumor suppression. Examples of tumor virus proteins and their respective activities are shown. All of these proteins have direct or indirect roles in the inhibition of major cellular tumor suppressors.
Table 1.2 DNA tumor virus proteins

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<td>HBV</td>
<td>HBx</td>
<td>transcriptional repression of p21\textsuperscript{waf1/cip1} transcriptional activation of cyclins A and E</td>
</tr>
<tr>
<td>HCV</td>
<td>NS3</td>
<td>transcriptional repression of p21\textsuperscript{waf1/cip1} mislocalization of p53</td>
</tr>
<tr>
<td></td>
<td>NS5A</td>
<td>modulation of p53 target transactivation</td>
</tr>
<tr>
<td>SV40</td>
<td>Large T</td>
<td>direct inhibition of p53 and pRb</td>
</tr>
<tr>
<td></td>
<td>Small T</td>
<td>direct inhibition of PP2A tumor suppressor</td>
</tr>
</tbody>
</table>
Figure 1.7. The mitotic checkpoint. Proper distribution of genetic material to each daughter cell in mitosis is dependent on the precise timing of sister chromatid separation. The anaphase-promoting complex/cyclosome (APC/C) serves as the central regulator of this process in mitosis and functions in a negative regulatory system that prevents progression of mitosis until all chromosomes have attached spindles and possess adequate tension so as to align at the metaphase plate. Signaling is accomplished by the Mad/Bub family of proteins, which continually relay from unattached kinetochore complexes to bind and inactivate the APC/C activator protein Cdc20. Once all kinetochores possess spindles, Mad/Bub signaling ceases and active APC/C$_{\text{Cdc20}}$ ubiquitinates several targets including mitotic cyclins as well as securin, the inhibitory subunit of the separase enzyme. Active separase then cleaves cohesin complexes that bind sister chromatids together allowing for their rapid retraction to the spindle poles, anaphase progression, and subsequent exit from mitosis.
Figure 1.7

Adapted from Peters et al. 2002 Mol. Cell
Figure 1.8. Chicken Anemia Virus (CAV). (A) Diagram of circular ssDNA genome of CAV. Transcription is initiated from a single start site (354bp) downstream of a non-coding region and produces a polycistronic, polyadenylated mRNA. Translation occurs from alternate start codons to generate the three CAV proteins VP1, VP2, and VP3. The coding region for VP3 is buried within that of VP2 and is translated from an alternate reading frame. (B) Schematic of VP3 protein domains. Sequence analysis reveals a putative nuclear export signal (NES) toward the N-terminus. Consensus sequence for canonical NES is shown. Basic patches at the C-terminus may function in nuclear import.
Figure 1.8

A

Chicken Anemia Virus (CAV), 2,319 bp

VP1 (51.6kDa)
VP2 (24.0kDa)
VP3 (13.6kDa)

non-coding region

putative NES

canonical NES

-X-(1-4)-(2-3)-X

basic regions

B

putative NES

canonical NES

-1-4-2-3-1-4-2-3-

basic regions
CHAPTER II

CELL TYPE-SPECIFIC LOCALIZATION OF CAV APOPTIN IS REGULATED BY MULTIFUNCTIONAL DOMAINS THAT CONTROL NUCLEO-CYTOPLASMIC SHUTTLING
Abstract

The Chicken Anemia Virus protein Apoptin selectively induces apoptosis in transformed cells while leaving normal cells unaffected. This selectivity is thought to be largely due to cell type-specific localization: in primary cells Apoptin is cytoplasmic, whereas in transformed cells the protein localizes to the nucleus. The basis for this cell type-specific localization remains to be determined. In this study, Apoptin is revealed to be a nucleo-cytoplasmic shuttling protein whose localization is mediated by an N-terminal nuclear export signal (NES) and a C-terminal nuclear localization signal (NLS). Both signals are required for cell type-specific localization, as Apoptin fragments containing either the NES or NLS fail to localize differently between transformed and primary cells. Significantly, cell type-specific localization can be rescued in trans by co-expression of the two separate fragments, which are able to interact through an Apoptin multimerization domain. Interestingly, this multimerization domain overlaps with the NES suggesting that these two activities may be functionally coupled in cytoplasmic retention in primary cell types. Factors present in transformed cells induce localization of Apoptin to the nucleus where a biochemically distinct, more soluble form of the protein exists. Previous reports have suggested that Apoptin phosphorylation at the C-terminus may control localization activity as well as induction of apoptosis. In contrast, this study shows that this phosphorylation event and region of the protein were dispensable for the achievement of cell type-specific localization and instead, the unique Apoptin NES domain was found to confer this activity. These data suggest that nucleocytoplasmic shuttling activity is necessary for proper Apoptin localization and apoptotic activity and
suggest that biochemical differences conferred by the Apoptin N-terminus provide the basis for cell type-specific localization between normal and transformed cells. Based on these results, a model is proposed for the differential localization of Apoptin between normal and transformed cells.
Introduction

Viruses employ diverse strategies to alter host cell functions and facilitate the viral life cycle. For this reason, many viral proteins target cellular pathways at critical points, such that many cellular activities can be altered with a minimal amount of virus-host factor interaction. For example, numerous DNA viruses are known to encode proteins that target and inhibit p53 and pRB; two of the key growth regulators in mammalian cells (see Chapter I, Figure 1.6)(93, 200). Thus, the study of virus-host interactions has been valuable in understanding a wide variety of cellular processes. For instance, apoptosis induced by viral proteins is a common mechanism to facilitate viral egress and promote viral spreading. Many animal viruses encode proteins that are potent inducers of apoptosis and in certain cases such activity is selective to transformed cells. One example is the Apoptin protein encoded by the Chicken Anemia Virus (CAV).

CAV exists as the only member of the family Circoviridae, genus Gyroviridae and is the etiologic agent of chicken infectious anemia, which results from large-scale apoptosis of cortical thymocytes and erythroblastoid cells in the bone marrow (84). The CAV genome encodes 3 proteins, the third of which (VP3 or Apoptin) was found to be responsible for induction of apoptosis. Two properties of CAV Apoptin make it a provocative protein to study for cancer-specific processes. First, Apoptin selectively induces apoptosis in transformed cells while leaving primary normal cells intact and unaffected (37). Transient or long-term expression of Apoptin in primary cells has no deleterious effects. Second, induction of apoptosis in transformed cells occurs regardless of the status of the p53 tumor suppressor (38). The p53 pathway is the major mechanism
by which cancer cells are destroyed by chemotherapy and radiotherapy (162). Over half
of all human tumors are mutated in the p53 gene rendering these cancer cells refractory to
such forms of therapy (155). Therefore, the study of Apoptin represents a system to study
novel p53-independent pathways to apoptosis in cancer cells and may also elucidate
cancer-specific processes.

The transformed cell-specific killing effects of apoptin are largely related to
differences in subcellular localization of the protein. In primary cells, Apoptin localizes
in the cytoplasm whereas in transformed cells, it is nuclear (37). This differential
localization of Apoptin is highly unique and the basis for this behavior is yet to be
determined. The study of cellular mechanisms regulating this localization may provide
valuable insights into transformed cell physiology. In the present study the basis of
Apoptin cell type-specific localization is addressed and a model for transformed cell-
selective killing is discussed.
Materials and Methods

Cells and Adenoviruses. Primary foreskin fibroblast (PFF), H1299 (from ATCC), and HA1-IM cells (S. Bacchetti, McMaster University, Hamilton, Canada) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS plus 10 mg/ml streptomycin and 10 U/ml penicillin (Sigma, St Louis, MO) at 37°C under 5% CO₂ (95% air). Apoptin adenovirus (Ad-Apwt) containing a single 5’ FLAG epitope was generated using the AdEasy XL Adenoviral Vector System (Stratagene) according to the manufacturer’s instructions. Ad-Ap-pmNES was generated by site directed mutagenesis of Apwt sequence as indicated followed by generation of FLAG epitope tagged adenovirus as indicated above. Viral supernatants were plaque purified, amplified, and titers determined by plaque assay. LacZ adenovirus (Ad-LacZ) was prepared as previously described (8). Cells were infected at approximately 80% confluence at an MOI of 35. Adenovirus was added to cells in a minimal volume of culture media and gently agitated at 37°C under 5% CO₂ for 1 h. Following infection, culture media was added and cells were incubated for indicated times.

Plasmid Constructions. GFP-Apoptin deletion mutants were constructed by PCR amplification of Apoptin sub-sequences, which were directionally cloned into the vector pEGFP-C1 (Clontech). GFP-Ap-pmNES and -pmNLS were generated by PCR site-directed mutagenesis. GFP-Ap-SV40NLS was made by PCR amplification of Apoptin amino acids 1-88 with simultaneous addition of SV40 LT NLS sequence (PKKKRKV) to the C-terminus followed by cloning into pEGFP-C1. DsRed-Apoptin fusions were
generated by PCR amplification of GFP-Apoptin templates followed by cloning into pDsRed1-N1 (Clontech, Palo Alto, CA). For the dsRed-Ap(82-121) construct, a start codon was added because the endogenous Apoptin 5’ sequence had been removed. GFP-Ap-RevNES was generated by replacing the wild type Apoptin NES sequence with an oligonucleotide linker containing the HIV-1 Rev NES. To generate the dsRed-dnRan fusion, a construct expressing dnRan (Dr. Lan Xu, University of Massachusetts Medical School, Worcester, MA) was PCR amplified and cloned in-frame into pDsRed1-N1. All constructs were confirmed by restriction digest analysis and DNA sequencing.

**Immunofluorescence and Fluorescence Microscopy.** Ad-Apwt-infected PFF and HA1-IM cells were fixed in 4% paraformaldehyde (in PBS), permeabilized in 0.5% TritonX-100 (in PBS) and stained with α-Flag M5 primary mAb (Sigma) followed by α-mouse Ig Texas Red-conjugated secondary Ab (Sigma).

For fluorescence microscopy, H1299 cells were transiently transected using Effectene reagent (Qiagen) and PFF transfections were performed using an Amaxa Nucleofector (Amaxa Biosystems). After 24 h, cells were fixed in 4% paraformaldehyde and stained with DAPI. Cells were mounted on slides and observed using a Zeiss Axiophot2 fluorescence microscope using Axiovision 4.2 software.

**Nucleo-cytoplasmic Shuttling Assays.** ~5 x 10⁵ PFF cells were transiently transfected with GFP-Apwt, and 24 h later treated with leptomycin B (LMB) at a final concentration
of 2.5 ng/ml. ~5 x 10^5 H1299 cells were transfected with GFP-Apwt or Rev-GFP, and 12 h later transfected with a construct expressing dsRed-dnRan.

**Heterokaryon Assays.** ~5 x 10^5 H1299 or PFF cells were transiently transfected with dsRed-Apwt or GFP-Apwt, respectively, by Amaxa nucleofection. Immediately following transfection, cells were either mixed or plated separately (for controls) in 6 well format on cover slips and left to recover for 12 h. Media was then removed and cells were washed twice in PBS and fused by exposure to a solution of 50% PEG 1000 in serum-free DMEM for 125 s at room temperature. PEG solution was then removed by washing with PBS and fresh medium was added. After overnight recovery, cells were fixed in 4% paraformaldehyde (in PBS) and stained with DAPI, and Apoptin localization was determined by fluorescence microscopy.

**Immunoprecipitations and Immunoblotting.** For multimerization experiments, ~1 x 10^7 H1299 cells were transiently transfected with GFP-Apoptin truncation mutants using Effectene. After 12 h cells were infected with Ad-Apwt and incubated for 24 h. Cells were harvested and lysed in Buffer X (50 mM Tris, pH 8.5, 250 mM NaCl, 1 mM EDTA, 1% NP40, Complete Mini tablet (Roche)) on ice for 20 min. Cell debris was removed by centrifugation and supernatants were incubated with 30 ml Ezview Red α-Flag M2 affinity beads (Sigma) at 4°C for 4 h. Beads were then washed in Buffer X and bound proteins were eluted by boiling in SDS sample buffer. For solubility analysis, ~1 x 10^7
H1299 or PFF cells were infected with Ad-Apwt followed by incubation for 48 h. Cells were then harvested and immunoprecipitated as described above.

For immunoblotting, samples were resolved by 15% SDS-PAGE and transferred to nitrocellulose. Blots were blocked with 5% milk in TBS-T and probed with either α-Flag M2 mAb (Sigma) or α-GFP mAb (Clontech) followed by appropriate HRP-conjugated α-Ig secondary Ab (Amersham Biosciences). Protein bands were visualized by chemiluminescence using SuperSignal Substrate (Pierce).
Results

Apoptin is a Nucleo-cytoplasmic Shuttling Protein. To optimize expression of Apoptin for proteomic studies, an adenovirus expressing FLAG-tagged Apoptin (Ad-Apwt) was constructed. Apoptin expression was confirmed by immunoblot analysis (see Chapter III). Previous studies have shown that Apoptin is nuclear localized in transformed cells and cytoplasmic in untransformed or primary cells (37). To determine the intracellular localization of Flag-tagged Apoptin, immunocytochemistry was performed using an anti-FLAG monoclonal antibody. Figure 2.1a shows that in primary foreskin fibroblasts (PFFs) Apoptin stained with a characteristic cytoskeletal-like pattern with nuclear exclusion, whereas in non-small cell lung carcinoma H1299 cells the protein was completely nuclear. Thus, FLAG-tagged Apoptin exhibits the characteristic differential localization in transformed and primary cells described previously (37). These results also suggest that the protein may be associated with cytoskeletal components or may have a filamentous structure in primary cells.

The ability of the CAV protein Apoptin to localize to either the nucleus or cytoplasm depending on cell type suggested the protein may undergo nucleo-cytoplasmic shuttling. To determine whether Apoptin shuttles in primary cells, primary foreskin fibroblasts (PFFs) were infected with Ad-Apwt virus and 24 hours later were treated with leptomycin B (LMB), a compound that specifically blocks Crm1-mediated nuclear export. If Apoptin shuttles in primary cells, then blocking nuclear export should result in the accumulation of Apoptin in the nucleus. Figure 2.1b shows that LMB treatment results in nuclear accumulation of Apoptin in PFF cells indicating that Crm1-dependent
nucleo-cytoplasmic shuttling is taking place. Interestingly, not all Apoptin protein was engaged in shuttling and much remained cytoplasmic and aggregated suggesting that this form of Apoptin may be stable or tightly associated with filamentous structures. For subsequent experiments, a plasmid was constructed with Apoptin fused to the C-terminus of green fluorescent protein (GFP-Apwt). To confirm shuttling behavior with this construct, PFF cells were transiently transfected with GFP-Apwt and the cells were treated with LMB as before. Figure 2.1c shows that LMB treatment also resulted in nuclear accumulation of GFP-Apwt in PFFs. A larger shuttling pool of Apoptin was found with GFP-Apwt following LMB treatment suggesting fusion of Apoptin with GFP at the N-terminus may have some effect on cytoplasmic retention in PFF cells.

To test whether Apoptin also shuttles in transformed cells, an assay was developed to monitor nucleo-cytoplasmic shuttling of a predominantly nuclear protein. A dominant-negative Ran GTPase mutant (dnRan), which blocks Ran-dependent nuclear import (92), was expressed H1299 cells. To validate this approach, a control experiment was performed monitoring the effect of dnRan on localization of HIV Rev, a well-characterized nucleo-cytoplasmic shuttling protein (87, 114, 115). Figure 2.1d shows, as expected, that a dsRed-dnRan fusion-protein localized to the nuclear periphery (87, 115) and that a Rev-GFP fusion-protein accumulated in the nucleolus (171). Co-expression of dsRed-dnRan and Rev-GFP resulted in loss of nuclear GFP signal, confirming that Rev-GFP exited the nucleus and was blocked for subsequent re-entry. Similarly, expression of dsRed-dnRan resulted in loss of nuclear GFP-Apwt, indicating that Apoptin shuttled in transformed cells. Collectively, the data of Figures 2.1.b-d indicate that Apoptin
localization is regulated by nucleo-cytoplasmic shuttling in both primary and transformed cells, and that nuclear import and export are mediated by common cellular trafficking machinery.

**Apoptin nuclear localization is mediated by a dominant transformed cell-specific activity.** To determine whether the transformed or primary cell contained a dominant Apoptin localization activity, a heterokaryon experiment was performed. PFF cells were transiently transfected with a plasmid expressing GFP-Apwt, and H1299 cells were transiently transfected with a plasmid expressing Apoptin fused to the C-terminus of dsRed (dsRed-Apwt). Twelve hours later cells were combined, fused with polyethylene glycol, and allowed to recover overnight. Figure 2.2a shows that when either PFF/GFP-Apwt or H1299/dsRed-Apwt cells were self-fused, the expected localization patterns were observed. However, fusion of PFF/GFP-Apwt cells with H1299/dsRed-Apwt cells resulted in the translocation of PFF-derived GFP-Apwt to the nucleus where it co-localized with dsRed-Apwt. These results indicate that transformed cells contain a dominant activity that confers Apoptin nuclear localization.

Localization of Apoptin was also monitored in HA-1 cells, a clonal population of human embryonic kidney cells transformed with SV40 early region DNA that bypass senescence and enter crisis (33). HA-1 cells were infected with Ad-Apwt and localization was monitored both before (passage 44) and after (passage 94) crisis. Figure 2.2b shows that Apoptin exhibited a filamentous, cytoskeletal-like cytoplasmic staining pattern in pre-crisis cells, whereas in post-crisis cells Apoptin localized predominantly to
the nucleus. Thus, the dominant activity in transformed cells that directs Apoptin to the nucleus appears early during the transformation process.

**Biochemical properties of Apoptin differ between primary and transformed cells.**

Others have previously reported that Apoptin forms large aggregates in primary cells (37). One hypothesis is that this aggregation may reflect a fundamental difference in the biochemical state of Apoptin in primary versus transformed cells. The immunoprecipitation experiment of Figure 2.2c shows that Apoptin was highly resistant to extraction from PFFs compared to H1299 cells in low ionic strength buffer. In fact, in primary cells the majority of Apoptin was not solubilized even under high ionic strength conditions (data not shown). Based upon the filamentous, cytoplasmic staining pattern observed in primary cells (see Figures 2.1a and 2.2b), Apoptin may be tightly associated with the cytoskeleton, which could explain the difference in solubility between primary and transformed cells. It is also possible that this solubility difference is related to Apoptin sub-cellular localization. For example, Apoptin may be generally insoluble in the cytoplasm but soluble in the nucleus. To test this possibility, PFF cells infected with Ad-Apwt were treated with LMB and subjected to immunoprecipitation as before. Figure 2.2d shows that LMB treatment had no effect on the degree of extraction indicating that forcing Apoptin to the nucleus of a primary cell is not sufficient to increase solubility.
**Functional characterization of Apoptin NES and NLS sequences.** Experiments were performed to characterize the specific sequence elements that contribute to nucleo-cytoplasmic shuttling. Previous studies have noted a putative NES comprising amino acids 37-46 in the N-terminus and a putative NLS comprising residues 70-121 in the C-terminus (shown in Figure 1.8b, Chapter I) (42, 176). To determine whether the putative N-terminal sequence was a functional NES, a mutant was constructed in which the core residues leucine-44 and leucine-46 were mutated to alanine (GFP-Ap-pmNES). Figure 2.3 shows that in PFFs GFP-Ap-pmNES mislocalized to the nucleus, indicating that the putative Apoptin NES is functional and that the NES activity of Apoptin is required for cytoplasmic accumulation in primary cells. The putative bipartite NLS contains two domains highly enriched in lysine and arginine (residues 86-88 and 116-118). An NLS mutant was constructed in which both trios of basic amino acids were mutated to alanine (Ap-pmNLS). This mutant mislocalized to the cytoplasm in H1299 cells, indicating the NLS is required for nuclear localization. Thus, Apoptin contains both a functional NES and NLS, consistent with the nucleo-cytoplasmic shuttling activity described above.

**Apoptin fragments containing either the NES or NLS fail to undergo cell type-specific localization.** The simplest explanation for the differential localization of Apoptin in primary and transformed cells is that one of the localization signals is subject to cell type-specific regulation. For example, the NES might be active in primary and inactive in transformed cells. To test this model the localization of GFP-fused Apoptin fragments containing either the NLS or NES were analyzed in primary and transformed cells.
cells. Figure 2.4 shows that GFP-Ap(42-88), which lacks both the NLS and NES, displayed a diffuse homogeneous localization pattern in both H1299 and PFF cells, similar to that of GFP alone. As expected, the GFP-Ap(1-48) and GFP-Ap(1-88) mutants, which contain the N-terminal NES but lack the C-terminal NLS, displayed a predominantly cytoplasmic localization pattern in PFFs. However, both mutants also localized to the cytoplasm in H1299 cells, indicating the NES is active in transformed cells. Conversely, the GFP-Ap(42-121) and GFP-Ap(82-121) mutants, which contain the C-terminal NLS but lack the N-terminal NES, localized to the nucleus in H1299 cells as well as in PFFs, indicating the NLS is active in primary cells. Thus, the NLS and NES are active in both primary and transformed cells. These results suggest that when uncoupled the NES and NLS function constitutively.

**N-terminal NES confers LMB sensitivity.** Next, experiments were performed to determine whether the elucidated export sequence at the N-terminus of Apoptin was responsible for the Leptomycin B sensitivity observed in Figure 2.1. To test this possibility, the Ap(1-48) and Ap(1-88) derivatives, which are constitutively cytoplasmic and lack any C-terminal localization sequences, were treated with LMB. Figure 2.5a shows that upon LMB treatment, GFP signal is dramatically increased in the nucleus compared to untreated control cells (quantifications are shown in Figure 2.5b). These results indicate that the export sequence contained within this region is a functional Crm1-dependent NES and can confer the export capability observed with Apoptin. To confirm that the NES sequence contained within this domain was required for shuttling
activity, the Ap-pmNES GFP mutant was tested for shuttling activity using the dnRan experiment (as in Figure 2.1d). Figure 2.5c shows that the GFP signal remains in the nucleus following introduction of dsRed-dnRan indicating that this mutant is defective for shuttling activity. Together, these results indicate that the function of the N-terminal NES sequence is required for Apoptin shuttling activity by virtue of the Crm-1 nuclear export receptor.

**Trans restoration of Apoptin cell type-specific localization through protein multimerization.** Previous studies have shown that recombinant Apoptin aggregates *in vitro* (102), raising the possibility that Apoptin functions as a multimer *in vivo*. As a first test of this possibility it was determined if cell type-specific localization could be restored in *trans* by co-expression of two Apoptin fragments, one containing the NLS and the other containing the NES. Figure 2.6a shows, as expected, that GFP-Ap-pmNLS, which lacks a functional NLS, localized to the cytoplasm of both H1299 cells and PFFs, whereas dsRed-Ap-pmNES, which lacks a functional NES, localized to the nucleus in both H1299 cells and PFFs. However, co-expression of GFP-Ap-pmNLS and dsRed-Ap-pmNES resulted in the localization of both proteins to the nucleus of H1299 cells and the cytoplasm of PFFs. These results suggest that Apoptin is a multimer *in vivo* and confirm that both localization signals are required for proper cell type-specific localization.

To confirm that Apoptin is a multimer *in vivo* and to map the multimerization domain a series of co-immunoprecipitation experiments were performed. H1299 cells were transfected with a series of GFP-fused Apoptin derivatives and 12 hours later
infected with Flag-tagged Ad-Apwt. Twenty-four hours after infection, Flag-Apoptin was immunoprecipitated and the immunoprecipitate analyzed by immunoblotting with an anti-GFP antibody. Figure 2.6b shows that only derivatives containing the N-terminal third of Apoptin [GFP-Apwt, GFP-Ap(1-48), GFP-Ap(1-88)] co-immunoprecipitated with the Flag-Apoptin, indicating that this region mediates protein multimerization.

To confirm the results of the co-immunoprecipitation experiments and to verify that multimerization is the basis for restoration of cell type-specific localization in trans, three additional Apoptin derivatives were analyzed in the trans-expression assay. Figure 2.6c shows that expression of dsRed-Apwt restored nuclear localization to the constitutively cytoplasmic GFP-Ap(1-88) mutant in transformed cells and maintained cytoplasmic localization in primary cells. By contrast, dsRed-Ap(82-121) failed to alter cytoplasmic localization of GFP-Ap(1-88) in transformed cells, indicating that the N-terminal multimerization domain is required for trans-association of the localization signals. These results show that the activity of a constitutively cytoplasmic mutant can be restored by expressing an NLS-containing C-terminal fragment in trans.

The specific Apoptin NES is required for cell type-specific localization. To determine if the specific Apoptin NES and NLS sequences were required for cell type-specific localization, heterologous localization signals were functionally substituted for Apoptin sequences. Figure 2.7a shows that replacement of the Apoptin NLS with that of SV40 large T antigen (Ap-SV40NLS) resulted in nuclear localization in H1299 cells and predominantly cytoplasmic localization in PFFs. Thus, the SV40 large T antigen NLS
can functionally substitute for the Apoptin NLS and the specific Apoptin sub-sequence is not critical for regulated localization behavior. By contrast, replacement of the Apoptin NES with that of Rev (Ap-RevNES), a well established, prototypical NES (75, 115, 187), resulted in a similar localization pattern in both H1299 and PFFs, indicating that the specific sequence of the Apoptin NES is critical for proper cell type-specific localization.

**Apoptin NES and multimerization domains overlap.** One explanation for the failure of the Rev NES to functionally substitute for the Apoptin NES is that the Apoptin NES provides an activity in addition to nuclear export. Because both the NES and multimerization domain map to the N-terminus, it was reasoned that the NES might be an essential part of the multimerization domain. To test this possibility, the two NES derivatives, Ap-pmNES and Ap-RevNES, were analyzed for their ability to multimerize in the co-immunoprecipitation assay. Figure 2.7b shows that compared to GFP-Apwt, the GFP-Ap-pmNES mutant showed a comparable (albeit slightly reduced) ability to interact with Flag-Apoptin. Significantly, GFP-Ap-RevNES, which contains a functional NES that differs at multiple residues from the Apoptin NES, failed to detectably co-immunoprecipitate with Flag-Apoptin. Interestingly, the intermediate level of multimerization observed with the Ap-pmNES mutant was sufficient to confer function in the *trans* assay; however, close inspection of Figure 2.6 (and data not shown) revealed that Ap-pmNES was less effective than wild type Apoptin in mediating *trans* localization, which presumably reflected the decreased multimerization efficiency.
Together, these results suggest that the Apoptin multimerization domain overlaps with the NES.
Discussion

In this report, it was shown that Apoptin is a nucleo-cytoplasmic shuttling protein whose localization is mediated by an N-terminal CRM1-dependent nuclear export signal (NES) and a C-terminal bi-partite nuclear localization signal (NLS). Interestingly, these domains impart Apoptin with particular sub-cellular trafficking behavior, as well as the biochemical properties and protein interactions that provide the basis for cell type-specific localization. Based upon the results presented here, a working model for how Apoptin localization is regulated between primary and transformed cells is discussed.

In primary cells, Apoptin forms large insoluble aggregates mediated by N-terminal multimerization activity providing a basis for cytoplasmic retention. It was found that although Apoptin shuttles in such cell types, this shuttling is restricted to a small soluble pool of Apoptin proteins most likely in equilibrium with the vast insoluble and sequestered population (Figure 2.1b). Activities present in transformed cells induce steady state levels of Apoptin to increase in the nucleus where a smaller, more soluble multimeric form can exist. In this case the majority of Apoptin is mobile and engaged in nucleo-cytoplasmic shuttling as well as events that will lead to the induction of apoptosis (Figure 2.1d).

Based on the experiments of Figures 2.4 and 2.6, it is apparent that Apoptin cell type-specific localization behavior is dependent on the presence of both localization domains. When uncoupled, each localization sequence is constitutively active suggesting that interplay between the two establishes different localization patterns depending on cell type-specific conditions. Moreover, the shuttling experiments of Figure 2.1 suggest
that changes in Apoptin biochemistry in transformed cells shift the equilibrium of shuttling toward higher steady-state levels in the nucleus. It is likely that changes in Apoptin conformation dictate which localization signal has greater effect: the NES region being dominant in primary cell types and the NLS region being dominant in transformed cells. This would explain the necessity for both localization domains to establish cell type-specific localization, as alternate regions of the protein would be needed for inhibition of the opposing sequence. Consistent with this model, recent studies have demonstrated that Apoptin localization sequence masking may occur at the C-terminus and that an NLS dominant form exists in transformed cells, whereas this sequence is less accessible in primary cell types (177).

Since CAV is often found to associate with other viruses that encode dominant oncogenes, including Marek’s Disease Virus and Avian Adenovirus (116, 167, 192), it is understandable that Apoptin has evolved a mechanism that senses transformation. The equilibrium dynamics of Apoptin nucleo-cytoplasmic shuttling provides a powerful mechanism to induce rapid changes in protein localization with minimal changes in cellular activities. Such rapid changes in protein localization and induction of apoptosis may be important for the timing of CAV life cycle and viral egress. Overlapping functional domains is a common theme among many viruses with compact genomes. The evolution of NES and NLS sequences that overlap with protein interaction domains is therefore not surprising and provides a means by which localization can be elegantly regulated by direct interaction. The existence of such a mechanism has been implicated in the regulation of at least one other viral protein, HIV-1 Rev. Like Apoptin, Rev
contains both an NES and NLS and shuttles between the nucleus and cytoplasm (114). The Rev NES is bound by the nuclear kinesin-like protein REBP (Rex/Rev effector binding protein) that, in turn, stimulates nuclear export by the CRM1 receptor (172). The Rev NLS overlaps with an RNA binding domain that contacts the RRE (Rev responsive element) RNA stem loop structure within the HIV-1 genome resulting in localization change (35). Interestingly, Rev NLS activity is sensitive to the multimerization state of the protein (36).

The multimerization state of Apoptin appears to vary in degree between cell types and may affect Apoptin’s localization and apoptotic activities. A higher state of multimerization in primary cells could explain the aggregation, insolubility and retention of Apoptin in the cytoplasm. It has proven difficult to isolate mutations that target multimerization without affecting export suggesting that these two activities may be very tightly coupled. For this reason it is unclear whether multimerization activity is required for Apoptin localization and activities.

The nature of the cell type-specific modification remains unclear. However, one suggestion has been that Apoptin is regulated through phosphorylation by a transformed cell-specific kinase. Previous studies have reported that Apoptin is differentially phosphorylated in transformed and primary cells on threonine-108 (151), which is located between the two halves of the bipartite NLS (see Appendix). Additionally, it has been suggested that an alternate, non-canonical export sequence may exist in this area and that phosphorylation at T108 could modulate Apoptin localization (140). By contrast, Figure 2.7 indicates that the C-terminal region containing the T108 phosphorylation site and
proposed alternate export region are dispensable for regulated localization: Apoptin derivatives in which this domain is substituted by heterologous sequences that lack this phosphorylation and putative export site localize normally. These data are consistent with other studies that show the Apoptin NLS region does not confer the transformed cell-specific localization observed with the wild type protein (176). Contrary to Wadia et al. however, no evidence was found that the cellular concentration of Apoptin has any effect on localization as overexpression studies by plasmid vector or adenovirus retain normal cell type specific localization. Instead, the results in this report clearly demonstrate that Apoptin’s regulated localization is solely dependent on the presence the N-terminal multimerization and export domain, which is LMB sensitive, and functional to confer the export properties observed for Apoptin. Derivatives lacking this region have neither the ability to undergo cell type-specific localization nor shuttling activity (Figures 2.5 and 2.7). This is in contrast to previous studies that report the N-terminal NES to be non-functional or to enhance nuclear entry (140).

Transformed cell-specific activities other than that of a kinase can be envisioned and have yet to be explored. For example, recent studies have found that the second of three CAV proteins, VP2, functions as a dual specificity protein phosphatase, the function of which is essential for CAV proliferation (135, 136). Cell type-specific regulation of exogenously expressed Apoptin may involve de-phosphorylation of key residues by a surrogate cellular phosphatase. Several putative Apoptin phosphorylation sites are adjacent to proline residues and are thus potential targets for peptidyl-prolyl isomerase activity as well. A transformed cell-specific isomerization event could result
in a conformational change, which may establish a form of Apoptin competent for
induction of apoptosis. Alternatively, Apoptin may be acted upon by a chaperone, which
could also result in a conformational change as well. Notably, increased chaperone
activity has been reported in cancer cells (58). Consistent with this idea, Apoptin has
been reported to be activated by the N-terminal J domain of SV40 large T antigen (193),
which binds to and stimulates activity of Hsp-70 family members (46). Identification of
the critical Apoptin modifying activity is expected to provide new insights into common
properties of transformed cells.
Figure 2.1. Apoptin shuttles between the nucleus and cytoplasm in a Crm1-dependent manner. (A) Immunofluorescence of PFF and H1299 cells 24 h post-infection with Ad-Apwt. Cells were stained with an α-FLAG antibody or DAPI (magnification 1000X). (B) PFF cells are infected with Ad-wtAp and 24 hours later are treated or not treated with leptomycin B (LMB) for 3 hours. Cells were fixed and Apoptin localization was monitored by immunofluorescence using α-Flag antibody. (C) PFF cells expressing GFP-Apwt were treated in the presence or absence of LMB. 3 h later, Apoptin localization was visualized by fluorescence microscopy for GFP, and nuclei were visualized by DAPI staining. (D) H1299 cells were transfected with a construct expressing dsRed-dnRan either alone (top panel) or 12 h following transfection with GFP-Apwt (bottom panel). As a control, H1299 cells were also transfected with either a GFP Rev-GFP fusion alone, Rev-GFP plus dsRed-dnRan, or GFP-Apwt (middle panels). 12 h following transfection, localization was monitored by fluorescence microscopy for dsRed or GFP and by DAPI staining.
Figure 2.1

A

B

C

D

PFF

H1299

Flag-wtAp

dsRed-dnRan

Rev-GFP

GFP-Apwt

dsRed-dnRan + Rev-GFP

GFP-Apwt

dsRed-dnRan + GFP-Apwt

Flag-wtAp

PFF

-LMB

+LMB

GFP

GFP + DAPI

Rev-GFP

dsRed-dnRan

dsRed-dnRan + GFP-Apwt
Figure 2.2. Apoptin nuclear localization is mediated by a dominant transformed cell-specific activity. (A) Heterokaryon assay. PFFs expressing GFP-Apwt and H1299 cells expressing dsRed-Apwt were either mixed or plated separately, incubated for 12 h, and subjected to polyethylene glycol-induced fusion. (B) Immunofluorescence of HA-1 cells infected with an adenovirus expressing Flag-tagged Apoptin (Ad-Apwt) pre-crisis (passage 44; upper panels) and post crisis (passage 94; lower panels). Cells were stained with an α-Flag antibody or DAPI. (C) The solubility of Apoptin in PFF and H1299 cell extracts was monitored by immunoprecipitation using an α-Flag affinity resin, and the presence of Apoptin in the immunoprecipitate (IP) (top) and whole cell extract (WCE) (bottom) was monitored by immunoblot analysis. (D) The solubility of Apoptin was monitored as above in PFF cells following either treatment or no treatment with LMB for 3 hours.
Figure 2.2

A. Heterokaryon Assay

B. HA-1 Cells

C. PFF/GFP-Apwt + H1299/dsRed-Apwt

D. HA-1 Cells

LMB - +

Flag IP WCE α-Flag
Figure 2.3. Apoptin contains functional NES and NLS sequences. (Top) Schematic diagrams of N-terminal GFP-tagged Apoptin NES and NLS mutants. (Bottom) H1299 and PFF cells expressing GFP-Apoptin mutants were monitored by fluorescence microscopy for GFP and DAPI staining.
Figure 2.3

![Diagram of GFP-tagged proteins and their localization in H1299 and PFF cells]
Figure 2.4. Apoptin fragments containing either the NES or NLS fail to undergo cell type-specific localization. (Top) Schematic diagrams of GFP-Apoptin deletion mutant derivatives. (Bottom) Fluorescence microscopy of GFP and DAPI signals in H1299 and PFFs expressing GFP-Apoptin derivatives.
Figure 2.4

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<td>GFP-Ap(82-121)</td>
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Figure 2.5 Apoptin NES sequence within the N-terminus confers LMB sensitivity and is required for nucleo-cytoplasmic shuttling activity. (A) H1299 cells expressing GFP-Ap(1-48) or GFP-Ap(1-88) were treated with LMB or left untreated. 3 h later, Apoptin localization was visualized by fluorescence microscopy for GFP, and nuclei were visualized by DAPI staining. (B) Quantitation of the nuclear/cytoplasmic GFP signal ratio in the presence and absence of LMB. (C) DnRan shuttling assay, performed as described in Fig. 2.1D using GFP-Ap-pmNES mutant.
Figure 2.5

A

H1299

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B

Signal (nuc/Cyto)

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C

H1299

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Figure 2.6. Apoptin localization signals are modular and can cooperate in trans to confer cell type-specific localization. (A) H1299 and PFF cells expressing GFP-Ap-pmNLS, dsRed-Ap-pmNES or both were monitored by fluorescence microscopy. (B) H1299 cells were transfected with GFP-Apoptin mutants and 12 h later infected with Ad-Apwt. Apoptin multimerization was monitored by immunoprecipitation of Flag-tagged Apoptin using α-Flag affinity resin followed by analysis of the immunoprecipitate (IP) for GFP-Apoptin by immunoblotting with an α-GFP antibody. Expression of the GFP-Apoptin mutants was also monitored in WCE. (C) H1299 and PFF cells were co-transfected with GFP-Ap(1-88) and either dsRed-Apwt, dsRed-Ap-pmNES or dsRed-Ap(82-121) and monitored by fluorescence microscopy.
Figure 2.6

A

GFP-Ap-pmNLS

dsRed-Ap-pmNES

H1299

PFF

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B

\[\alpha-\text{FLAG IP} \alpha-\text{GFP immunoblot} \]

WCE \(\alpha\)-GFP

C

GFP-Ap(1-88)

dsRed-Apwt

dsRed-Ap(82-121)

dsRed-Ap-pmNES

H1299

PFF

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Figure 2.7. An overlapping Apoptin NES/multimerization domain is required for cell type-specific localization. (A) Fluorescence microscopy of H1299 and PFFs expressing GFP-Apoptin derivatives in which either the NLS was replaced by the SV40 NLS (top) or the NES was replaced by the HIV-1 Rev NES (bottom). (B) Apoptin multimerization was monitored as described in Figure 2.6b.
Figure 2.7

A

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GFP-Ap-SV40NLS
GFP-Ap-RevNES

GFP-Apwt GFP-Ap-RevNES GFP-Ap-pmNES

WCE

Enumerable tags:
- GFP
- GFP + DAPI
- Ap-SV40NLS
- Ap-RevNES
- Apoptin NES
- Apoptin NLS
- HIV-1 Rev NES
- SV40-LT NLS
- P-S-K-R...K-R-R
- L-P-P-L-E-R-L-T-L-D

B

Enumerable tags:
- GFP-Apwt
- GFP-Ap-pmNES
- GFP-Ap-RevNES
- α-FLAG IP
- α-GFP immunoblot
- WCE α-GFP
CHAPTER III
CAV APOPTIN ASSOCIATES WITH THE ANAPHASE-PROMOTING
COMPLEX/CYCLOSUME TO INDUCE G2/M CELL CYCLE ARREST AND
APOPTOSIS IN THE ABSENCE OF P53
Abstract

The majority of human cancers lack the tumor suppressor p53, rendering them refractory to conventional chemotherapeutic agents. It is therefore critical to identify p53-independent apoptotic pathways. The Chicken Anemia Virus Apoptin protein induces apoptosis in transformed cells in the absence of p53 by a mechanism that remains to be elucidated. Here using affinity-purification and mass spectroscopy it was found that in vivo, Apoptin is associated with APC1, a subunit of the anaphase-promoting complex/cyclosome (APC/C). The APC/C is required to establish a mitotic cell-cycle checkpoint, and its inhibition results in G2/M arrest and apoptosis. Expression of wild type Apoptin in transformed cells inhibits APC/C function and induces G2/M arrest and apoptosis, whereas Apoptin mutants that are unable to associate with APC1 have no effect. In p53 null cells, ablation of APC1 by RNA interference induces a G2/M arrest and apoptosis analogous to that observed following Apoptin expression. Furthermore, Apoptin was found to induce the formation of PML bodies and to recruit APC/C subunits to these nuclear structures. Thus, these results explain the ability of Apoptin to induce apoptosis in the absence of p53 and suggest a mechanism involving sequestration and inhibition of the APC/C. This study is consistent with previous reports that mechanisms of apoptosis involving G2/M arrest and APC/C modulation lead to p53-independent apoptosis, and suggests that the APC/C may be an attractive target for the development of anti-cancer drugs.
**Introduction**

Apoptosis is a physiological form of cell death that is required during normal development and plays a key role in controlling disease by mediating the elimination of cancerous or virus-infected cells. Many animal viruses have been found to regulate apoptosis (reviewed in (152, 166)). Inhibition of apoptosis can maximize viral replication efficiency and help evade an immune response. Conversely, induction of apoptosis near the end of virus replication facilitates viral egress.

Development of novel and effective cancer therapies depends upon the discovery of agents that selectively destroy tumor cells while leaving normal cells intact. Several viruses have been demonstrated to have such selective intrinsic oncolytic activity or have been engineered to become oncolytic (reviewed in (90, 91)). The Chicken Anemia Virus (CAV) Apoptin protein is a 13.6 kDa protein that can induce apoptosis in a variety of human malignant cell lines (196). Two properties of Apoptin-induced cell death are particularly intriguing: first, Apoptin does not induce apoptosis in normal (untransformed) cells; and second, Apoptin-induced cell death is not dependent upon the p53 tumor suppressor (37, 38). Thus, Apoptin represents a potential agent for the treatment of tumors that have lost their p53 status and are therefore refractory to many cancer therapies. Apoptin has shown efficacy in treating human xenografted tumors in mice and is currently being evaluated as a gene therapy agent to selectively destroy cancer cells (169).

The molecular mechanism by which Apoptin induces apoptosis is largely unknown. Several studies have demonstrated that nuclear localization of Apoptin is
required for induction of apoptosis (42, 67), suggesting the cellular target of Apoptin is a nuclear protein. In this study, *in vivo* Apoptin is shown to be associated with subunit 1 of the anaphase-promoting complex/cyclosome (APC/C).

This study examines the consequences of an Apoptin interaction with the APC/C. Functional analysis reveals that Apoptin expression results in inhibition of APC/C activity specifically in transformed cells. This inhibitory action results in G2/M cell cycle arrest and induction of p53-independent apoptosis. These data reveal a mechanism for Apoptin p53-independent apoptosis and suggest inhibition of the APC/C as a novel potential target area for the development of anti-cancer drugs.
Materials and methods

Cells and adenoviruses. Saos-2, H1299, and primary foreskin fibroblast cells (PFFs) were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum plus 10 mg/ml streptomycin and 10 U/ml penicillin (Sigma) at 37°C under 5% CO2 (95% air).

Apoptin adenovirus (Ad-Apwt), Ad-Ap-pmNES, and LacZ adenovirus (Ad-LacZ) were prepared as previously described (Chapter II). All cells were infected at approximately 80% confluence at an MOI of 35. Adenovirus was added to cells in a minimal volume of culture media and gently agitated at 37°C under 5% CO2 for 1 h. Following infection, culture media was added and cells were incubated for indicated times.

Plasmid constructions. Flag-Apoptin deletion constructs were sub-cloned from the GFP-Apoptin deletion panel (previously described in Chapter II) via EcoRI and BamHI and directionally cloned into the p3XFLAG-myc-CMV-26 vector (Sigma) to construct an in-frame N-terminal fusion to the FLAG epitope. Stop sequences were generated to exclude the C-terminal myc tag. Ap-pmNLS was constructed as previously described (Chapter II). Ap-pmNLS2 constructs were generated by site-directed mutagenesis of indicated sequences followed by directional cloning via EcoRI and BamHI into both the pEGFP-C1 (Sigma) or p3XFLAG-myc-CMV-26 vectors for N-terminal fusions. CFP-PML was a kind gift from Dr. Claude Gazin (UMass Medical School). Constructs were verified by restriction digest analysis and DNA sequencing.
**Immunofluorescence.** H1299 cells were transiently transfected using Effectene reagent (Qiagen) and after 16 hr, fixed in 4% paraformaldehyde and stained with either α-Cdc27 mAb (Santa Cruz) or α-PML mAb (Santa Cruz) followed by Cy3 conjugated α-mouse 2 Ab. Cells were mounted on slides and observed using a Ziess Axio phot2 fluorescence microscope using Axiovision 4.2 software.

**Immunoprecipitations.** For affinity purification, ~8 x 10^7 cells were infected with FLAG-tagged Ad-Apwt. After 24 h, cells were washed in PBS, harvested by scraping, and lysed in Buffer X [50 mM Tris, pH 8.5, 250 mM NaCl, 1 mM EDTA, 1% NP40, Complete Mini tablet (Roche)] on ice for 20 min. Following centrifugation, lysates were pre-cleared with Protein A/G-agarose for 1 h followed by incubation with FLAG-agarose at 4°C for 4 h. Beads were washed in Buffer X and eluted using FLAG-peptide. Eluates were resuspended in 1X SDS sample buffer and boiled for 5 min. Proteins were resolved by SDS-PAGE and visualized by silver staining using the SilverQuest kit (Invitrogen). Bands were excised and submitted for MALDI-TOF mass spectrometry to the Proteomic Mass Spectrometry Lab at UMass Medical School.

For co-immunoprecipitations, ~2 x 10^7 cells were infected with Ad-Apwt or Ad-LacZ and harvested and lysed as described above. Following centrifugation, supernatants were incubated with 20 μl equilibrated EZview Red α-Flag M2 affinity beads (Sigma) at 4°C for 4 h. Beads were washed in Buffer X and bound proteins were eluted in 1X SDS sample buffer by boiling for 5 min.
For APC1 association domain mapping, \(~1 \times 10^7\) cells were transiently transfected with 3XFLAG-Apoptin truncation mutants using Effectene reagent. After 48 h, cells were processed as described for the co-immunoprecipitation experiments.

**Gel filtration chromatography.** H1299 cells (\(~2 \times 10^7\)) were either mock- or Ad-Apwt-infected, and after 24 h cells were washed in PBS, harvested by scraping, and lysed in Buffer A [20 mM Tris, pH 7.5, 100 mM NaCl, 20 mM β-glycerophosphate, 0.2% NP-40, 10% glycerol, 0.5 mM DTT, Complete Mini Tablet (Roche)] on ice for 30 min. Lysates were sonicated for \(~10\) sec, centrifuged for 1 h at 100,000g, and \(~500\) mg of total protein was injected into a Pharmacia FPLC apparatus and separated on a Superose 6 10/30 (Pharmacia) column. Fractions (500 μl) were collected and precipitated with 20% trichloroacetic acid (TCA) on ice for 1 h. Precipitates were then centrifuged at 13,000g for 15 min, washed with \(~20^\circ\)C acetone, dried, resuspended in 1X SDS sample buffer and boiled for 5 min.

**Immunoblotting.** Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were blocked with 5% milk in TBS-T and probed with either α-FLAG M2 monoclonal (Sigma), α-APC1 polyclonal (affinity purified a-peptide serum), α-Cdc27 monoclonal (Santa Cruz); α-cyclin B1 (Upstate Biotech); α-Plk (Zymed Laboratories, Inc.), α-cyclin E (Upstate Biotech); or α-tubulin (Sigma) antibodies followed by appropriate HRP-conjugated α-Ig secondary antibody (Amersham
Biosciences). Protein bands were visualized by chemiluminescence using SuperSignal Substrate (Pierce).

**Apoptosis and cell cycle assays.** To generate kill curves, Ad-Apwt- Ad-Ap-pmNES and Ad-LacZ-infected H1299, Saos-2 and PFF cells were harvested by trypsinization, washed in PBS, and monitored for cell viability by trypan blue exclusion. For additional cell viability assays, H1299 and PFF cells infected with Ad-Apwt, Ad-pmNES or Ad-LacZ were harvested, washed in PBS, and stained with ViaCount reagent (Guava Technologies), and viability was quantified using a Guava Personal flow cytometer. Data points were collected as percent cell viability per 5000 events. Apoptotic assays using GFP-Apoptin deletion mutants were performed by transfecting the deletion panel into H1299 cells, and 4 d later, cells were fixed with 4% paraformaldehyde and stained with DAPI. Percent apoptosis was scored as the percent of 100 GFP positive cells showing apoptotic morphology. Results were collected in a blind study by at least three individuals in two separate experiments. Data was graphed after subtraction of GFP background; all mutant samples are shown as percent apoptosis of wild type. For Annexin V analysis, cells were stained using the Annexin V-PE Apoptosis Detection Kit-I (BD Pharmingen) and analyzed using a Guava Personal flow cytometer (Guava Technologies, Inc.). Data points were collected as percent Annexin V positive cells per 5000 events.

For cell cycle analysis, cells were harvested by trypsinization, fixed and stained with propidium iodide, and analyzed by FACS. To establish cell synchrony, H1299 cells
were treated with 100nM nocodazole for 12 hours followed by mitotic shake-off.
Detached cells were collected, washed and re-plated without nocodazole. Procedure was repeated 3 times.

**RNA interference.** Double-stranded RNAs (ds-RNAs) were synthesized in vitro using a 500 bp PCR fragment as the template. Primer sequences, containing T7 priming sites, were as follows: APC1,

5'-GCGTAATACGACTCCTAGGGGAGAAAAGGAGTAAGTGAAATTGG-3' and 5'-GCGTAATACGACTCCTAGGGGAGAAAAGGAGTAAGTGAAATTGG-3';

Lamin A/C,

5'-GCGTAATACGACTCCTAGGGGAGAAAAGGAGTAAGTGAAATTGG-3' and 5'-GCGTAATACGACTCCTAGGGGAGAAAAGGAGTAAGTGAAATTGG-3'.  *In vitro* transcription was performed using the T7 Megascript Kit (Ambion), and the ds-RNA products were cleaved using the Dicer siRNA Generation Kit (Gene Therapy Systems, Inc.). The pool of diced-siRNAs was then transfected into cells using Oligofectamine (Invitrogen) as described previously (55).
Results

Apoptin induces apoptosis specifically in transformed cells in the absence of p53. To demonstrate Apoptin p53-independent and cell type-specific killing ability, both normal and p53-null transformed cells were infected with Ad-Apwt (Chapter II) and monitored over 4 days. Figure 3.1a shows that following infection with Ad-Apwt, p53 null SAOS2 osteosarcoma cells underwent pronounced apoptosis after 24 hours, and most cells were dead by 72 hours. Little or no death was observed in cells infected with a control adenovirus expressing LacZ (Ad-LacZ). Similar results were obtained in H1299 cells, a p53 negative non-small cell lung carcinoma cell line. By contrast, Apoptin expression in normal PFF cells had no apoptotic effect, as was the case with Ad-LacZ infected controls. Apoptin protein expression was confirmed by immunoblot assay using an α-FLAG monoclonal antibody (Figure 3.1b). Transformed cell death was concurrent with Apoptin expression.

Apoptin co-immunoprecipitates with subunit 1 of the anaphase-promoting complex.
To identify Apoptin-associated cellular proteins, extracts were prepared from Ad-Apwt infected H1299 cells and Apoptin was purified using α-FLAG affinity resin. Apoptin and associated proteins that bound to the affinity column were separated by SDS-PAGE and visualized by silver staining. MALDI-TOF mass spectroscopic analysis revealed that a major Apoptin-associated polypeptide was APC1, the largest subunit of the APC/C and an essential component of the mitotic checkpoint apparatus (Figure 3.2a). Apoptin also co-immunoprecipitated with HSP-70, which is known to associate with over-expressed
proteins, as well as α-tubulin, β-tubulin and β-actin, suggesting an association with filamentous networks, consistent with the immunocytochemistry results previously described (Figures 2.1a and 2.2b).

To confirm the Apoptin-APC1 association, Apoptin was immunoprecipitated from Ad-Apwt-infected H1299 cells with the α-FLAG antibody and the immunoprecipitate analyzed for APC1 by immunoblotting. Figure 3.2b shows that APC1 was present in the Apoptin immunoprecipitate but not in the immunoprecipitate of a control LacZ protein. By contrast, Figure 3.2c shows that APC1 was not present in the immunoprecipitate of PFF cells indicating that the Apoptin-APC1 association is specific to transformed cells.

**Apoptin expression in transformed cells induces G2/M cell-cycle arrest by inhibition of APC/C function.** The association of Apoptin with APC1 raised the possibility that Apoptin expression could affect cell-cycle progression. To address this issue, H1299 and PFF cells were infected with Ad-Apwt or Ad-LacZ and analyzed by fluorescence activated cell sorting (FACS). Figures 3.3a-b show that following infection with Ad-Apwt, H1299 cells began to accumulate in G2/M after 12 h, whereas the cell-cycle profile of PFF cells was unaffected. Although FACS analysis of Apoptin-expressing H1299 cells clearly showed a 4N DNA content, morphological examination did not reveal a classical mitotic-arrested appearance but rather a condensed nuclear morphology that is a hallmark of apoptosis. The onset of apoptosis was rapid, as evidenced by the appearance of a prominent sub-G1 peak concomitant with G2/M accumulation.
To determine whether cell cycle arrest is a prerequisite for induction of apoptosis, H1299 cells were synchronized by repeated rounds of mitotic shake-off and were infected with Ad-Apwt in parallel with asynchronous cells. After 24 hours, cells were harvested and analyzed for cell viability. Figure 3.3c shows that at 24 hours asynchronous H1299 cells underwent a marginal amount of apoptosis similar to that observed in Figure 3.1a. Synchronous cells, by contrast, showed a significant increase in the percent of apoptotic cells and underwent cell death simultaneously as a large population. These data suggest that Apoptin induced apoptosis is activated in response to the state of the cell cycle and additionally, that the G2/M arrest observed with Apoptin expression may be a prerequisite for cell death.

**Apoptin expression results in disruption of the APC/C.** To verify that Apoptin inhibited APC/C function, an independent assay was used to measure APC/C activity. APC/C catalyzes the ubiquitination of several substrates including cyclin-B1 and Polo-like kinase (Plk), whose degradation by the proteasome results in anaphase progression and mitotic exit (reviewed in (134)). If APC/C is inhibited, these substrates are not degraded and remain in the nucleus. Thus, stabilization of APC/C substrates is indicative of cyclosome dysfunction (189). To test whether Apoptin expression resulted in stabilization of APC/C substrates, H1299 cells were infected with Ad-Apwt, Ad-LacZ or mock-infected, and 24 hours later levels of mitotic APC/C substrates were determined by immunoblotting. As a positive control, cells were arrested in G2/M by nocodazole treatment. Figure 3.4a shows that cyclin-B1 was stabilized in Apoptin-infected and
nocodazole-treated cells compared to Ad-LacZ-infected cells. In contrast, protein levels of cyclin E, a G1 cyclin that is not an APC/C substrate, were slightly decreased by Apoptin expression or nocodazole treatment. RT-PCR analysis confirmed that Apoptin did not affect cyclin B1 or Plk mRNA levels (Figure 3.4b). These data suggest that Apoptin inhibits cyclosome function leading to G2/M arrest and apoptosis.

To gain insight into how Apoptin might inhibit APC/C activity, gel filtration analysis of the APC/C was performed in mock- or Ad-Apwt-infected H1299 cells. Fractions from the gel filtration column were analyzed by immunoblotting for APC1 or another APC/C subunit, Cdc27 (APC3). The results of Figure 3.4c reveal two dramatic effects of Apoptin expression on the APC/C. First, following Apoptin expression, both APC1 and Cdc27 eluted later consistent with lower native molecular weights, strongly suggesting disruption of the APC/C. In fact, in Apoptin-expressing cells some APC1 migrated at the size expected for a free subunit (~200 kDa). Second, in Apoptin-expressing cells Cdc27 degradation products appeared in the lower molecular weight range. Collectively, these results suggest a model in which Apoptin binds to APC1, leading to disruption of the APC/C and the resultant degradation of some APC/C subunits. Finally, it is noteworthy that despite its small size (13.6 kDa), essentially all Apoptin migrated at a native molecular weight of ~200 kDa to greater than 1 MDa, consistent with the possibility that Apoptin is associated with one or more large multi-subunit complexes.
The Apoptin NLS and nucleo-cytoplasmic shuttling are involved in APC1 interaction and required for induction of apoptosis. To delineate the region of Apoptin required for association with APC1, a panel of FLAG-tagged Apoptin deletion mutants (Figure 3.5a) was transiently expressed in H1299 cells. Forty-eight hours following transfection, Apoptin was immunoprecipitated with α-FLAG antibody, and the immunoprecipitate analyzed for APC1 by immunoblotting. Figure 3.5b shows that APC1 was present only in immunoprecipitates of Apoptin derivatives that contained the C-terminal domain (amino acids 82-121).

To investigate whether association with APC1 is required for Apoptin-mediated cell death, GFP-derivatives of the Apoptin deletion mutants were transiently expressed in H1299 cells. Four days following transfection, cells were fixed, stained with DAPI, and analyzed by fluorescence microscopy for apoptotic morphology. Figure 3.5c shows that the ability of mutants lacking the C-terminal (APC1-associating) region to induce cell death was significantly reduced. Interestingly, Apoptin mutants Ap(82-121) and Ap(42-121) retained a relative greater portion of wild-type killing activity suggesting that the C-terminus alone may have some capacity for induction of apoptosis. Collectively, the results of Figures 3.5b-c indicate that the C-terminal domain of Apoptin is necessary (and possibly sufficient) for induction of apoptosis and that association with APC1 is responsible for Apoptin-mediated G2/M arrest and cell death.

1 Similar results were obtained using electroporated Flag-Apoptin deletion mutants and cell viability analysis via flow cytometry (not shown), however, apoptotic capacities for the deletion mutants compared to wild type Apoptin are less obvious by this method. This difference may be attributed to the use of GFP fusions as well as chemical transfection methods, which might result in spurious effects.
The association of APC1 with the C-terminus of Apoptin prompted examination of whether NLS sequences within this domain were involved in APC1 association. To investigate the role of the NLS region, several candidate mutations were constructed (not all shown) including one in which lysine-116, arginine-117 and arginine-118 were mutated to alanine (Ap-pmNLS2; Figure 3.6a). The majority of this mutant protein retained nuclear localization in H1299 cells (Figure 3.6b). Additionally, the Ap-SV40NLS mutant, which localized exclusively to the nucleus in H1299 cells (previously described in Chapter II, Figure 2.7a), was also analyzed to verify that N-terminal sequences did not take part in APC1 association when sent to the nucleus (unlike in Figure 3.5, where they localize to the cytoplasm). To determine the ability of these two mutants to induce cell death in transformed cells, GFP-Apwt, GFP-Ap-pmNLS2 and GFP-Ap-SV40NLS were transiently expressed in H1299 cells. Three days after transfection, cells were fixed, stained with DAPI, and analyzed by microscopy for apoptotic morphology. As expected, cells expressing GFP-Apwt underwent pronounced apoptosis whereas the ability of the Ap-pmNLS2 and Ap-SV40NLS mutants to induce apoptosis was severely reduced (Figure 3.6c, top).

To understand why the Ap-pmNLS2 and Ap-SV40NLS mutants failed to induce apoptosis in transformed cells even though they localized to the nucleus, their ability to interact with APC1 was monitored. A triple Flag-tagged version of each mutant was transiently transfected into H1299 cells and immunoprecipitated with an α-Flag antibody, and the immunoprecipitate analyzed for APC1 by immunoblotting. Interestingly, APC1 was present in the immunoprecipitate from wild type Apoptin, but not in that of the Ap-
pmNLS2 or Ap-SV40NLS mutants (Figure 3.6c, bottom) despite the presence of both these mutants in the nucleus. These observations are consistent with the results in Figure 3.5 and suggest that the NLS sequence overlaps with the domain required for association with APC1. Furthermore, these results indicate that nuclear localization in the absence of APC1 association is not sufficient to induce apoptosis.

To clarify the involvement of the NES and shuttling activity in Apoptin-induced cell death, the ability of the Ap-pmNES mutant (Chapter II), which contains a wild type C-terminal domain and localizes to the nucleus in both transformed and primary cells (Chapter II, Figure 2.3), to interact with APC1 and induce apoptosis was investigated. Surprisingly, despite its nuclear localization, this mutant exhibited greatly reduced ability to interact with APC1 (Figure 3.6d, bottom) and failed to induce apoptosis in transformed and primary cells (Figure 3.6d, top). Thus, the shuttling activity of Apoptin may be required for interaction with APC1 and induction of apoptosis.

**Loss of APC1 induces G2/M arrest and apoptosis in the absence of p53.** The results described above suggest a model whereby Apoptin inhibits APC/C function resulting in G2/M arrest followed by apoptosis. A prediction of this model is that inhibition of APC1 through other means should have a similar effect. To test this prediction, the consequence of depleting APC1 by RNA interference (RNAi) was analyzed in H1299 cells. Figure 3.7a demonstrates that transfection of short interfering RNAs (siRNAs) directed against APC1 resulted in specific and near complete reduction of APC1 protein levels, whereas a non-specific control siRNA directed against Lamin A/C had no effect.
Transfection of an APC1 siRNA resulted in a dramatic reduction in cell density (Figure 3.7b) and viability (Figure 3.7c) compared to the Lamin A/C siRNA-transfected and mock-transfected controls. Annexin V-FITC staining confirmed, as expected, that cell death was due to apoptosis (Figure 3.7d). Figure 3.7e shows that transfection of an APC1 siRNA, but not control siRNAs, resulted in G2/M accumulation and the appearance of cells in the sub-G1 region. Quantification of the FACS data indicates that nearly 50% of APC1 siRNA-transfected cells were present in G2/M compared with approximately 15% for the Lamin A/C siRNA- or mock-transfected controls (Figure 3.7e, right panel). Thus, RNAi-mediated depletion of APC1 results in a phenotype analogous to that observed following Apoptin expression.

**Apoptin co-localizes with the APC/C within PML bodies in the nuclei of transformed cells.** Next, to verify Apoptin’s co-localization with APC/C complexes in transformed cells, immunofluorescence was performed for the APC/C subunit Cdc27 (APC3), a core subunit routinely used as a marker for APC/C localization (81, 145). H1299 cells were transiently transfected with a plasmid expressing dsRed-Apwt and following a 12 hr incubation, fixed and stained with an α-Cdc27 antibody. Figure 3.8a shows that dsRed-Apwt co-localized with Cdc27 in the nucleus of transformed cells. We failed to detect Cdc27 immunofluorescence in the presence of the Ap-pmNLS2 mutant, which does not associate with the APC/C and fails to induce apoptosis (Figure 3.6b-c) and G2/M arrest (data not shown). This result is consistent with previous reports showing
that various APC/C subunits, including Cdc27, are not detected by immunofluorescence in interphase or non-mitosis arrested cells (80, 81, 86).

It has recently been reported that Apoptin associates with PML nuclear bodies (140). To verify this result, cells were transfected with either dsRed-Apwt or, as a control, dsRed-Ap-pmNLS2 and stained with an α-PML antibody. Figure 3.8b confirms that wild-type Apoptin co-localized with PML bodies within the nucleus of transformed cells. Significantly, in cells transfected with dsRed-Ap-pmNLS2, PML immunofluorescence was undetectable, consistent with previous studies showing that in H1299 cells, PML nuclear body formation occurs only under conditions of apoptotic stimulus (44). Thus, Apoptin induces the formation of PML nuclear bodies in transformed cells.

By inference, the immunofluorescence experiments of Figures 3.8a-b suggest that in the presence of Apoptin, APC/C co-localizes with PML nuclear bodies. To provide more direct evidence for co-localization of Cdc27 and PML in the presence of Apoptin, plasmids expressing either dsRed-Apwt or dsRed-Ap-NLS2 were co-transfected into H1299 cells with a plasmid expressing PML fused to the C-terminus of cyan fluorescent protein (CFP-PML). Twelve hours following transfection, cells were fixed and stained with α-Cdc27 antibody. Figure 3.8c shows that, as expected, dsRed-Apwt, Cdc27 and CFP-PML co-localized in the nucleus of transformed cells. Thus, in the presence of Apoptin, the APC/C becomes sequestered in PML bodies.
Discussion

Most human cancers have mutations in the p53 gene rendering them refractory to existing therapies (17, 82, 161). Novel approaches for inducing programmed cell death in cancers that lack functional p53 would represent a significant advance. The results in this study suggest that CAV Apoptin induces G2/M arrest and apoptosis in p53 null transformed cells through association with and disruption of the APC/C.

Inhibition of the APC/C may represent a major tumor suppressor mechanism (reviewed in (73). For example, the endogenous APC/C inhibitor RASSF1 is mutated in some human cancers (6). Moreover, dysfunction of the APC/C and mitotic checkpoint function has been suggested to contribute to transformation and could lead to properties in cancer cells such as unscheduled proliferation and aneuploidy. In addition to Apoptin, several viral proteins have been found to interact with the APC/C and deregulate its activity. The adenovirus E4orf4 protein also arrests cells in G2/M (95) and induces apoptosis specifically in transformed cells in the absence of p53 (110). Yeast genetics experiments suggest that E4orf4-induced cell cycle arrest is mediated by the APC/C, most likely through its Cdc16 subunit (95). More recently, the HTLV-1 Tax protein has been found to deregulate APC/C activity through association with the Cdc20 activator protein (106). Thus, inhibition of the cyclosome complex may represent a convergently evolved viral cytopathic mechanism and suggests an underlying role for the APC/C as a nodal point in cell cycle control and tumor suppression. The evolution of these viruses to target the APC/C may involve cellular arrest in mitosis to facilitate viral replication. For example, mitotic cells become rounded and lift from the surrounding substrate, which
may provide a means whereby infected cells can become mobilized to areas of uninfected cells and more efficiently disseminate the virus.

APC1 is suggested to be essential in assembly and regulation of the cyclosome complex (96, 174). The preparative IP/mass spectroscopic analysis performed in this study identified APC1 but not other APC/C subunits suggesting that Apoptin may associate with free APC1 and not the intact APC/C. These results suggest a model in which Apoptin induces either a failure to assemble an APC/C or disassembly of an existing one and a resultant lack of APC/C activity.

Several previous observations are consistent with the notion that apoptotic signaling from the mitotic checkpoint apparatus functions independently of p53. For example, drugs that suppress microtubule dynamics, such as paclitaxel (Taxol), which promote mitotic arrest and cell death are generally unaffected by p53 status (45). In fact, cancer cells that are p53 negative are sensitized to paclitaxel-mediated killing (173, 178). Other drugs that target terminal steps of the pathway, such as degradation of mitotic cyclins, have also been shown to be p53-independent. For example, the proteasome inhibitor PS-341 (Bortezomib), which is being evaluated for clinical use, induces apoptosis in cells lacking functional p53 (191). The mechanism of Apoptin-induced cell death proposed here is therefore consistent with previous observations that the mitotic-checkpoint apparatus can stimulate apoptosis independent of p53 status. Reports have indicated that the taxane class of chemotherapeutics as well as other G2/M arresting drugs, including Bortezomib, induce and/or stabilize BIM leading to onset of apoptosis in a p53-independent manner (164). The results presented here indicate that Apoptin may
act on a similar pathway as a mechanism of p53-independent apoptosis in transformed cells suggesting a role for BIM mediated apoptosis in tumor suppression.

The results of this study demonstrating Apoptin-mediated induction of PML nuclear body formation are consistent with those of previous studies showing co-localization of Apoptin with PML nuclear bodies (140). Moreover, general induction of PML nuclear bodies in H1299 cells has been previously demonstrated by a number of groups (including (44)), which have shown that these cells respond dramatically to various types of apoptotic stimuli by increasing both the size and number of PML bodies. PML nuclear bodies play an integral role in apoptotic processes resulting from a variety of stimuli and have been proposed to serve as a “hub” for nuclear apoptotic reinforcement (16). Cells derived from PML-/- knockout mice are virtually resistant to apoptosis stimulated by intrinsic as well as extrinsic stimuli, indicating a strong connection between PML body formation and induction or execution of programmed cell death (16). Interestingly, PML nuclear bodies have been implicated in the execution of p53-independent (as well as p53-dependent) apoptotic programs (181, 182, 195), an important requisite for Apoptin induced cell death.

PML nuclear bodies are potential hubs for the control and regulation of proteins by post-translational modification (e.g., sumoylation, acetylation and ubiquitination) and by degradation (7, 97, 99). For example, many cellular and viral sumoylated proteins are targeted to PML nuclear bodies (153). In many cases, however, the functional significance between protein modification and PML localization remains unclear. Interestingly, studies in yeast have shown that the mitotic functions of the APC/C are
modulated by sumoylation and ubiquitination (49). Thus, the Apoptin-mediated recruitment of APC/C components (or complexes) into PML nuclear bodies suggests a functional connection between Apoptin-APC/C interaction, cyclosome inhibition and induction of apoptosis. Abrogation of APC/C activity through targeting to PML nuclear bodies provides a method by which APC/C could be sequestered and inhibited, and apoptotic programming of nuclear origin could ensue.
Figure 3.1. Apoptin induces apoptosis specifically in transformed cells. (A) Saos-2 and H1299 cells were infected with adenovirus expressing FLAG-Apoptin (Ad-Apwt) or LacZ (Ad-LacZ), and cell death was monitored for 96 h post-infection by trypan blue exclusion. (B) Immunoblot analysis using α-FLAG antibody to monitor Apoptin protein expression in Saos-2 cells.
Figure 3.1

A

B

0 6 12 24 48 72 96

0 10 20 30 40 50 60 70 80 90 100

% Cell survival

Hours post-infection

LacZ (SAOS-2)
Ad-Ap (SAOS-2)
LacZ (H1299)
Ad-Ap (H1299)
LacZ (PFF)
Ad-Ap (PFF)

FLAG-Ap
Figure 3.2. Apoptin is associated \textit{in vivo} with APC1 in transformed cells. (A) Affinity purification of Apoptin-associated proteins from H1299 cells infected with Ad-Apwt. Proteins were separated by SDS-PAGE and visualized by silver staining. Microsequenced bands are indicated. (B) Apoptin was immunoprecipitated from Ad-Apwt-and Ad-LacZ-infected H1299 cells using \( \alpha \)-FLAG antibody, and the immunoprecipitates were analyzed for APC1 by immunoblotting with a polyclonal \( \alpha \)-APC1 antibody. (C) Apoptin was immunoprecipitated from Ad-Apwt-infected PFF or H1299 cells using \( \alpha \)-FLAG antibody, and the immunoprecipitates were analyzed for APC1 by immunoblotting. Immunoblotting for FLAG-Apoptin was performed on whole cell extracts.
Figure 3.2

A

Ad-LacZ
Ad-Apwt

kDa
97
66
45

APC1
Hsp70
β-actin
α-tubulin
β-tubulin

Apoptin

B

co-IP

APC1
FLAG-Ap

C

co-IP

APC1
FLAG-Ap

immunoblot

PFF/Ad-Apwt
H1299/Ad-Apwt
Figure 3.3. Apoptin expression induces G2/M cell cycle arrest specifically in transformed cells. (A) Cell cycle analysis of H1299 cells infected with either Ad-LacZ or Ad-Apwt at 0, 12, 24 and 36 h post-infection. (B) Percent of H1299 or PFF cells in G2/M were quantified. (C) H1299 cells were synchronized and compared to an asynchronous population 24 hours following Ad-Apwt infection. Levels of apoptosis were measured using ViaCount reagent on a Guava personal flow cytometer.
Figure 3.3

A

Ad-LacZ

Ad-Apwt

DNA content

Cell number

0 h

12 h

24 h

36 h

0 h

12 h

24 h

36 h

B

H1299

% Cells in G2/M

0

20

40

60

80

100

0

10

20

30

40

50

Ad-LacZ

Ad-Apwt

PFF

% Cells in G2/M

0

10

20

30

40

50

0

12

24

36

Hours post-infection

C

% Apoptosis

ASYNC

SYNC

1

2

3

4

5

6

7

8

9

10

11

12

13

14
Figure 3.4. Apoptin induces APC/C dysfunction via complex dissociation. (A) Immunoblot analysis of APC/C substrates cyclin B1 and Polo-like kinase (Plk), and a non-APC/C substrate, cyclin E, in H1299 whole-cell lysates 24 h following infection with Ad-Apwt or Ad-LacZ, or in cells treated with nocodozole (noc) for 12 h. Tubulin levels were monitored as a loading control. (B) RT-PCR analysis of cyclin B1 or Plk mRNA levels in H1299 cells 24 h following infection with Ad-Apwt or Ad-LacZ, or in cells treated with nocodozole (noc) for 12 h. GAPDH was monitored as a loading control. (C) Gel filtration analysis of the APC/C complex in mock- or Ad-Apwt-infected H1299 cells. Fractions from the gel filtration column were analyzed by immunoblotting for FLAG-Apoptin, APC1 or another APC/C subunit, Cdc27. Positions of molecular weight markers are indicated.
Figure 3.4

A

noc Ad-LacZ Ad-Apwt

cyclin B1
Plk
cyclin E
tubulin

B

noc Ad-LacZ Ad-Apwt
cycB1
Plk
GAPDH

C

fraction #: 15 17 19 21 23 25 27 670 29 31 33 35 158 37 44 41 17 kDa

mock

Ad-Apwt

APC1
Cdc27
FLAG-Ap

APC1
Cdc27
FLAG-Ap
Figure 3.5. The C-terminal domain of Apoptin is required for association with APC1 and apoptosis. (A) Schematic representations of N-terminal FLAG- or GFP-tagged Apoptin deletion mutants. (B) H1299 cells were transfected with FLAG-Apoptin deletion constructs, and 48 h later Apoptin was immunoprecipitated with α-FLAG antibody and the immunoprecipitate analyzed for APC1 and Apoptin by immunoblotting. (C) H1299 cells were transfected with GFP-Apoptin deletion constructs and 4 d later fixed and stained with DAPI and analyzed by fluorescence microscopy for GFP expression associated with apoptotic morphology. The percent apoptosis in cells expressing GFP alone was taken as background and subtracted from all samples. All mutant samples are shown as percent apoptosis of Apwt.
Figure 3.5

A

FLAG or GFP

Apwt

Ap(1-48)

Ap(42-88)

Ap(82-121)

Ap(1-88)

Ap(42-121)

B


α-APC1

α-FLAG

C

% Apoptosis

GFP-Apwt

GFP-Ap(1-48)

GFP-Ap(42-88)

GFP-Ap(82-121)

GFP-Ap(1-88)

GFP-Ap(42-121)
Figure 3.6. The Apoptin NLS and nucleo-cytoplasmic shuttling are involved in APC1 interaction and required for induction of apoptosis.  

(A) Schematic diagrams of Apoptin NLS mutations.  

(B) Fluorescence microscopy of H1299 cells expressing GFP-Ap-pmNLS2.  

(C) (Top) Apoptosis assays. H1299 cells were transfected with GFP-Apwt, GFP-Ap-pmNLS2 or GFP-Ap-SV40NLS and after 72 h, fixed, stained with DAPI and analyzed by fluorescence microscopy for GFP expression associated with apoptotic morphology. The percent apoptosis in cells expressing GFP alone was taken as background and subtracted from all samples. All mutant samples are shown as percent apoptosis of GFP-Apwt.  

(Bottom) APC1 association. H1299 cells expressing Flag-tagged versions of Apoptin mutants were immunoprecipitated with an α-Flag antibody and the immunoprecipitate analyzed for APC1 and Apoptin by immunoblotting.  

(D) (Top) PFF or H1299 cells were infected with Ad-LacZ, Ad-Apwt or Ad-Ap-pmNES, and 72 h later cell viability was quantified by flow cytometry.  

(Bottom) APC1 association.
Figure 3.6

A

Flag or GFP

Ap-pmNLS2

Ap-SV40NLS

B

H1299

GFP

DAPI

GFP-Ap-pmNLS2

C

% Apoptotic nuclei

0

20

40

60

80

100

GFP-Apwt

GFP-Ap-pmNLS2

GFP-Ap-SV40NLS

D

% Cell viability

0

20

40

60

80

100

PFF

H1299

Ad-LacZ

Ad-Apwt

Ad-Ap-pmNES

Flag IP

α-APC1

α-Flag

Flag IP

α-APC1

α-Flag
Figure 3.7. Depletion of APC1 by RNA interference induces G2/M arrest and apoptosis in the absence of p53. (A) Immunoblot analysis of H1299 cells transfected with 0, 0.05 and 0.5 μg siRNAs directed against either Lamin A/C or APC1. Tubulin was monitored as a loading control. (B) Crystal violet staining of H1299 cells following two rounds of transfection with Lamin A/C or APC1 siRNAs, or mock-transfected. (C) Cell viability assays of H1299 cells 48 h following transfection of siRNAs, or mock-transfected. (D) Annexin V-FITC staining to monitor apoptosis in siRNA-transfected cells. (E) Cell cycle analysis (left) and quantification of the percentage of cells arrested in G2/M (right) of siRNA-transfected cells.
Figure 3.7

A. Lamin A/C siRNA and APC1 siRNA

B. Mock, Lamin A/C siRNA, APC1 siRNA

C. Nucleated cells vs. Cell Death

D. % Annexin V positive

E. % Cells in G2/M

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Figure 3.8. Apoptin co-localizes with APC/C within PML bodies in the nuclei of transformed cells. (A, B) H1299 cells were transiently transfected with a plasmid expressing either dsRed-Apwt or dsRed-Ap-pmNLS2 and following a 12 hr incubation, fixed and stained with α-Cdc27 antibody (A) or an α-PML antibody (B). (C) H1299 cells were co-transfected with plasmids expressing either dsRed-Apwt or dsRed-Ap-NLS2 and a plasmid expressing CFP-PML. 12 hr following transfection, cells were fixed and stained with α-Cdc27 antibody.
Figure 3.8

A

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C

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CHAPTER IV

CONCLUSIONS AND PERSPECTIVES
Conclusions

The results of this study have yielded insight into the mechanism of tumor cell-selectivity of the CAV Apoptin protein as well as provided a novel p53-independent pathway to apoptosis in transformed cells. Since CAV is often found to co-infect cells with several other transforming viruses, it is understandable to find that this virus carries a protein activity that senses and responds to the transformation state of the cell.

Several Apoptin characteristics are influenced by early events in cellular transformation including the degree of self-association of the protein, intracellular solubility, and sub-cellular trafficking (Chapter II). In primary cell types, Apoptin is functionally inert owing to the large degree of cytoplasmic aggregation, low steady-state level of shuttling protein, and inaccessibility of the nucleus. Transformation dramatically increases the soluble shuttling pool of Apoptin protein, and resultant steady-state levels of protein increase in the nuclear compartment. Presumably a modification that affects aggregation and N-terminal function could lead to these overall changes in protein behavior. Those modifications previously discussed, including modulation of phosphorylation and/or conformational changes, are formally possible. Conformational changes that would dampen the activity of the N-terminal export/multimerization domain and expose the NLS region in the C-terminus could lead to such an equilibrium shift (see Figure 4.1b). Consistent with these ideas, recent studies have suggested that Apoptin conformational changes may occur in transformed cells to unmask an otherwise inaccessible NLS at the C-terminus (177). Studies involving masking of the NES and Crm1 binding have yet to be performed, but may reveal a similar strategy for regulation
at the N-terminus. Notably, regulated masking of the NLS is expected to have the additional consequence of affecting APC1 association via this region as well.

Steady state localization of Apoptin is controlled by NES and NLS sequences that must be coupled either intra- or intermolecularly to function in a cell type-specific fashion. Nuclear-cytoplasmic shuttling activity originating from the coupled activity of these localization sequences is surprisingly critical for most Apoptin activities including regulated localization behavior, APC/C association, and induction of apoptosis. This shuttling activity occurs in both normal and transformed cells and allows for the rapid translocation of protein from one sub-cellular compartment to another. The smaller shuttling pool of Apoptin in primary cell types may involve stochastic conformational changes in a sub-population of Apoptin molecules. It is not likely that shuttling in primary cell types has a definitive role to play in cytoplasmic retention or Apoptin inactivity. By contrast, the shuttling pool of Apoptin is much larger in transformed cell types as indicated by rapid and complete nuclear exclusion in import blocking experiments (Chapter II), indicating the vast majority of Apoptin is mobile. Consistent with this idea, Apoptin in transformed cells is readily immunoprecipitated as opposed to in primary cells where it is mostly insoluble.

Apoptin localization sequences have evolved as overlapping protein-protein interaction domains (Figure 4.1a), a strategy adopted by other compact and multifaceted viral proteins (e.g. HIV-1 Rev, Chapter II). It is possible that the activity of these localization sequences is modulated by interaction with each respective protein association as is the case with Rev. Stimulation as well as inhibition of localization
sequences has been observed in response to protein interaction (35, 83, 88, 141, 150, 180). For example, activity at the NES may be positively or negatively responsive to the degree of Apoptin multimerization. Indeed, additional observations indicate that in situations of constitutive Apoptin NES activity (through truncation or mutation of the NLS region) dramatic increases in cytoplasmic aggregation are observed (data not shown). Behavior similar to this may take place for the APC/C interaction with the Apoptin NLS region as well. Association with the APC/C is sensitive to Apoptin shuttling capability which suggests that continual access to the cytoplasm is necessary in transformed cell types for this interaction to occur. As many APC/C subunits are found predominantly in the cytoplasm during interphase (80, 81, 170), including APC1 (unpublished observations), it is possible that Apoptin-APC1 association may first take place there (see Figure 4.1c). The equilibrium of shuttling may be shifted upon APC/C binding and stimulate nuclear re-uptake, at which point the APC/C may be released, and subsequent rounds of shuttling can take place.

Localization of Apoptin to the nucleus is required for induction of apoptosis in transformed cells (Chapter I, Table 4.1). Interestingly however, forcing Apoptin to the nucleus of primary cell types by addition of supernumerary NLS sequences is not sufficient to induce apoptosis (67). Additionally, Apoptin mutations leading to constitutive nuclear localization (e.g. point mutants of the NES) are also insufficient to kill transformed cells. In either case, however, normal Apoptin shuttling activity is altered or prevented. Thus, the requirements for Apoptin killing activity are more complex than bulk nuclear localization of the protein. Instead, it is likely that a critical
steady state level of Apoptin protein is required in the nucleus resulting from a change in shuttling equilibrium. Continued cytoplasmic access and APC/C accessibility may therefore be important requisites for Apoptin induced cell death.

It is clear that for certain capacities the specific Apoptin NLS region is dispensable. Functional replacement of the NLS with heterologous localization sequences can be performed without consequence on cell type-specific localization. However, the capacity for induction of apoptosis is lost, most likely due to interruption of APC/C association. By contrast, the function of the specific Apoptin NES region is critical for regulated localization as well as apoptosis induction. Cytoplasmic retention and aggregation as well as export function are mediated by the activity of the Apoptin NES domain. These properties are responsible for maintenance of Apoptin in the inactive state in primary cells and are negatively affected upon transformation. In contrast to other studies (discussed in Chapter II), this suggests that regulation of Apoptin activity and localization between primary and transformed cell types involves modulation of the N-terminal NES region. It is possible that several potential phosphorylation events proximal to the NES region may play a role in regulating functions, however the number and placement of these sites is controversial (see Appendix).

In addition to shuttling activity, the induction of apoptosis in transformed cells by Apoptin correlates with association with the APC/C (see Table 4.1). The absence of other subunits in preparative co-immunoprecipitation experiments with Apoptin suggests that association with APC1 may either occur in free form separate from the remaining subunits or simply reflect the greater IP efficiency observed with direct APC1 binding.
Additional experiments (data not shown) indicate that Apoptin does co-purify with other APC/C subunits including cdc27 (APC3) and cdc16 (APC6) albeit at relatively lower levels than that of APC1. This is interesting given that current data indicates that APC1 alone may exist at sub-stoichiometric levels relative to other subunits within the complex (133). FPLC analysis of Apoptin expressing cells demonstrates APC/C dissociation and subunit degradation after 24 hours (Chapter III). This indicates that APC/C dysfunction occurs temporally prior to any appreciable levels of apoptosis. G2/M cell cycle arrest, however, is concurrent with APC/C dissociation and dysfunction suggesting that inhibition of APC/C function by Apoptin is causative for both cell cycle arrest and apoptosis. Consistent with this idea, independent disruption of APC/C function by ablation of APC1 mRNA results in G2/M arrest and apoptosis with similar temporal progression and kinetics to that of Apoptin expression (Chapter III). These data suggest that interruption of APC1 function can lead to overall APC/C dysfunction as well as indicate this as a plausible mechanism for the induction of apoptosis by Apoptin.

The molecular mechanism for APC/C disruption by Apoptin remains unclear. Yeast genetic experiments have implicated APC1 in the assembly and regulation of the cyclosome (see Chapter III discussion), however mechanistic data is not yet available. APC1 is phosphorylated on greater than 40 residues where particular groups of residues are modified in particular cell cycle stages suggesting this as a potential regulatory mechanism for this subunit. Additionally, it is known that cyclosome ubiquitination function is affected by the status of phosphorylation on several subunits (including Cdc20 and Cdh1 activators) (76, 96). One potential model would involve Apoptin affecting the
phosphorylation status of APC1, which may alter APC/C activation, subunit assembly, or complex stability. Interestingly, the mechanism proposed for the Adenovirus E4orf4-mediated inhibition of APC/C\(^{Cdc20}\) involves recruitment of Protein Phosphatase-2A to the APC/C and potential alteration of phosphorylation (95).

Recent studies suggest that the activity of the APC/C may be more complex than originally believed; specifically, APC/C function is not merely restricted to anaphase and kinetochore signaling. Rather, there are at least two major versions of the complex: the anaphase version of the APC/C exists in a form bound to the activator cdc20 (APC/C\(^{Cdc20}\)), which is necessary for coordination of aforementioned mitotic events, whereas another less understood complex exists mainly in G1 that binds to the activator Cdh1 (APC/C\(^{Cdh1}\)) (reviewed in (134)). Complete exit from mitosis is facilitated by APC/C\(^{Cdc20}\)-dependent destruction of Cyclin B, which begins in metaphase and continues throughout anaphase while sister chromatids separate and migrate to the spindle poles. Studies in Drosophila have shown that Cyclin B destruction occurs spatially in a wave emanating from APC/C activity at the centrosomes, proceeding down the spindles, and eventually arriving at the cell equator (81). In most cell types this destruction is concurrent with the degradation of the cdc20 activator as well, which is replaced by the cdh1 G1 activator protein for the APC/C. APC/C\(^{Cdh1}\) localized to the centrosome can presumably diffuse outward and catalyze the ubiquitination of residual Cyclin B in the cytosol (80, 143). APC/C\(^{Cdh1}\) activity is maintained throughout G1 and functions primarily to keep levels of mitotic cyclins low. Despite this activity, Cyclin A levels eventually rise in preparation for S-phase entry. Recent studies indicate that APC/C\(^{Cdh1}\)
autonomously regulates itself in G1, allowing for inactivation when all substrates have been destroyed. Inactivation allows for the accumulation of Cyclin A effectively coupling mitotic activity to G1 inactivation (145).

In addition to autoregulatory activity in G1, the APC/C has also been implicated in the direct regulation of S-phase entry. Skp2 and its cofactor Cks1 are substrate-targeting subunits of the E3 SCF ubiquitin ligase complex that regulates S-phase entry by ubiquitination and subsequent degradation of the p21 and p27 CKIs. This allows the release of E2F from pRb and gene activation leading to G1/S transition. APC/C^Cdhl has recently been found to ubiquitinate and target for destruction both Skp2 and Cks1, effectively inhibiting SCF function, preventing unscheduled entry into S-phase and further maintaining the G1 state. Inactivation of APC/C^Cdhl either by expression of non-degradable forms of Skp2 or ablation of Cdh1 by RNA interference induces premature entry into S phase (13, 14, 184). Collectively, these studies suggest that the APC/C, in addition to controlling mitosis, may represent an autonomous master regulator of the metazoan cell cycle.

A role for the viral manipulation of the APC/C^Cdhl complex is now becoming evident. Studies of CMV have suggested that this virus may act on the Cdh1-activated APC/C specifically in G1 resulting in a release of APC/C-dependent SCF inhibition and entry into S-phase (188). This reveals a novel mechanism for the forced induction of S-phase by a virus and reinforces the idea that the APC/C could represent a convergently evolved nodal target of many viruses to manipulate the cell cycle at various stages.
Whether Apoptin inhibition of the APC/C is restricted to the mitotic complex or involves G1 APC/C modulation remains unclear.

Recent studies have succeeded in producing low-resolution structural maps of the yeast and mammalian APC/C complexes using cryo-electron microscopy (62, 133). As mentioned before, it appears that APC1 exists in either sub-stoichiometric levels relative to all other subunits or may exist in only certain APC/C complexes. Studies in drosophila have indicated that several distinct APC/C complexes may exist in the cell in addition to the cdc20 and cdh1 activated forms (81). The cyclosome, like many other large multi-subunit complexes in the cell, may also be regulated at the level of subunit composition where each sub-complex may have a distinct site of action and/or substrate specificity. It is possible that Apoptin may act on only those APC/C complexes which possess an APC1 subunit and have specific ubiquitination functions aside from, or in addition to, anaphase progression. Consistent with this, Apoptin arrested cells do not present typical metaphase chromosomes expected for inhibition of only the mitotic checkpoint functions of the APC/C. Instead what is observed are cells arrested with intact nuclear membranes that rapidly exhibit apoptotic morphology (data not shown). Surprisingly, this somewhat unexpected phenotype is also what has been observed for mutation of certain proteins that modulate APC/C activity upstream in late G2 and early mitosis (104). It is well established that mitotic A-type cyclins and the kinase Nek2A are degraded during prometaphase prior to chromatin condensation in an APC\textsuperscript{Cdc20}-dependent manner, indicating that an APC/C is already active early in mitosis (43, 47, 61, 69, 156). These data have spawned suggestions that the APC/C\textsuperscript{Cdc20} (or distinct sub-complexes) may also
promote a novel late G2 checkpoint that can arrest cells before entering mitosis. Thus, it is clear that with the wide array of phenotypes observed by modulation of many different APC/C effectors that cyclosome functions are diverse and numerous with respect to the cell cycle. Apoptin interaction with one or several of these complexes is expected to result in unique multifaceted phenotypes beyond simple metaphase arrest.

Recent structural analysis of the APC/C has indicated that it may also operate as a multimer. Native gel analysis of purified APC/C from yeast reveals at least two differently migrating forms that correlate with monomeric and dimeric species of the complex (133). The dimeric species has been shown to harbor ubiquitination processivity in vast excess of that of the monomeric form suggesting that this may be the predominant species of APC/C in vivo. Consistent with these observations, close inspection of the FPLC profile in Chapter III, Figure 3.4c also indicates that the APC/C peaks at two migration points which could correlate to these monomeric and dimeric forms. This raises the interesting possibility that Apoptin could act on the APC/C by preventing formation of processive dimers. The effects of this type of activity on cyclosome complex stability or cell cycle have yet to be determined. Interestingly, it has been suggested that the sub-stoichiometric levels of APC1 may also reflect that only one of these subunits may be present for each APC/C dimer (133). In this case APC1 could serve to nucleate cyclosome dimers and/or bridge them together, lending credence to why Apoptin should have evolved to target APC1 over other subunits.

Increasing evidence is favoring the idea that a block in APC/C function, particularly in mitosis, leads to apoptosis independently of the actions of p53 (Figure 4.2
Drug targeting of those activating stimuli upstream of the APC/C, including the use of MIAs, induces G2/M cell cycle arrest and apoptosis in the absence of p53 (118). Likewise, newer drugs that block the actions of the proteasome downstream of APC/C function also induce G2/M arrest and apoptosis regardless of p53 status (3-5). Studies have shown that apoptotic signaling from mitotically arrested cells involves stabilization of the BH3-only protein Bim (103, 118, 142). Interestingly, some cancers that have acquired resistance to MIAs show a lack of Bim stabilization but can be re-sensitized by treatment with proteasome inhibitors such as Bortezomib that block active Bim degradation (164). These two classes of drug have been shown to act synergistically when used in concert, supporting a method of action involving the same pathway. Given the similarities in G2/M arrest, p53-independence, and function of the APC/C, it is likely that the killing action of Apoptin involves this same pathway as well (see Chapter III). These studies of Apoptin suggest that the APC/C is a nodal point in the regulation of cell cycle and apoptosis and raise the insightful possibility that drug targeting of the APC/C itself may prove to be effective for the selective killing of transformed cells.
Perspectives

Most organisms have been found to occupy a particular niche in which co-evolution has occurred with one or many other organisms. Viruses are no exception: transformed cells can also be viewed as a reservoir for the evolution of certain viral behaviors or protein activities. In this regard, those viruses that have selected for activities that are specific to cancerous cells can exploit beneficial aspects of transformation at the cellular level. Targeting of the APC/C by viruses including CAV, HIV, CMV, HTLV and Adenovirus represents a newfound field deserving much attention (73). The convergent evolution of these viruses to target this cellular complex suggests that there is an evolutionary advantage to be gained in doing so (see Figure 4.2). The role of the APC/C is now being understood to be global in the regulation of many aspects of the cell cycle, and several possible advantages can be envisioned through viral manipulation of one or various forms of this complex. It is possible that one strategy of these viruses is to simultaneously stimulate S phase and block mitotic functions through inhibition of both the Cdc20 and Cdh1 activated forms of the cyclosome. With a minimal amount of protein-protein interaction the virus could achieve a situation of unabated genome replication while at the same time increasing the concentration of viral genomes (and resultant progeny) by blocking cell division. Alternatively, targeting of individual forms of the APC/C has also been observed. For example, CMV manipulation of Cdh1 activator in G1 results in activation of this APC/C and selective progression of S phase independently of the mitotic complex. Evidence also suggests that positive APC/C regulation can occur in response to different proteins produced by these viruses. In the
case of HTLV-1, coordinated manipulation of the cdc20 activator protein results in rapid progression of mitosis.

It is evident from clinical trials involving Bortezomib and Gleevec that the development of drugs based on the inhibition of specific protein activities holds the potential for far greater specificity and minimal collateral effects. It is possible that targeted inhibition of the APC/C may result in tumor cell killing with higher specificity than that of the current drugs that target peripheral areas of APC/C regulation. Specific transformed cell targeting of the cyclosome by Apoptin is achieved through differential localization, which is not readily available for a small molecule inhibitor. However, recent studies indicate that the development of many cancers involves the aberrant expression of APC/C subunits and/or downregulation or mutation of endogenous APC/C inhibitors (18, 132, 159, 160, 183). Increased activity of the APC/C in transformed cell types coupled with innate cellular sensitivity to the regulation and levels of this complex could provide a large dosage-dependent difference for drug inhibition of normal versus transformed cell APC/C.

Alternatively, it is possible that the essential nature of the APC/C and its functions will result in a global inability to inhibit this complex in any situation. Experiments must be performed to address the therapeutic efficacy of differential inhibition of the complex between normal and cancerous cells. Quantification of primary and transformed cell death following modulation of endogenous APC/C inhibitors may provide clues to whether treatment using this complex is possible. Additionally, the exact nature of interaction of Apoptin with the various potential species of APC/C is unknown. If one or
more of these distinct complexes is found to differ in its activity, abundance, or subunit composition between normal and transformed cells it might be possible to specifically target such species for inhibition while leaving others undisturbed. Future detailed investigation of the APC/C in its various forms and functions throughout the cell cycle will be integral to these endeavors.

Continued study of Apoptin as well as of other viral proteins that possess unique properties in transformed cells holds the promise of identifying many novel targets that may be of use in treating cancer. The availability of such candidates, coupled with evolving target-based drug development techniques have hope of providing anti-cancer drugs similar to Gleevec, but with usefulness in treating a broad range of cancers specifically and effectively.
Figure 4.1. Multifunctional localization signals mediate the cell type-specific localization of Apoptin. (A) Schematic diagram of Apoptin’s localization signals and overlapping protein interaction domains. (B) A model for the cell type-specific localization of Apoptin. In primary cells, Apoptin favors an NES dominant conformation and aggregates by virtue of multimerization capacities present in the same domain. In transformed cells, Apoptin favors an NLS dominant conformation, possibly via unmasking of the C-terminus (177), allowing for higher steady state levels of nuclear protein. (C) A model for the transformed cell-specific killing activity of Apoptin. In transformed cells, Apoptin shuttling is shifted toward nuclear accumulation. The localization of many APC/C subunits is predominantly cytoplasmic outside of mitosis; Apoptin may either transport APC/C subunits from the cytoplasm to the nucleus, or associate with APC/C subunits already present in the nucleus. Apoptin-mediated recruitment of APC/C into PML nuclear bodies in transformed cells leads to inactivation of APC/C function and subsequent induction of G2/M arrest and apoptosis.
Figure 4.1

A

Apoptin multimerization

Apoptin

NES
NLS

APC/C

APC1

B

Primary cell

Transformed cell

Ap (multi.)

NLS

Cytoplasm

Nucleus

C

Transformed Cell

Apoptin

APC/C

PML body

APC/C sequestration and inactivation

G2/M arrest

Apoptosis
Figure 4.2. Inhibition of the mitotic checkpoint leads to G2/M arrest and apoptosis independently of p53. Several promising cancer therapeutics target critical players in the establishment of mitotic checkpoint control. These various inhibitors have been shown to induce G2/M cell cycle arrest followed by the induction of apoptosis in a manner independent of the p53 tumor suppressor. At the center of these activities is the APC/C whose function may mediate the effects of microtubule inhibiting agents (MIA’s) and possibly inhibitors of the 26S proteasome as well. The APC/C is beginning to be understood as a critical node in the overall regulation of the cell cycle. Several viral proteins including Adenovirus e4orf4 protein and the CAV protein Apoptin are known to induce G2/M arrest and have been shown to do so via inhibition of the APC/C. These viral proteins may inhibit both of the two known forms of the APC/C, which differ by either the cdc20 or cdh1 activators. Effects observed with such viral inhibitors may involve a common pathway leading to the induction of apoptosis.
Figure 4.2

APOPTOSIS

G2/M arrest

MIAs (eg. Taxol)

Apopltin E4Orf4 vpr?

proteasome inhibitors (eg. Bortezomib)

p53

oncogene DNA damage

microtubule dynamics

Cdc20

APC/C

Proteasome
Table 4.1 Apoptin Mutants and Phenotypes. Various Apoptin mutations generated throughout this study are listed with known localization, protein association, and apoptotic phenotypes. Those phenotypes not determined (N/D) are notated where relevant.
### Table 4.1

**Apoptin Mutants and Phenotypes**

<table>
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<th>Mutant</th>
<th>Localization</th>
<th>Localization</th>
<th>Shuttling</th>
<th>Multimerization</th>
<th>APC1 Association</th>
<th>Apoptotic Activity</th>
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<td>Primary Cell</td>
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<td>*+/-</td>
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Denoted activities are relative to wtAp

* Results averaged from GFP and Flag fusion experiments (see footnote 2, Chapter III)

† Actual quantification is greater than that shown (see Appendix)
APPENDIX

ANALYSIS OF APOPTIN REGULATION BY PHOSPHORYLATION
Introduction

Efforts to isolate the transformed cell-specific activity(ies) that act upon Apoptin have been conducted throughout this study as well as in others, however, none have yet been successful. Current data indicates that the most likely candidate for this activity is one that modulates phosphorylation (i.e. kinase or phosphatase). The few studies that have reported on investigation of such an activity have suggested the existence of a transformed cell-specific kinase that potentially modifies Apoptin at the C-terminus (151, 193). Specifically, phosphorylation of the single residue T108 has been suggested to take place only in transformed cell types and is absent in primary cells. This phosphorylation has been proposed to activate nuclear entry as well as the apoptotic activity of Apoptin. In the same studies, the ablation of phosphorylation at this site by mutagenesis of the target threonine residue was shown to nullify any such activation and translocation.

During the execution of the work presented in the previous chapters, a significant amount of time was devoted to verification and clarification of Apoptin cell type-specific phosphorylation. Efforts were made to dissect the specific functional consequences of T108 phosphorylation on localization and apoptotic activity, however many of the results obtained were contradictory to various aspects of the published literature. Those results that were in agreement with what has previously been reported remain open to interpretation. This section serves to introduce some of the unpublished findings elucidated from these efforts and to illustrate areas of consistency and inconsistency from previously published results.
Materials and methods

Cells and adenoviruses. H1299 and primary lung fibroblast cells (MRC-5) were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum plus 10 mg/ml streptomycin and 10 U/ml penicillin (Sigma) at 37°C under 5% CO₂ (95% air).

Ad-Apwt, Ad-Ap-pmNES, and Ad-LacZ were prepared as previously described (Chapter II). All cells were infected at approximately 80% confluence at an MOI of 35. Adenovirus was added to cells in a minimal volume of culture media and gently agitated at 37°C under 5% CO₂ for 1 h. Following infection, culture media was added and cells were incubated for indicated times.

Plasmid constructions. GFP- and Flag-Apoptin phosphorylation site point mutations were generated by PCR-mediated site directed mutagenesis of indicated residues followed by cloning into either the pEGFP-C1 (Clontech) or p3XFLAG-myc-CMV-26 (Sigma) vectors via engineered EcoRI and BamHI restriction sites. All constructs were confirmed by DNA sequencing.

Metabolic labeling and two-dimensional electrophoresis. Thirty-six hours following infection with either Ad-Apwt, Ad-pmNES or Ad-LacZ virus, cells were washed in PBS and placed into DMEM (10% FBS) without phosphate for 20 min. Media was then replaced with a minimal volume of phosphate lacking DMEM supplemented with ~1.5mCi ³²P-orthophosphoric acid. Cells were labeled for 2 hours, washed 3x in PBS,
and harvested by scraping. Cells were then lysed in RIPA buffer [150 mM NaCl, 1% NP40, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, 5 mM EDTA, Complete Mini tablet (Roche)] and subjected to immunoprecipitation (see below).

For two-dimensional electrophoresis, recovered immunoprecipitates were first subjected to isoelectric focusing (pH range of 6.0-10.0) using a Zoom Runner system (Invitrogen; see commercial protocol for details). The second dimension was accomplished via SDS-PAGE (gradient 6-15%) (Invitrogen). Gels were dried, and labeled protein was visualized by autoradiography.

**Immunoprecipitations.** Following 9,000 x g centrifugation for 30 min., supernatants were incubated with 20 μl equilibrated EZview Red α-Flag M2 affinity beads (Sigma) at 4°C for 4 h. Beads were washed in RIPA buffer, and bound proteins were eluted in 1X sample resuspension buffer (Invitrogen).

**Apoptosis assays.** For cell viability assays, H1299 and PFF cells transfected with Flag-Apoptin phosphorylation site mutants were harvested, washed in PBS, and stained with ViaCount reagent (Guava Technologies). Viability was quantified using a Guava Personal flow cytometer. Data points were collected as percent cell viability per 5000 events. For H1299 cells, data was graphed as fold X increase of wild type activity, where Apwt was set to 1. For MRC-5 cells, data was graphed as percent Apoptosis.
Results

Apoptin phosphorylation state differs between primary and transformed cells.

Previous reports have suggested that Apoptin may be controlled by phosphorylation (151, 193). Differences between the primary or transformed cell phosphorylation status of Apoptin could represent a potential regulatory mechanism. Therefore, the phosphorylation status of Apoptin between primary and transformed cells was examined by metabolic $^{32}$P-ortho-phosphate labeling. H1299 and MRC-5 primary lung fibroblast cells were infected with either Ad-Apwt or Ad-LacZ virus and after 36 hours were labeled with $^{32}$P-orthophosphoric acid. Cell extracts were then subjected to Flag immunoprecipitation followed by SDS-PAGE and autoradiography to visualize labeled Apoptin protein. Contrary to previous reports, Figure 5.1a shows that compared to Ad-LacZ infected cells, Apoptin is robustly phosphorylated in primary as well as transformed cells. Similar results were obtained using normal PFF cells (data not shown).

The lack of any appreciable difference in phosphorylation status between normal and transformed cells prompted examination of the patterns of Apoptin phosphorylation in these two cell types. To address this, H1299 and MRC-5 cells were again infected with Ad-Apwt virus and subjected to metabolic labeling and immunoprecipitation as before. Two-dimensional electrophoresis was then performed on the resultant IP material by first dimension isoelectric focusing followed by second dimension gradient SDS-PAGE. Proteins were then visualized by autoradiography as before. Figure 5.1b shows that Apoptin phosphorylation status differs greatly between normal and transformed cell types as the degree of phosphorylation is more extensive in transformed cells.
Additionally these results suggest that at least 4 separate phosphorylation events occur in H1299 cells as evidenced by labeled foci separated by the second dimension. Together these results suggest that although Apoptin is phosphorylated in both normal and transformed cell types, the residues that are phosphorylated differ between these two cell types.

**T108 region modulates Apoptin behavior.** Previous studies have suggested that T108 phosphorylation specifically activates Apoptin. To verify such observations, several point mutants were constructed in both GFP and Flag vector backgrounds and are detailed in Figure 5.2a. As a test for the necessity of T108 phosphorylation, this site was substituted with alanine. A T108E mutation was also constructed similarly to previous reports that suggest this mutation to simulate phosphorylation (193). T108 is part of a triplet of threonine residues within the C-terminus of Apoptin. Given the evidence of Figure 5.1b for multiple phosphorylations, a mutant was also constructed where threonines 106, 107 and 108 were mutated to alanine.

Surprisingly, Figure 5.2b shows that expression of GFP-Apoptin phosphorylation mutants into both MRC-5 and H1299 cells results in nuclear localization in all cases, as compared to GFP-Apwt, which maintains the expected cell type-specific localization behavior.

To assess the apoptotic potential of these proteins, Flag epitope tagged versions of each mutant were transiently expressed in both H1299 and MRC-5 cells and after 72 hours, cell viability was quantified by flow cytometry. Figure 5.2c shows that relative to
Flag-Apwt, the Flag-Ap(T108E) mutation had approximately 7-fold greater apoptotic activity in H1299 cells. Flag-Ap(T106,107,108A) showed negligible activity, however, Flag-(T108A) expression yielded levels of apoptosis approximately 2-fold higher than that of Flag-Apwt. These results indicate that mutation of T108 to either alanine or glutamic acid is able to increase the apoptotic activity of Apoptin, especially in the case of the latter.

As expected, Flag-Apwt showed negligible apoptotic activity in MRC-5 cells. Similarly, the Flag-Ap(T108A) and Flag-Ap(T106,107,108A) mutants showed no appreciable amount of apoptosis, despite the nuclear localization observed in Figure 5.2b. Interestingly, the Flag-Ap(T108E) mutant showed a robust ability to induce apoptosis in MRC-5 cells.
Discussion

The results of Figure 5.1 indicate that, in contrast to previous reports, kinase activity(ies) responsible for Apoptin phosphorylation is not restricted to transformed cells but occurs in normal cells as well. These results also indicate that T108 is not the only residue that is phosphorylated. Apoptin may have as many as four phosphorylated residues in transformed cells suggesting that alternate phosphorylations may contribute to (or control) the activity of Apoptin. Several attempts to identify such residues by mass-spectrometry were unsuccessful, possibly due to the labile nature of these phosphorylations. Additionally, T108 phosphorylation specifically was not observed in these attempts. Other groups have attempted to verify T108 phosphorylation in transformed cells by metabolic labeling and mass-spectrometry and have also reported inability to verify this modification (176).

Consistent with what has been reported, however, substitution of T108 does affect the killing capacity of Apoptin. Interestingly, modification of T108 to either alanine or glutamic acid was able to increase apoptotic activity, however T108A appears to have retained cell-type specificity and was hyperactive to a lesser degree than was T108E. Given the unpredictable nature of amino acid substitution, it is formally possible that the T108E mutation in this case is acting as a loss of function mutant as apposed to the anticipated phosphorylation site “mimic”. Thus, given the data obtained from the T108A mutant, it is possible that blocking phosphorylation at this site has activating potential. Additionally, activation may also be related to conformational changes within this area in response to the amino acid substitutions. Introduction of charged residues such as
glutamic acid into this region may alter the local geometries of the C-terminus or may have protein-wide consequences. This would be understandable as Apoptin is a small protein with multiple overlapping functional regions. Through these studies it is at least clear that the nature of Apoptin phosphorylation and its consequences on protein function are more complex than is suggested in the literature.
Figure 5.1. Apoptin phosphorylation status differs between primary and transformed cells. (A) 24 hours after introduction of either LacZ or wtAp adenoviruses (or mock infected samples) Apoptin phosphorylation status was monitored in MRC-5 and H1299 cells by metabolic $^{32}$P ortho-phosphate labeling for 3 hours followed by immunoprecipitation of Apoptin using a-Flag affinity resin. Immunoprecipitate was separated by SDS-PAGE and phosphorylated proteins were visualized by autoradiography. Apoptin protein levels were monitored by $\alpha$-Flag western blot of whole cell extracts prepared in parallel. (B) 2-dimentional gel electrophoresis of $^{32}$P ortho-phosphate labeled Apoptin protein recovered from (A) in either H1299 or MRC-5 cells.
Figure 5.1

A

MRC-5

H1299

P\text{\textsubscript{32}}\_Flag

\text{\textsubscript{32}}P

B

pH 10 6

H1299

MRC-5

Ad-wtAp \text{\textsubscript{32}}P
Figure 5.2. Mutation of putative C-terminal phosphorylation region of Apoptin modulates sub-cellular localization and killing activity. (A) Schematic of Apwt vs. Apoptin phosphorylation site mutations. (B) Sub-cellular localization of GFP-Apoptin phosphorylation site mutants compared to GFP-Apwt in both H1299 and MRC-5 cells. (C) Analysis of apoptotic capacity of Flag-Apoptin phosphorylation site mutants in either H1299 or MRC-5 cells. H1299 cell viability is shown as fold increase over Apwt activity (which is normalized to 1). MRC-5 cell viability is shown as % apoptosis.
Figure 5.2

A

FLAG or GFP
S-L-I-T-T-P-S
GFP-Apwt
GFP-Ap(T108E)
GFP-Ap(T108A)
GFP-Ap(T106,107,108A)

B

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C

Fold X of Apwt

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REFERENCES


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