MOLECULAR MECHANISMS OF E1A-INDUCED APOPTOSIS IN THE HEART

BY KAREN M. ENS

A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment

of the Requirements for the Degree of

MASTER OF SCIENCE

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Abstract

The molecular mechanisms that regulate E1A-mediated apoptosis remain poorly defined. Evidence suggests important roles for the ubiquitously expressed transcription factor nuclear factor kappa beta (NFKB) with respect to E1A, and the mitochondria with respect to apoptosis. E1A's effect on mitochondrial function has not been formerly investigated.

Methods: Neonatal ventricular myocytes were induced to express E1A and then assayed for the incidence of apoptosis. NFKB-dependent transcriptional activity was assessed by luciferase assay, which was normalized with a βgal assay. The effect of E1A on post-translational modification of NFKB was examined by western blot analysis and electromobility shift assay. Mitochondrial perturbations including cytochrome c and smac release and permeability transition pore opening were monitored by western blot analysis and fluorescent microscopy respectively. Following overexpression of IKKB and BNIP3ΔTM (deficient in mitochondrial membrane integration), the effect on mitochondrial defects and apoptosis were examined.

Results: Compared to control, E1A induced a 5-fold increase ($P<0.05$) in apoptosis of neonatal ventricular myocytes. The p300 binding domain of E1A repressed NFKB-dependent gene transcription (2.3-fold decrease) by directly effecting the transactivation domain of NFKB (p65) at the level of transcription, as opposed to its normal regulation at the post-translational level. NFKB-DNA binding was not inhibited. Mitochondrial perturbations including opening of PT pore and release of Cytochrome c and Smac into the cytosol were observed in

myocytes expressing E1A, and were inhibited by adenoviral delivery of BNIP3ΔTM. Moreover, apoptosis was rescued in E1A-expressing myocytes if IKKB or BNIP3ΔTM was overexpressed as well.

Conclusions: The results of this study have identified two modes by which E1A induces apoptosis of neonatal ventricular myocytes. (1) By repressing NFKB-dependent genes, and (2) by inducing BNIP3 integration into mitochondrial membranes, triggering the apoptotic cascade. We have also identified a method for rescuing these effects; overexpressing IKKB and BNIP3ΔTM by adenoviral delivery.

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List of Abbreviations

E1A	-Adenovirus early region one A
NFKB	-Nuclear factor kappa beta
Smac	-Second mitochondria derived activator of caspase
DIABLO	-Direct IAP-binding protein with low pI
IKKB	-Inhibitor of kappa beta kinase
BNIP3	-Bcl-2 19 kDa interacting protein 3
PT pore	-Permeability transition pore
IKBα	-Inhibitor of kappa beta
TNFα	-Tumor necrosis factor alpha
IL-1β	-Interleukin-1 beta
AIF	-Apoptosis inducing factor
Caspase	-Cysteine aspartic-acid specific protease
Rb	-Retinoblastoma product
ROS	-Reactive oxygen species
SIMPS	-Soluble intermembrane proteins
VDAC	-Voltage-dependent anion channel
ANT	-Adenine nucleotide translocator
Apaf-1	-Apoptotic protease activating factor 1
IAP	-Inhibitor of apoptosis
ICE	-Interleukin-1β converting enzyme
FADD	-Fas-associated protein with death domain
DED	-Death effector domain

CARD	-Caspase recruitment domain
DMEM	-Dulbecco's modified Eagle's medium
FBS	-Fetal bovine serum
PBS	-Phosphate buffered saline
CMV	-Cytomegalovirus promoter
PVDF	-Polyvinylidene difluoride
ECL	-Enhanced chemiluminescence
Luc	-Luciferase
$\Delta\Psi M$	-Mitochondrial membrane potential
calcein-AM	-Calcein-acetoxymethylester
NLS	-Nuclear localization signal
HEK293	-Human embryonic kidney cells 293
TUNEL	-TdT mediated deoxy-triphosphate nick end labeling
TdT	-Terminal deoxynucleotidyl transferase
MEKK-1	-Mitogen activated protein kinase kinase kinase 1
DFSF	-DMEM F-12 serum free

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I. Introduction

After birth cardiac muscle cells lose their capacity for proliferative growth and exit the cell cycle (2). Subsequent growth of the post-natal heart occurs by cardiac hypertrophy rather than hyperplasia (3). Therefore, the inadvertent loss of cardiac muscle cells during disease states may contribute to ventricular remodeling and negatively impact upon cardiac performance. Evidence suggests that myocyte cell death occurs involves apoptosis, but the degree of involvement varies between different reports (9, 38, 42-49).

Apoptosis, is the term used to describe programmed cell death. This death pathway is genetically programmed within cells, and is an evolutionarily conserved event, which occurs in many species, illustrating the importance of apoptosis for normal function and development. Normal development of tissues and organs in multi-cellular organisms depends on apoptosis to remove cells that are no longer required. At the fetal stage, apoptosis ensures the correct number of cells is positioned in the fetus (10). Tissue regression via apoptosis, causes digit formation on the hands and feet (22). It is crucial for organism survival to maintain homeostasis, by balancing cell life and cell death. Excessive apoptosis can be detrimental to cells and whole organisms. Dysregulation of apoptosis can result in tumorigenesis, autoimmunity, hematopoietic deficiencies, infertility (24), and neurodegenerative diseases such as Alzheimer's disease (25), Parkinson's disease (26), and Huntington's disease (27).

Apoptosis is an energy-dependent process, and is morphologically characterized by cell shrinkage, chromatin condensation, DNA fragmentation,

membrane blebbing, and phagocytosis by neighbouring cells (10). During the entire apoptotic process, the body's immune system continues to function as per usual, because the membrane doesn't actually rupture, as it does during necrosis (11).

In mammalian cells, the transcription factor; nuclear factor kappa beta (NFKB), comprised of 50 kDa and 65 kDa protein subunits, bound to the cytoplasmic inhibitor protein IKBα (54), is a key anti-apoptotic player. Activation of NFKB by cytokines such as TNFα or IL-1β (55, 56) involves the N-terminal phosphorylation and degradation of IKBα by the ubiquitination-proteasome pathway (58-63). This permits NFKB to translocate to the nucleus and direct gene transcription (64-66).

Mitochondria play a big role in regulating the apoptotic signaling pathway (198). Apoptotic signals cause perturbations to mitochondria, resulting in the loss of $\Delta\Psi m$, from the opening of a large multi-protein conductance channel referred to as the permeability transition (PT) pore (171), and the release of mitochondrial proteins into the cytosol, such as Cytochrome c, AIF (apoptosis inducing factor), and Smac/DIABLO (second mitochondria derived activator of caspase/direct IAP-binding protein with low pI) (158-161). Their association with specific cellular factors, including dATP triggers the activation of caspases, and finally ending in apoptosis of the cell (158, 199).

The link between the apoptotic signal and mitochondrial defects can be attributed in part to the work of pro-apoptotic Bcl-2 family members. In general, this family contains at least one Bcl-2 homology (BH) domain (BH1, BH2, BH3,

and/or BH4). All anti-apoptotic members contain the BH4 domain (136), including Bcl-2 itself. The mechanism by which Bcl-2 prevents apoptosis involves NFKB activation. Bcl-2 activates MEKK-1, which further activates IKKB. IKBα is phosphorylated and degraded, facilitating NFKB activation (142-144). Pro-apoptotic members lack BH4, and contain BH3. Bid cleavage by caspase-8 produces tBid, which translocates from the cytosol to mitochondria. There it causes a change in Bax conformation followed by insertion into the membrane. Bax can also be cleaved by calpain, which produces an 18 kDa fragment capable of inserting into the membrane, facilitating cytochrome c release (155, 156). This insertion appears critical since release of mitochondrial proteins soon follow.

BNIP3 is one of the more recently identified Bcl-2 pro-apoptotic members. Recently, Regula *et al.* (150) demonstrated that BNIP3 mitochondrial membrane insertion was involved in hypoxia-mediated apoptosis, and rescue could be achieved with BNIP3ΔTM, which is a carboxyl terminal transmembrane deletion mutant of BNIP3, unable to integrate into the mitochondrial membrane.

The 12S E1A gene product of the Adenovirus early region one protein (E1A) is a dual function protein that can promote cell cycle re-entry and transformation of rodent cells in the presence of oncogenic Ras or adenovirus E1B 19 kDa proteins. This property has largely been attributed to the inactivation by E1A of the tumor suppressor protein Rb and transcriptional co-activator protein p300. Alternatively, a less defined role of E1A is its ability to provoke apoptosis of cells. In this regard, we have previously shown that 12S E1A mediates apoptosis in ventricular myocytes (89). The mode by which E1A

provokes apoptosis in cells is poorly defined but it may impinge upon key components of the cell death pathway that are antagonized or neutralized by E1B 19 kDa or Bcl-2 proteins.

Therefore, **the main goal of my research studies are: (I) to verify that E1A induces apoptosis in neonatal ventricular myocytes. (II) to identify the molecular mechanisms and signaling pathways involved in E1A-mediated apoptosis.** The specific aims of the study are: (1) To determine the impact of 12S E1A protein on the NFKB signaling pathway of neonatal ventricular myocytes with respect to cell death. (2) To determine if E1A-mediated apoptosis involves mitochondrial perturbations. (3) To determine if Bcl-2 pro-apoptotic member; BNIP3 plays a role in E1A-mediated apoptosis. (4) To evaluate the impact of compensatory mechanisms to rescue E1A-mediated apoptosis.

I. Literature Review

A. Heart Failure

The heart is a simple pump, receiving and ejecting blood to body tissues. However, if the heart is unable to pump sufficient blood to meet the metabolic needs of the body (1) the syndrome known as heart failure arises. The problem stems from the fact that cardiac myocytes lose their ability to divide shortly after birth (2). Therefore, if heart failure occurs due to a variety of cardiovascular diseases, with ischemic, hypertensive, toxic, and inflammatory results, the heart is unable to repair itself. For this reason, heart failure is one of the leading causes of morbidity in North America. In an attempt to maintain cardiac output, the heart adapts compensatory mechanisms, such as hypertrophy rather than hyperplasia of myocytes (3), and dilation and enhanced activity of the sympathetic nervous system and renin angiotensin system (4). Ironically, these short-term compensatory responses eventually become detrimental, and themselves contribute to the process of heart failure. The molecular mechanisms involved in myocardial dysfunction and clinical heart failure are poorly understood, but include abnormal calcium handling (5), impaired excitation-contraction coupling (6), ATP synthesis (7), and oxidative stress (8). There is increasing evidence suggesting that cell death in the heart involves apoptosis (9, 38, 42-49).

B. Apoptosis

B.1 Definition

In 1972, the term "apoptosis" emerged to describe a highly ordered, conserved mode of cell death (10). Unlike necrosis, which is cell death that follows severe injury or interference with the nutrition of tissues, and is characterized by cell swelling, membrane rupture, and a triggered immune response, all independent of ATP, apoptosis is ATP-dependent and occurs without membrane rupture, and therefore doesn't trigger the body's immune response (11). Cells undergoing this programmed cell death have a characteristic morphology: shrinkage of the cell, DNA fragmentation, membrane blebbing, nuclear condensation, and finally phagocytosis by neighbouring cells (10), all of which can be visualized by electron microscopy (12). However, the apoptotic process begins long before the morphological changes can be observed. First, there is an initiation phase, where a pro-apoptotic stimulus activates the apoptotic molecular pathway. Second, there is an effector phase, where the pathway becomes fully activated. Last, there is the degradation phase which can be visualized (13, 14).

B.2 Biochemical hallmarks of apoptosis

In order to identify typical morphological features of apoptosis, a number of biochemical techniques can be used. DNA fragmentation occurs by endogenous DNases which cut the double-stranded DNA into 180-200 base pair fragments (15). The DNA is isolated and subjected to gel electrophoresis,

where apoptosis can be detected as a distinct ladder of fragmented DNA (16). Necrosis would be visualized as a smear of degraded DNA due to random cutting by degradative enzymes (17). However, this method is not very sensitive, and cannot detect small quantitative differences, but it is useful to strengthen other methods of detecting apoptosis.

Morphologic criteria are more reliable methods for evidence of apoptosis. Fluorescent microscopy can be used to visualize nuclear morphology. Hoechst 33258 is a fluorescent dye that stains the nuclei of isolated cells (18). Normal cell nuclei stain blue and appear round and full, while apoptotic cell nuclei appear fragmented and smaller. It should be noted that this technique may be subjective when analyzing nuclear morphology, however the overall pattern is usually obvious.

A biochemical assay known as TUNEL (TdT mediated deoxy-triphosphate nick end labeling) involves the polymerization of fluorescein labeled dUTP nucleotides to free 3'-OH DNA ends by a TdT (terminal deoxynucleotidyl transferase). This technique relies on the fact that apoptosis yields single-stranded breaks in high molecular weight DNA and in double-stranded, low molecular weight DNA fragments, exposing the 3'-OH ends (19). The fluorescein labeled DNA can be visualized using fluorescence microscopy or flow cytometry. TdT will incorporate the fluorescein label to any free 3' end, therefore it should be noted that this is not only a feature unique to apoptosis.

B.3 Good versus Evil

In order to live, we must have death. Apoptosis is a method by which unwanted or damaged cells or tissue can be removed from the body (20,21). Apoptosis is a crucial part of normal embryogenesis by removing excess cells, and genetically damaged cells from the developing embryo (22). Cells that are no longer required are also removed by apoptosis. For example, digit formation on the hands and feet occurs because apoptosis causes the tissue in between the digits to regress (22). In fact, insufficient removal of cells is associated with proliferative disorders such as cancer (23). Physiologically, apoptosis is responsible for the removal of autoreactive T cells and thymic involution, and for renewing mature cells, such as leukocytes (15,40-41). Alternatively, dysregulation of apoptosis can have detrimental results, including tumorigenesis, autoimmunity, hematopoietic deficiencies, infertility (24), and neurodegenerative diseases, including Alzheimer's disease (25), Parkinson's disease (26), and Huntington's disease (27).

B.4 Apoptosis in heart failure

Terminally differentiated cells, such as myocardial or neuronal cells do not normally undergo apoptosis. However, apoptosis can be induced by hypoxia (28,29), oxidative stress (30), ischemia-reperfusion (31-33), post-infarction (34-36), and in patients who are in end-stage heart failure (37,38). In fact, as hypothesized by Bing in 1994, apoptosis may be the link between chronic pressure overload and heart failure (9). Kajstura *et al.* (1996) later demonstrated

that apoptosis preceded necrosis after myocardial infarction in rats (34). The following year, Saraste *et al.* verified these results in human hearts following acute myocardial infarction (39).

In 1996, Narula *et al.* examined explanted human hearts that had either idiopathic dilated cardiomyopathy or ischemic cardiomyopathy. Techniques used to detect apoptosis (electrophoresis of end-labeled DNA and histochemically by in-situ end-labeling with dUTP), both revealing the involvement of apoptosis. They concluded that myocyte death occurred due to an apoptotic process in patients with end-stage cardiomyopathy (38).

Although it is known that apoptosis is involved in myocyte cell death, the degree of involvement varies between reports from several investigators (38, 42-49).

C. Genetic regulators of apoptosis

C.1 Tumor suppressor proteins; p53 and Rb/E2F1

P53 is a transcription factor that regulates cell cycle control and apoptosis. It acts as a sensor, waiting for signals that cause stress to the cell, and then responds by facilitating adaptive and protective responses. Stresses such as damage to DNA and gamma irradiation cause phoshorylation followed by accumulation and activation of p53 (119). The result for the cell will either be apoptosis or growth arrest if DNA repair is possible (120, 121). P53 itself is strictly regulated to keep it in check until it is actually needed to avoid catastrophe (111). Greater than 50% of all human malignancies are due to genetic abnormalities in the p53 gene or its regulation (113). The importance of

p53 is illustrated by a classic study which showed that fibroblasts from human tumors lacking p53, also were resistant to apoptosis (112-114). When p53 was replaced back into the fibroblasts, normal growth arrest and apoptosis occurred (112, 115, 116). Kirshenbaum *et al.* were among the first to show that overexpression of p53 induced apoptosis of ventricular myocytes (117). Furthermore, studies by Long *et al.* determined that hypoxia-mediated apoptosis of cardiomyocytes may work by increasing p53 protein expression (118).

E2F family member E2F1 activation provokes apoptosis and initiates S-phase progression (122). E2F1 S-phase transcriptional activity is regulated by the retinoblastoma gene product (Rb) (123), which is a tumor suppressor protein. Loss or inactivation of Rb allows for release and activation of E2F1 (124, 125). Several studies have shown that E2F1 mediates apoptosis through different mechanisms. E2F1 has been linked to p53-dependent apoptosis (126), and p53-independent apoptosis involving the p53 homologue p73 (127, 128). Studies by Chen *et al.* (129) and Tanaka *et al.* (130) suggest that E2F1 regulates anti-apoptotic transcription factor nuclear factor kappa B (NFKB) activity by stabilizing IKB and thus inhibiting nuclear translocation. Tanaka *et al.* (130) also reported that E2F1 induces accumulation of reactive oxygen species (ROS) which enhances serum-deprived apoptosis in NIH3T3 and Saos-2 cells. Furthermore, Shelat *et al.* (131) published that overexpression of E2F1 induces caspase-3 activity followed by apoptosis in coronary vascular smooth muscle cells.

C.2 Bcl-2 family proteins

The Bcl-2 family of proteins consists of both inhibitors and promoters of apoptosis. The road to the discovery of the Bcl-2 family began in 1986, when Ellis and Horvitz discovered that two loci, ced-3 and ced-4 were essential for apoptosis during development of Caenorhabditis elegans, and a third locus, ced-9 could prevent apoptosis (137). Bcl-2 was originally identified as a gene that undergoes a chromosome translocation breakpoint (t14:18) in human follicular lymphoma (138), rendering bcl-2 inactive and thus allowing continual cell growth, and apoptotic resistance. It was later discovered that ced-9 was a functional and structural homolog of Bcl-2 (139). It is believed that it is the ratio of pro- versus anti-apoptotic Bcl-2 family proteins that decides whether a cell will live or die (132). Anti-apoptotic members include Bcl-2, Bcl-XL, Bcl-W, BFl-1, Mcl-1, and A1. Pro-apoptotic members include Bax, Bak, Bad, Bid, Bim, Bik, Bcl-Xs, Bok_s, BNIP3, Nix, and Hrk. Most members contain a hydrophobic C-terminus known as the transmembrane (TM) domain, which allows for insertion into intracellular membranes, particularly into the outer mitochondrial membrane (133-135). Four conserved domains, termed Bcl-2 homology (BH) domains, have been identified amongst the different Bcl-2 family member proteins: BH1, BH2, BH3, and BH4. Each family member possesses at least one but not all BH domains. However, all anti-apoptotic members contain the BH4 domain (136), which is located near the N-terminus. In contrast, all pro-apoptotic members lack BH4, except Bcl-Xs. The

BH3 death domain was originally identified in Bax (136), but has since been identified in the other pro-apoptotic members.

The mechanism by which Bcl-2 prevents apoptosis is not entirely known. However, it appears that the blockage occurs early in the apoptotic pathway because none of the typical morphological changes occur (140). Kirshenbaum *et al.* formally tested whether Bcl-2 would prevent apoptosis in cardiac myocytes provoked by p53 and TNFα, and discovered that it does. Mechanistically, Kirshenbaum *et al.* discovered that Bcl-2 functions via its BH4 domain which interacts with Raf-1, activating MEKK-1, which further activates IKKB. IKKB then phosphorylates IKBα, which causes its degradation, thereby activating NFKB, and allowing it to translocate to the nucleus. Therefore, the anti-apoptotic function of Bcl-2 works by ultimately activating NFKB (141-144). Another anti-apoptotic function for Bcl-2 is its role in an antioxidant pathway at sites of free radical generation. Bcl-2 rescued cells from H_20_2- and menadione-induced oxidative deaths (145).

BNIP3 is a novel pro-apoptotic member of the Bcl-2 family, that was initially identified as an adenovirus E1B 19-kd interacting protein (146, 147). It appears that BNIP3 may provoke apoptosis by disrupting mitochondrial function (148, 149). Recently, Regula *et al.* demonstrated that the carboxyl-terminal transmembrane domain of BNIP3 is necessary for mitochondrial insertion, and BNIP3ΔTM is sufficient to rescue hypoxia-induced apoptosis of neonatal cardiomyocytes by preventing endogenous BNIP3 protein from integrating into the mitochondrial membrane (150). The question of what causes BNIP3 to

integrate into the mitochondrial membrane remains unanswered. However, a look at insertion of other pro-apoptotic members may provide some insight.

In liver, tBid translocates from the cytosol to the mitochondria following Bid cleavage by caspase-8. It appears that it localizes to preferred contact sites that are rich in cardiolipin (151). Bid is also cleaved by calpain following ischemia/reperfusion in rabbit cardiomyocytes, although the fragments are different (152). Both cleavages mediate cytochrome c release into the cytosol.

Bax is usually in a conformation that prevents the protein from integrating into membranes. However, if there is an apoptotic signal, Bax inserts into mitochondrial membranes. Bax activation and insertion occurs a number of different ways. tBid can change Bax conformation, revealing the transmembrane signal, resulting in Bax insertion. However, Bid is not required, as demonstrated with Bid-null mice that are exposed to TNFα and E1A, which results in insertion as well (153). Also, calpain has been shown to cleave Bax to an 18 kDa fragment, which inserts into the membrane, facilitating cytochrome c release (155, 156).

C.3 Mitochondria

Mitochondria are traditionally thought of as the "powerhouse" of the cell. The mitochondrial respiratory chain together with the H+ATPase are localized in the inner mitochondrial membrane where they generate ATP molecules to control the energy requirements within the cell. The inner membrane is impermeable to ions, which allows the production of an electrochemical gradient ($\Delta\Psi m$) which

drives ATP synthesis (156). The mitochondrion has its own genome, consisting of 16 kb of circular DNA, which encodes less than 1% of mitochondrial proteins, all found in the inner membrane. The remaining mitochondrial proteins are synthesized in the cytosol from nuclear DNA, and then imported into the mitochondria. More recently, research has focused on the role that the mitochondria play in apoptotic cell death. Perturbation to mitochondria cause opening of a large non-specific ion channel called the permeability transition (PT) pore, which causes the $\Delta\Psi m$ to collapse (171), starting the apoptotic process (156, 157). The PT pore spans the inner and outer mitochondrial membranes, and is made up of a voltage-dependent anion channel (VDAC), an adenine nucleotide translocator (ANT), cyclophilin D, a benzodiazapene receptor, and hexokinase-1 that together form a channel that can allow the passage of molecules that are $\leq$1.5 kD (172, 173). Known inhibitors of the PT pore include cyclosporins and bongkrekic acid. Opening of the PT pore results in swelling of the matrix. The outer membrane will rupture because the inner membrane is folded into cristae, and therefore has a very large surface area compared to the outer membrane. Leaky mitochondria allow the release of a handful of soluble intermembrane proteins (SIMPS) into the cytoplasm. SIMPS include Cytochrome c (158), AIF (apoptosis inducing factor) (159), and Smac/DIABLO (second mitochondria derived activator of caspase/ direct IAP-binding protein with low pI) (160, 161). SIMPS in the cytoplasm trigger the activation of caspases and caspase-dependent pathways, resulting in apoptosis. Normally, Cytochrome c is bound to the inner mitochondrial membrane. An apoptotic stimulus causes its

release into the cytoplasm, where it triggers the assembly of a complex called the apoptosome, made up of dATP, Apaf-1 (apoptotic protease activating factor 1), and caspase-9 (163, 164). The active apoptosome recruits and activates pro-caspase-9, which is a prolific initiator caspase. The cell is protected from this deadly trigger while it is being synthesized and transported to the mitochondria because cytochrome c does not become active until after the addition of a haem group which takes place once it is in the mitochondria (174). Interestingly, Heiskanen *et al.* (175) revealed that $\Delta\Psi m$ loss began after Cytochrome c release had been initiated.

AIF are released from the mitochondria following an apoptotic signal. Some researchers suggest that AIF is released prior to cytochrome c (165, 166), and this causes Cytochrome c release, as well as chromatin condensation, and loss of $\Delta\Psi m$. Cytochrome c release leads to caspase activation as AIF appears to be caspase-independent (167).

The inhibitor-of-apoptosis (IAP) proteins suppress cell death by inhibiting the activity of mature, active caspases (168). Once Smac/DIABLO has been released into the cytosol, it promotes apoptosis by binding to IAPs, and thereby eliminates their inhibitory effect (169, 170).

Who is ultimately in control, the mitochondria or the cell? This question is argued from both sides, and each have evidence to support its claims. It appears that Bcl-2 family members residing in the cytosol or outer mitochondrial membrane, regulate the release of SIMPS; anti-apoptotic members inhibit their release, while pro-apoptotic members promote their release (162), suggesting

that the cell is in control. Others believe that the loss of $\Delta\Psi m$ resulting in mitochondrial membrane permeabilization is responsible for the subsequent release of SIMPS, thereby putting the mitochondria in control of the fate of the cell.

C.4 Caspases

Ced-3 is the product of a gene responsible for mediating cell death in the nematode *Caenorhabditis elegans* (137). The mammalian homologue of ced-3 was identified as Interleukin-1 β converting enzyme (ICE) (176) now referred to as caspase-1 or cysteine aspartic-acid specific protease. Over twenty members have been identified thus far. They specifically cleave proteins at a site following an aspartic acid amino acid (177). All caspases are initially inactive zymogens, that are activated once they are proteolytically processed at the P1 position of internally conserved Asp residues (178). The inactive zymogen is made up of an N-terminal domain, a large subunit (~20 kD), and a small subunit (~10 kD). Upon cleavage and activation, the large and small subunits heterodimerize (179).

Specifically, caspase-1 does not play a role in cell death, but other members have a distinct role in apoptosis, as either initiators or effectors. Upstream initiator caspases include; caspase-8, -9, and –10. Downstream effector caspases include; caspase-3, -6, and –7 (180). Initiator caspases are activated when a pro-apoptotic signal triggers a specific cofactor to bind to the procaspase, resulting in activation. For example, the cofactor for procaspase-8 is FADD (Fas-associated protein with death domain) with binding at the DED

(death effector domain) (181). Cofactor for procaspase-9 is APAF-1 with binding at the CARD (caspase recruitment domain) (182). Initiator caspases activate effector caspases, resulting in apoptosis.

Other roles of caspases include; inactivating anti-apoptotic proteins, cleaving nuclear lamins (contributing to chromatin condensation), and disrupting cytoskeleton regulation (183).

D. Adenovirus E1A

The first viral gene to be transcribed in Adenovirus is early region one protein (E1A). The primary E1A transcript is differentially spliced to yield different messages. Two major transcripts are 12S and 13S gene products, which translate into 243 and 289 amino acid proteins, respectively (87). Conserved regions CR1 and CR2, and the amino-terminus of E1A are essential for protein-protein interactions and biological activity of E1A. The retinoblastoma gene product, Rb binds to a region comprising both CR1 and CR2 (85-86), whereas the transcriptional coactivator, p300 binds to a region comprising the amino-terminus of E1A and CR1 (82). E1A-Rb binding allows the release of transcription factor E2F, which promotes cells to re-enter the cell cycle and initiate DNA replication. E1A-p300 binding causes the accumulation and stabilization of p53, inducing apoptosis (90-91). The 13S gene product contains an additional domain CR3, which encodes a domain necessary for viral replication (86). This makes 12S E1A the better choice and a useful tool for

studying the underlying mechanisms that regulate cellular growth and development (85-86).

Alternatively, a less defined role of E1A is its ability to provoke apoptosis of cells in the absence of E1B, an Adenovirus anti-apoptotic viral protein (88). Kirshenbaum and Schneider confirmed that 12S E1A, in the absence of E1B, induces apoptosis in cardiac myocytes (89). 12S E1A + E1B and R2GC124G (mutant E1A that cannot bind Rb or p300) + E1B did not show signs of apoptosis, similar to uninfected cells.

The mode by which E1A provokes apoptosis in cells is poorly defined, however it has been shown that E1A sensitizes cells to various stimuli, such as ionizing irradiation, DNA-damaging agents, serum starvation and tumor necrosis factor alpha (TNFα), making them more vulnerable to apoptosis (92-93). Independent studies have demonstrated that E1A mediates sensitization of radiation-induced apoptosis and TNFα- induced apoptosis through inhibition of NFKB activity (92, 94-95). Furthermore, Cook *et al.* suggests that E1A-Rb binding in mouse fibroblasts, blocks the NFKB dependent activation response to TNFα, at the transcription level by altering p65 activity (95). E1A has also been shown to repress basal transcription from the human immunodeficiency virus type1 (HIV-1) long terminal repeat (LTR). Inhibition requires the N-terminal domain of E1A, specifically amino acids 4 to 25 are crucial for repression (96). Interestingly, it was found that the HIV LTR promoter contained NFKB binding sites between –106 and –77, which can mediate E1A inhibition of transcription (97).

E. Nuclear factor kappa B

NFKB is a transcription factor expressed in a wide variety of cells. In 1986, Sen and Baltimore first identified NFKB as a nuclear protein that binds to the oligonucleotide sequence GGGACTTTCC, of the intronic enhancer element of the K light chain gene, and activates its gene transcription (50). Further studies revealed that NFKB was a dimeric protein, made up of NFKB subunits p50 (NFKB1)/p105 and p65 (RelA). Other members include p52 (NFKB2)/p100, RelB, and c-Rel (51). NFKB can be composed of many homo- and heterodimeric combinations of these family proteins, and each combination yields different transcription factor responsibilities (52). Most dimers are transcriptional activators, with the exception of p50/p50 and p52/p52 homodimers, which are transcriptional repressors (53). The most common form of NFKB is p50/p65. All of the NFKB/Rel proteins have a Rel homology domain (RHD) at the N-terminus consisting of a 300 conserved amino acids. The RHD is responsible for DNA binding, dimerization, and contains a nuclear translocation signal (NLS) (54). Inactive NFKB remains in the cytoplasm because it is tightly controlled by IKBα (inhibitor of kappa B), which interacts via a noncovalent bond with NFKB at the RHD, preventing NFKB from translocating to the nucleus, and regulating transcription (54). NFKB can be activated in response to a variety of stimuli including ionizing radiation, hypoxia, phorbol ester, and proinflammatory cytokines such as tumor necrosis factor –alpha (TNFα) and interleukin-1 (IL-1) (55,56). Ultimately, the stimuli activate IKB kinase (IKKB) (57), which then

phosphorylates IKBα at two conserved serines (Ser-32 and Ser-36) in the N-terminal domain (58-60), marking it for ubiquitination at lysines 21 and 22 (60, 63), and degradation by the 26S proteosome (61-62). This reveals the NLS, thereby allowing NFKB to translocate to the nucleus and initiate transcription (64-66). NFKB has been shown to activate genes which encode cell adhesion molecules (67-69), heat shock proteins (70), inducible nitric oxide synthase (71), and inflammatory response genes (72-74). Recently, an anti-apoptotic function for NFKB has been described, activating inhibitors of apoptosis proteins (cIAP) (75-76), A20 (77), and IEX-1L (78).

Studies have shown that IKKB is crucial for activation and phosphorylation of IKBα. Li *et al.* demonstrated a huge increase in apoptosis in the liver of IKKB-/- mouse embryos resulted in death at E12.5 (79-80). Studies by Beg *et al.* with RelA-/- mouse embryos showed similar results, the embryos dies at E14.5 due to liver degeneration (81). Therefore, loss of IKKB results in a greater decrease in NFKB activity than the loss of RelA alone.

P300 is a coactivator that was first identified based on its interaction with adenovirus E1A (82). As a coactivator, it interacts with many transcriptional activators such as CREB/ATP, c-Jun, c-myb, YY1, myo-D, c-Fos, and steroid receptors (83). The N-terminal region of p300 interacts with the transcriptional activation domain of NFKB, p65 (84).

F. Gene Transfer into Cardiomyocytes

The ability to transfer foreign DNA into cultured cells has substantially increased our understanding of signaling pathways. Chemical methods such as using DEAE-dextran and calcium phosphate and lipofection, are particularly cytotoxic to cardiac myocytes, with transfer efficiency of 3-10% in neonates and even worse in adult heart cells (98). Alternative techniques include electroporation which is a process that can transiently open pores by using an electrical pulse so that crude plasmid DNA can easily pass directly into the cell (99), direct injection of naked or plasmid DNA into tissue (100), and projectile bombardment which propels DNA-coated microprojectiles into target cells by using a gun powder discharge to gain momentum (101). The problem with these techniques is that the percentage of transfected cells is low because cardiomyocytes are extremely difficult to transfect.

More recently, viral vectors have been used as potential gene transfer systems. Certain viruses are highly efficient at nucleic acid delivery while avoiding an immune response by the infected host, and without causing any pathogenic effect. Retroviral and Adenoviral vectors have been developed and used for delivery of foreign DNA (102, 103). Retroviruses, although they can integrate into the genome of infected cells, require replicating cells for transduction. Adenoviruses on the other hand can infect replicating and non-replicating cells with high efficiency. Kirshenbaum *et al.* (1993) demonstrated that recombinant adenovirus could successfully transfer genes into both neonatal and adult cardiac muscle cells, with >95% efficiency (98, 105).

Construction of recombinant adenoviral vectors begins with the modification of adenoviral serotype 5 (Ad5) (106). Ad5, which contains linear, double-stranded DNA ~36 kb, is made incapable of replication because the early region gene (E1) necessary for viral replication is removed and replaced with exogenous, foreign DNA (102). On a side note, reconstructed adenovirus can replicate its own viral DNA in human embryonic kidney cells (HEK293) because these cells have been immortalized by stabily expressing complementary E1 genes in trans (E1A and E1B). In 1991, Dr. F. Graham and colleagues engineered a two plasmid system for the generation of adenoviral vectors capable of transferring foreign DNA (102, 107). The adenovirus genome, minus E1 gene is packaged into a 40 kb plasmid, containing a bacterial origin of replication and an ampicillin resistant gene. A "shuttle vector" contains overlapping adenoviral sequences with the cDNA insert of interest, replacing a deleted E1 gene. The viral rescue is attained upon co-transfection of these two plasmids into HEK293 cells. By homologous recombination in vivo, the foreign cDNA insert replaces the deleted E1 region, to produce a self-replication defective adenovirus capable of transiently expressing foreign genes of interest into replicating as well as non-replicating cell types.

The development of safe, efficient gene delivery systems used to over-express regulatory proteins in cardiac myocytes, could have tremendous benefits to the area of cardiovascular research, improving our understanding of signaling pathways, and ultimately in treating cardiac disease. Already, adenoviral and

parvoviral vectors are successfully being used in phase I clinical trials for gene therapy of cystic fibrosis and hemophilia B (104, 108).

III. Materials and Methods

Neonatal myocyte isolation and cell culture

Neonatal ventricular myocytes were isolated from 2 day old Sprague-Dawley rat hearts, and maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (DF) containing 10% fetal bovine serum (FBS), as previously described (89). Neonatal rats were sacrificed by cervical dislocation and immediately placed in 70% ethanol. Hearts were then excised from the rats and minced in cold Phosphate Buffered Saline (PBS), to remove red blood cell elements and cellular debris. Cells were digested with collagenase CLSII, trypsin, and Dnase. Neonatal ventricular myocytes were isolated using Percoll gradient centrifugation, which produced four distinct bands consisting of; myocytes, fibroblasts, red blood cells and debris, and a 'red phase'. To minimize contamination from non-myocytes, cells were preplated for one hour. Myocytes were then submitted to primary culture at a density of 1×10^6 cells per 35 mm dish. After overnight incubation at 37°C, the media was removed and myocytes were transferred to serum free media and utilized for experimental protocols.

Recombinant Adenoviruses

Adenoviruses were propagated and titered using replication defective adenovirus in human embryonic kidney (HEK) 293 cells that contain and express the genes necessary for adenoviral replication (E1A and E1B). Cells were harvested by freeze thawing, and were purified by ultracentrifugation in cesium chloride, as previously reported (117). The E1A virus is a "12S E1A-E1B" virus

containing a deletion between nucleotides 1769 and 3322 ligated into Ad5dl309 (184). CR3, which is required for transactivation of certain adenovirus early promoters is absent from the E1A virus. The IKKB virus was engineered in our lab (Ens K, 2001) by first inserting the coding sequence for IKKB into the PCA3 plasmid using HindIII/EcoRV restriction enzyme sites. The IKKB coding sequence was amplified by polymerase chain reaction (PCR), and HindIII/EcoRV linkers were incorporated at that time. The transgene was rescued into virus by cotransfecting the IKKB PCA3 construct with the PJM17 plasmid consisting of the viral genome in 293 cells, by methodology described by Kirshenbaum (98). To confirm the presence of the IKKB gene in the engineered adenovirus, viral DNA was isolated and the gene was amplified by PCR. IKBα (S32/36A) denotes the recombinant adenovirus that encodes a human IKBα protein with serine to alanine mutations at residues 32 and 36. The IKBSA virus was engineered in our lab by first inserting the IKB mutant gene into PCA3 plasmid using HindIII/XbaI restriction enzyme sites. Viral rescue was performed in the same way as the IKKB virus. The cDNA for the carboxyl terminal transmembrane domain deletion mutant (BNIP3ΔTM) has been described previously (148), and recombinant adenovirus encoding the transmembrane deletion mutant was generated as previously reported (105). To control the effects of viral infection alone, we used the adenovirus designated AdCMV that contains the CMV enhancer-promoter with an empty expression cassette.

Plasmid Constructs

To analyze cardiac gene expression we utilized plasmids containing promoter elements in front of the luciferase gene. To measure transcriptional activity of NFKB we used a luciferase reporter construct containing 3 tandem NFKB 'cis acting' binding sites, denoted 3xkbluc. To measure transcriptional activity of NFKB, independent of DNA binding, we used a luciferase reporter construct containing Gal-4 binding sites, and co-transfected myocytes with a plasmid construct comprised of the Gal-4 DNA binding domain fused to the p65 transactivation domain of NFKB. Plasmid constructs driven by the cytomegalovirus promoter (CMV), encoding E1A, NFKB, RG2 (point substitution mutant of E1A), Δ2-36 (N-terminal deletion mutant of E1A), p300, and Rb were used in these experiments.

To control for variability in transfection efficiency between conditions, luciferase activity was normalized to β galactosidase (CMV βgal) activity and expressed as relative light units.

Neonatal myocyte transfections

For transfection of neonatal myocytes, cells were incubated in DMEM containing DEAE-dextran, 5 μg of luciferase reporter plasmid DNA, 2 μg of CMV βgal, and 5 μg of plasmid DNA for 40 minutes at 37°C. Following incubation, media was removed, and cells were shocked with DMEM/10% DMSO. The cells were then incubated for 24 hours in DF/10% FBS , and then an additional 24 hours in DFSF media.

Electromobility Gel Shift Assay

Nuclear extracts of neonatal ventricular myocytes were prepared by scraping cells in 400 μL of lysis buffer (10 mM Tris ph 8.0, 60 mM KCl, 1 mM EDTA, 0.1% NP40, 1 mM DTT (dithiothretol) and protease inhibitors) as previously described (142). The cells were then centrifuged and resuspended in a buffer containing 20 mM Tris pH 8.0, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol. A ^{32}P radiolabelled oligonucleotide probe 5' AGTTGAGGGGACTTTCGCAGGC 3' was used as a template for the gel shift experiments. DNA binding reaction mixture contained 10 μg nuclear extract in 50 μl 10 x HGE, 50 μl 1.2 mg/ml PdIdC, 50 μl 10% NP40, 25 μl 0.1 M DTT, 25 μl 10 mg/ml BSA, 100 μl labeled probe. DNA-protein complexes were resolved on a native 5% polyacrylamide gel in 1 x Tris Borate – EDTA pH 8.0.

Mitochondrial and cytosolic fractionation

For detection of mitochondrial proteins Cytochrome c and Smac into the cytosol, myocytes were fractionated into mitochondrial and cytosolic compartments. Myocytes were infected for 24 hours, and harvested at 48 hours in 200 μl of isotonic buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1mM PMSF, pH 7.5). Following a 15 minute incubation on ice to allow cell swelling, myocytes were dounce homogenized with 20 strokes on ice, then centrifuged twice at 750 x g for 10 minutes at 4°C. Following aspiration of the supernatant and centrifugation at 10,000 x g at 4°C, the mitochondrial pellet was resuspended in 50 μl of buffer A.

To ensure the removal of all mitochondrial contamination, the supernatant was centrifuged at 100,000 x g at 4°C.

Alkaline extraction of mitochondrial fraction

To isolate mitochondria from neonatal myocytes, cells were scraped in HIM buffer containing 2.0% w/v bovine serum albumin (200 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH, 1 mM EGTA, pH 7.5). The crude pellet was disrupted and resuspended in HIM buffer. The homogenate was centrifuged to obtain the mitochondrial fraction and resuspended in 250 mM sucrose, 10 mM HEPES-KOH, 1 mM ATP, 5 mM sodium succinate, 0.08 mM ADP, 2 mM K_2HPO_4. To assess integration of BNIP3 into mitochondrial membranes, mitochondria were subjected to alkali extraction method using 0.01 M Na_2CO_3 (pH 11.5) to dissociate and solubilize proteins that are not membrane integrated as previously reported.

Western blot analysis

For detection of total protein in neonatal myocytes, cells were harvested in RIPA lysis buffer (1% Triton X, 0.1% sodium dodecyl sulfate (SDS) 140 mM NaCl, 10 mM Tris, and 1% deoxycholate) containing protease inhibitors. For detection of mitochondrial and cytosolic fractions, and mitochondrial integrated proteins, cells were prepared as described above. Following denaturation for 5 minutes at 100°C, protein was resolved on a 15% sodium dodecyl sulfate-polyacrylamide gel at 200 volts for 1.5 hours and electrophoretically transferred

to either a polyvinylidene difluoride (PVDF) (Roche Diagnostics, Laval, Quebec) membrane, or a nitrocellulose (Amersham Biosciences, Baie d'Urfe, Quebec) membrane at 100 volts for one hour. Membranes were blocked for one hour in blocking buffer (Roche Diagnostics) in TBS-TWEEN (150 mM NaCl, 50 mM Tris-HCl, 0.3% Tween-20, pH 7.4) and incubated with primary IgG antibodies at a specified dilution for 3 hours up to overnight at 4°C. Following three washes at 10 minutes with 1 x TBS-TWEEN, membranes were incubated with specific secondary antibodies for one hour at 4°C. Bound proteins were then detected by a chemiluminescence reaction using horseradish peroxidase conjugated antibody with enhanced chemiluminescence (ECL) reagents (Amersham Biosciences). For detection of IKBα, a rabbit anti-IKBα antibody was used at 1:1000 (Santa Cruz, Santa Cruz, California). For detection of BNIP3, a murine antibody directed toward BNIP3 (generously provided by Dr. A. H. Greenberg) was used at 1:10,000. To verify even integrated protein loading, an antibody directed towards VDAC was used at 1:5000 (Oncogene, San Diego, California). For detection of Smac, a murine anti-Smac antibody was used at 1:1000 (Calbiochem, San Diego, California). For detection of Cytochrome c, a murine anti-Cytochrome c antibody was used at 1:5000 (BD Biosciences, Mississauga, Ontario), and to verify even protein loading, a murine antibody directed towards sarcomeric α-actin M4401 (1.0 μg/ml; Sigma, Oakville, Ontario) was used at 1:5000. All western bands were quantified using the GS-800 Calibrated Densitometer (Bio-Rad, Mississauga, Ontario).

Assays of apoptosis

1.) DNA ladder

Neonatal myocytes were harvested, lysed, and the genomic DNA resolved on a 1% agarose gel for the analysis of nucleosomal DNA fragmentation (89).

2.) Hoechst 33258 staining

Fluorescent microscopy was utilized to visualize nuclear changes associated with cells undergoing apoptosis. Neonatal myocytes were grown on 12 mm glass coverslips. For the identification of cardiac nuclei, myocytes were fixed in 70% ethanol and stained with (1 μg/ml) Hoechst dye 33258 (Molecular Probes, Eugene, Oregon) for the visualization of nuclear morphology. Slides were then washed with phosphate buffered saline (PBS) and mounted on glass slides (117).

Mitochondrial permeability transition (PT) pore

To moniter changes in mitochondrial permeability transition (PT), neonatal myocytes were loaded with 0.4 μM calcein-acetoxymethylester (Molecular Probes) which will stain the mitochondria of viable cells. In the presence of 5 mM cobalt-chloride (Fisher, Fair Lawn, New Jersey), the cytoplasmic signal is quenched. Viable mitochondria will be visualized as green organelles against a dark background, while cells having an increase in membrane permeability will leak the calcein-AM from the mitochondria and appear colorless (185). Following

dye loading, cells were incubated for 30 minutes at 37°C. Cells were imaged on an Olympus AX-70 Research microscope and analyzed using Image-Pro plus software to compare relative intensities for each condition relative to control cells.

Statistical Analysis

Multiple comparisons between groups were determined by one-way ANOVA. Unpaired two tailed student t-test was used to compare mean differences between groups. Differences were considered to be statistically significant to a level of $p<0.05$. In all cases, the data was obtained from at least n=3 to 4 independent myocyte isolations.

IV. Results

E1A causes apoptosis of neonatal ventricular myocytes

To verify that the E1A protein (Figure 1) is sufficient to provoke apoptosis of ventricular myocytes, post-natal myocytes were infected with recombinant adenovirus that encodes 12S E1A and assessed for the incidence of nucleosomal DNA damage and apoptosis (Figure 2). In contrast to control cells, cells expressing E1A displayed a 5-fold increase ($p<0.05$) in apoptotic cell death as indicated by an increase in nucleosomal DNA laddering and Hoechst 33258 staining for nuclear morphology (panels A, B, and C). Control cells are uniformly stained blue, while cells expressing E1A display fragmented and condensed nuclei (panel B). Results in panel B, are summarized in panel C, as represented by a histogram of the data. The data indicate that E1A provokes apoptosis of neonatal ventricular myocytes, which agrees with earlier work in cardiac myocytes (89) and in striated skeletal muscle (88).

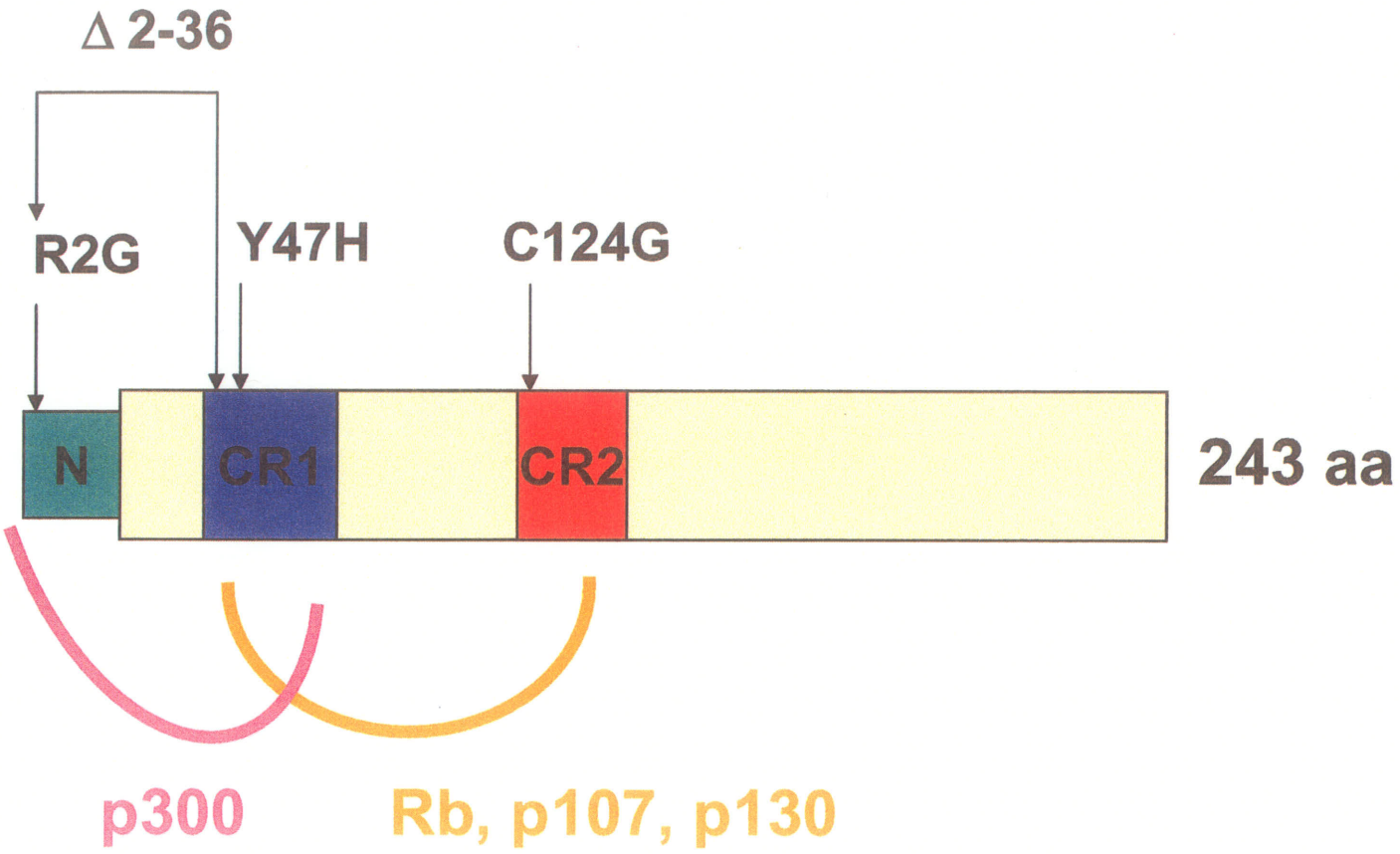

Figure 1. Characterized domains of 12S E1A and its cellular protein interactions

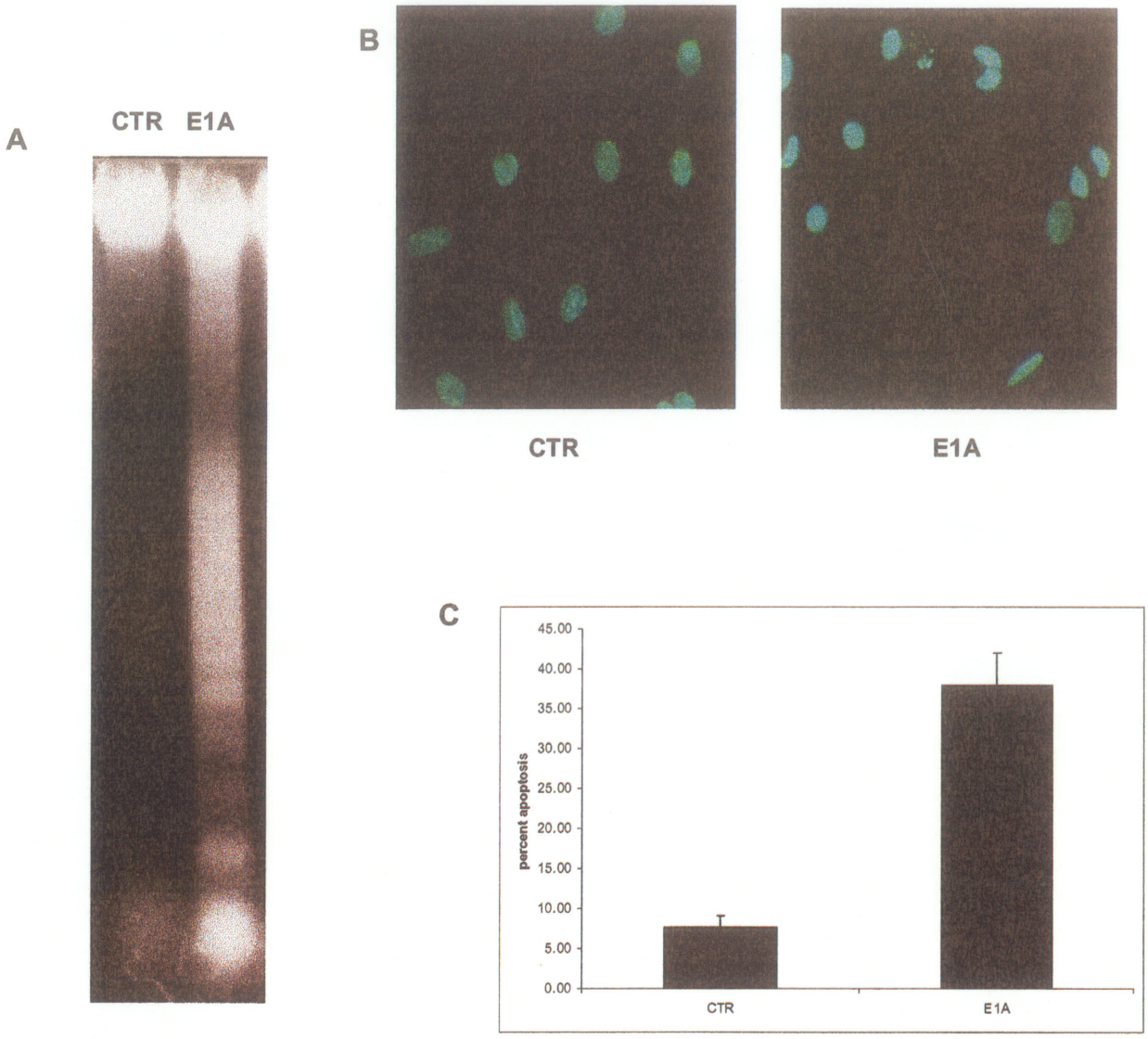

Figure 2. E1A provokes apoptosis of ventricular myocytes. Panel A, myocytes were infected with E1A resulting in nucleosomal DNA laddering. Panel B, Images of Hoechst 33258 stained myocytes viewed with an epifluorescence microscope to show nuclear morphology. Panel C, Histogram of data shown in panel B. Data were obtained from at least n=3 independent myocyte isolations, counting at least >200 cells per condition.

E1A represses NFKB-dependent gene transcription in neonatal myocytes

To study the impact of E1A on the NFKB signaling pathway (Figure 3), neonatal ventricular myocytes were transfected with a luciferase reporter construct that contains three tandem NFKB binding sites (Figure 4). Here, we demonstrate that E1A produced a 2.3 –fold decrease in NFKB-dependent gene transcription, while NFKB produced a 9.5 –fold increase in luciferase reporter gene activity. Co-expression of E1A and NFKB results in a rescue of expression with a 2.8 –fold increase in NFKB-dependent gene transcription. Results were normalized with a βgalactosidase assay performed with the same samples.

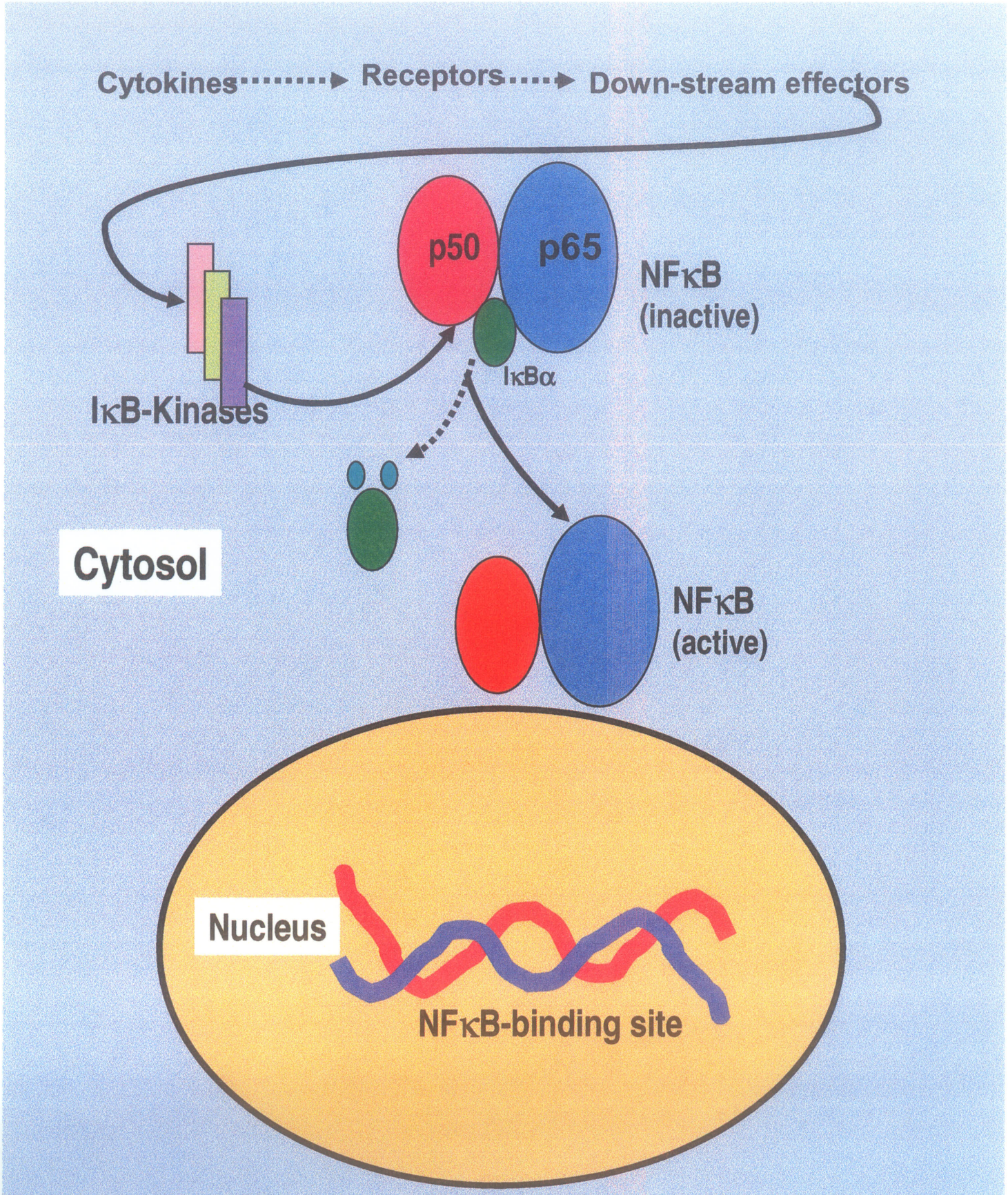

Figure 3. Model for NFKB activation in ventricular myocytes.

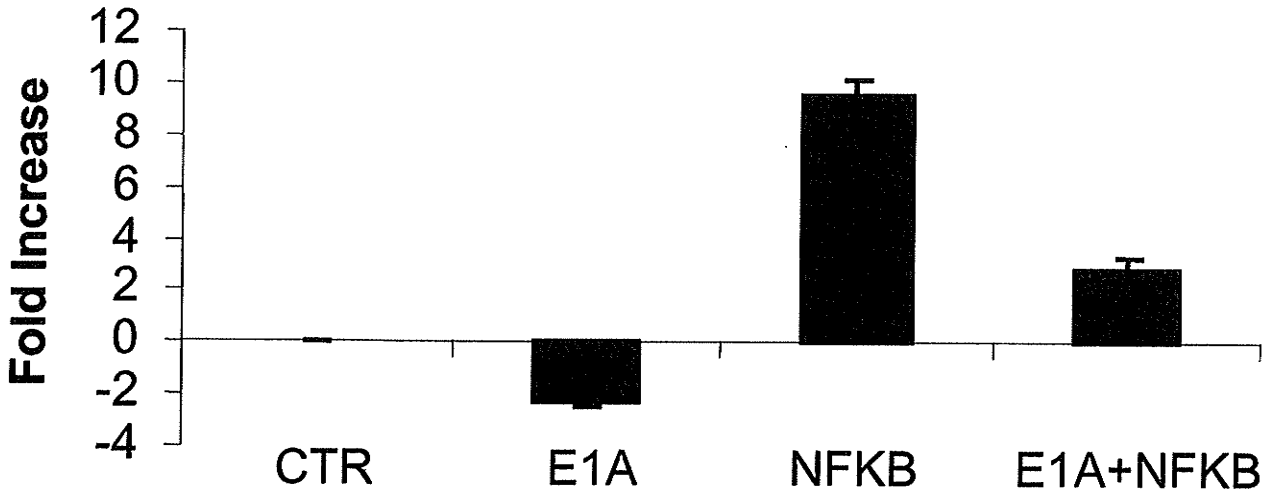

Figure 4. E1A represses NFKB-dependent gene transcription in ventricular myocytes. Cells were transfected with luciferase reporter plasmids containing NFKB binding elements (NFKB luc). 48 hours later, luciferase activity was determined for n=3 experiments with 3 replicates per condition

E1A at the post-translational level

Since NFKB is normally regulated at the post-translational level in the cytoplasm by IKBα, we determined whether the observed decrease in NFKB binding activity is related to changes in IKBα expression levels. Protein extracts of ventricular myocytes expressing E1A and IKKB were analyzed by western blot analysis, and probed with a rabbit antibody directed toward IKBα. As shown in Figure 5, IKBα expression levels were not altered by E1A. These observations suggest that E1A may be regulating NFKB pre-transcriptionally.

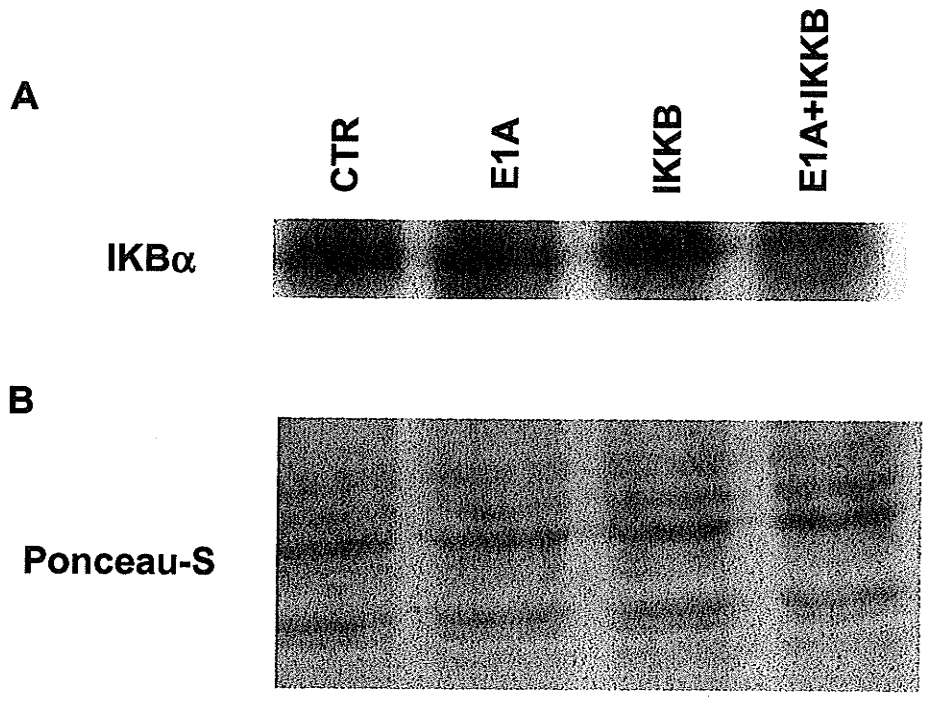

Figure 5. E1A does not alter IKBα expression levels. Panel A, cell lysates from neonatal ventricular myocytes infected with E1A and IKKB were analyzed by western blot. IKBα protein was detected by rabbit anti-IKBα antibody. Panel B, Ponceau-S stained filter to demonstrate equivalent protein loading.

E1A effects NFKB at the pre-transcriptional level

To further delineate the stage at which E1A mediates repression of NFKB-dependent expression, we used an electromobility gel shift assay (EMSA) with HEK 293 nuclear proteins (Figure 6). This technique measures NFKB-DNA binding activity directly by using a radiolabelled ^{32}P oligonucleotide probe containing an NFKB binding site. The probe is incubated with nuclear extracts from the cells, and run on a non-denaturing polyacrylamide gel. Gel shift experiments revealed that E1A is not regulating NFKB post-translationally. Consistent with the lack of effect of E1A on IKBα activity, E1A had no apparent effect on NFKB protein activation in the cytosol as indicated by the EMSA (lane 3), which was not different from uninfected control cells. IKKB was used as a control, since it is known to activate the NFKB pathway, which causes NFKB to translocate to the nucleus, indicated by a shift (lane 4). When E1A and IKKB were both expressed in HEK 293 cells, the shift caused by IKKB was not effected, suggesting that NFKB-DNA binding is not inhibited by E1A (lane 5). To demonstrate DNA-protein specificity, nuclear proteins were hybridized with p65 and E1A antibodies. Lane 6 reveals that p65 is directly binding to the NFKB oligonucleotide as indicated by a supershift. Lane 7 confirms that E1A is not directly binding to the DNA-NFKB protein complex, indicated by no supershift.

To further test whether E1A represses the transcription potential of NFKB, independent of binding to the promoter, we used the Gal4 protein fused to p65, the transactivation domain of NFKB. Myocytes were transfected with a luciferase reporter gene that contains the Gal4 DNA binding domain (Gal4luc) (Figure 7).

This way we could look at the effect of E1A on NFKB independent of the binding domain. VP16 is the transactivation domain for the Herpes Simplex Virus, and it is a very strong transcriptional activator. It is fused to Gal4 as a positive control for the assay. We see a 15,000 –fold increase in expression, as expected (data not shown). The 50 –kd protein subunit of NFKB is the binding domain, and the p65 –kd protein subunit is the transactivation domain. The p65 –kd subunit was fused to Gal4, and we see a 4000-fold increase in expression. When we co-transfected ventricular myocytes with Gal4p65 and E1A, there was almost complete repression, a low 40-fold increase compared to the 4000-fold increase without E1A. This repression is independent of DNA binding.

To study this further we looked at different E1A mutants to determine which part of the E1A protein was responsible for the repression. Y47H is a tyrosine to histidine mutation at position 47 and is located in the CR1 domain, effecting Rb binding. There is not a significant change from the wild type E1A, indicating that this region is not likely responsible for the repression of NFKB-dependent gene transcription (data not shown). R2G is an arginine to glycine mutation at position 2 located in the amino-terminus of E1A, which would slightly effect p300 binding. There is a small increase (600-fold) in expression. The most interesting mutation was the deletion mutation of amino acids 2-36, wiping out almost the entire N-terminus of E1A and significantly effecting E1A's ability to bind p300. This E1A mutation had the most regained expression, with a 3000-fold increase. In an attempt to rescue E1A repression, p300 was co-transfected with E1A and Gal4p65. An increase in expression from 40-fold to 90-fold was

observed. When Rb was co-transfected, there was only a 60-fold increase in expression. Therefore, the N-terminus of E1A represses transcription through the transactivation domain of NFKB, via p300 interaction, and this is independent of the binding domain.

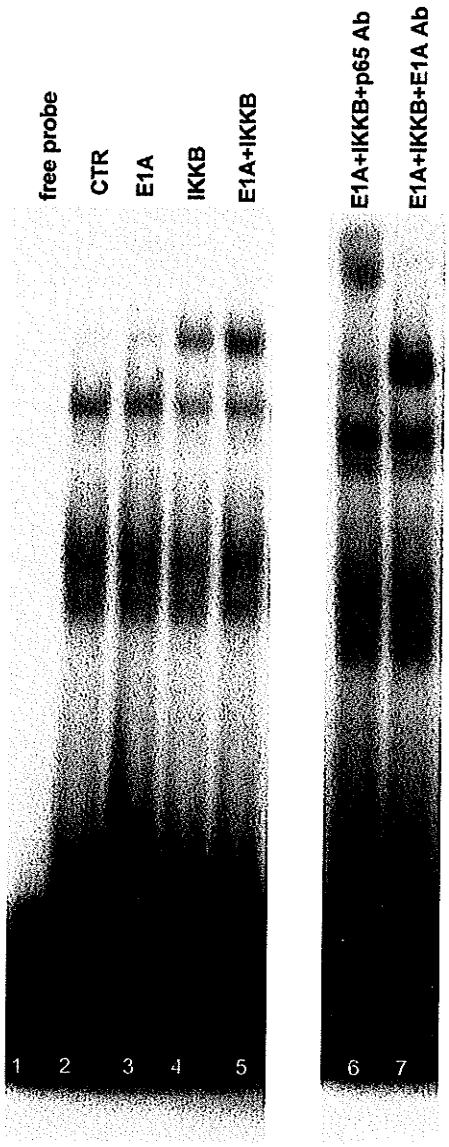

Figure 6. Electromobility gel shift assay of nuclear protein from human embryonic kidney (HEK) 293 cells. Equal amounts of nuclear protein extract from Hek 293's were prepared and analyzed for NFKB binding sites. Supershift analysis is shown in the last two lanes; myocyte nuclear extract was incubated with a goat antibody directed toward the p65 subunit of NFKB.

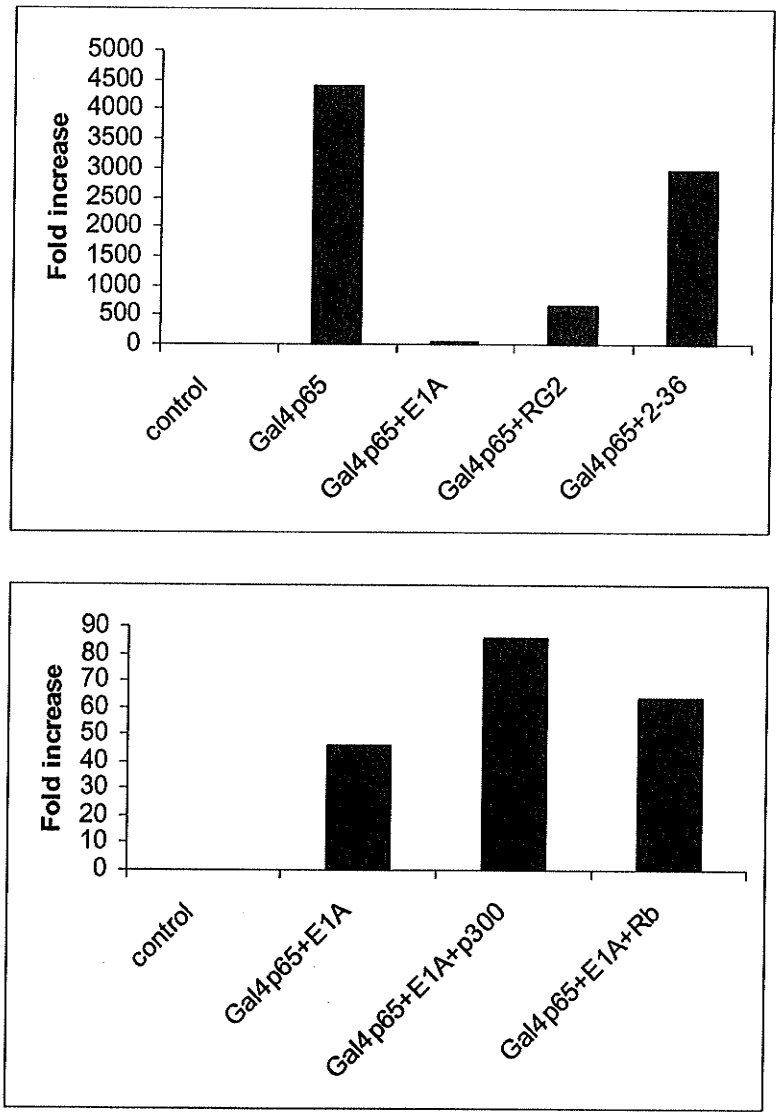

Figure 7. The N-terminal domain of E1A represses transcription through the transcription domain of NFKB, independent of the binding domain in ventricular myocytes. Cells were transfected with luciferase reporter plasmids containing Gal4 binding elements (Gal4 luc). 48 hours later, luciferase activity was determined.

IKKB rescues apoptosis induced by E1A

A hallmark of apoptosis is DNA fragmentation. Figure 8 shows that ventricular myocytes infected with E1A resulted in apoptosis as seen by the ladder effect. We activated the NFKB pathway by overexpressing IKKB and we now observe rescue of the cells from apoptosis.

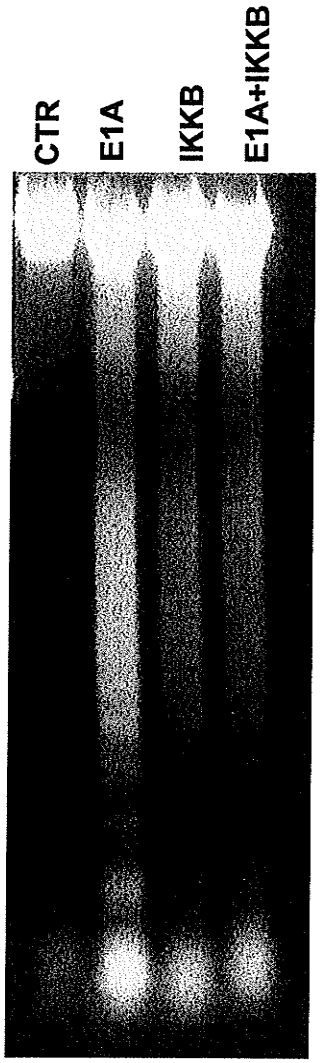

Figure 8. E1A triggers apoptosis of ventricular myocytes, which is rescued by IKKB. Nucleosomal DNA laddering of ventricular myocytes is seen in cells infected with E1A; IKKB is able to rescue apoptosis provoked by E1A.

E1A disrupts mitochondrial function

To test the effect of E1A on mitochondrial function, we tested whether E1A induces Cytochrome c and Smac release, by isolating control myocytes and E1A expressing myocytes, into cytosolic and mitochondrial compartments. Proteins were separated by western blot, and analyzed for the presence of cytochrome c and Smac in the cytosol (Figure 9, Panel A). In contrast to control cells, E1A provoked significant translocation of both proteins into the cytosol. Results in panel B are summarized in panel C, as represented by a histogram of the data.

Since perturbations to mitochondria resulting from opening of the mitochondrial permeability transition (PT) pore have been suggested to be an underlying feature of the apoptotic program, we addressed whether E1A-mediated apoptosis involves perturbations to mitochondria including PT pore changes. For these experiments, we loaded the myocytes with calcein-AM in the presence of cobalt-chloride. Calcein-AM is non-fluorescent until the AM ester is removed inside living cells. Calcein-AM emits a green fluorescence if it has been taken up inside a viable cell. The cobalt-chloride quenches the signal in the cytosol. When visualizing cells, green fluorescent mitochondria over a dark background is observed. If the PT pore is open, the images appear colorless due to calcein leakage into the cytosol, and subsequent quenching. As shown in Figure 9, panel B, control cells displayed punctate green staining mitochondria, indicative of the PT pore in a closed configuration. However, a marked reduction in the mitocondrial fluorescence was observed in cells expressing E1A, a finding consistent with the opening of the PT pore.

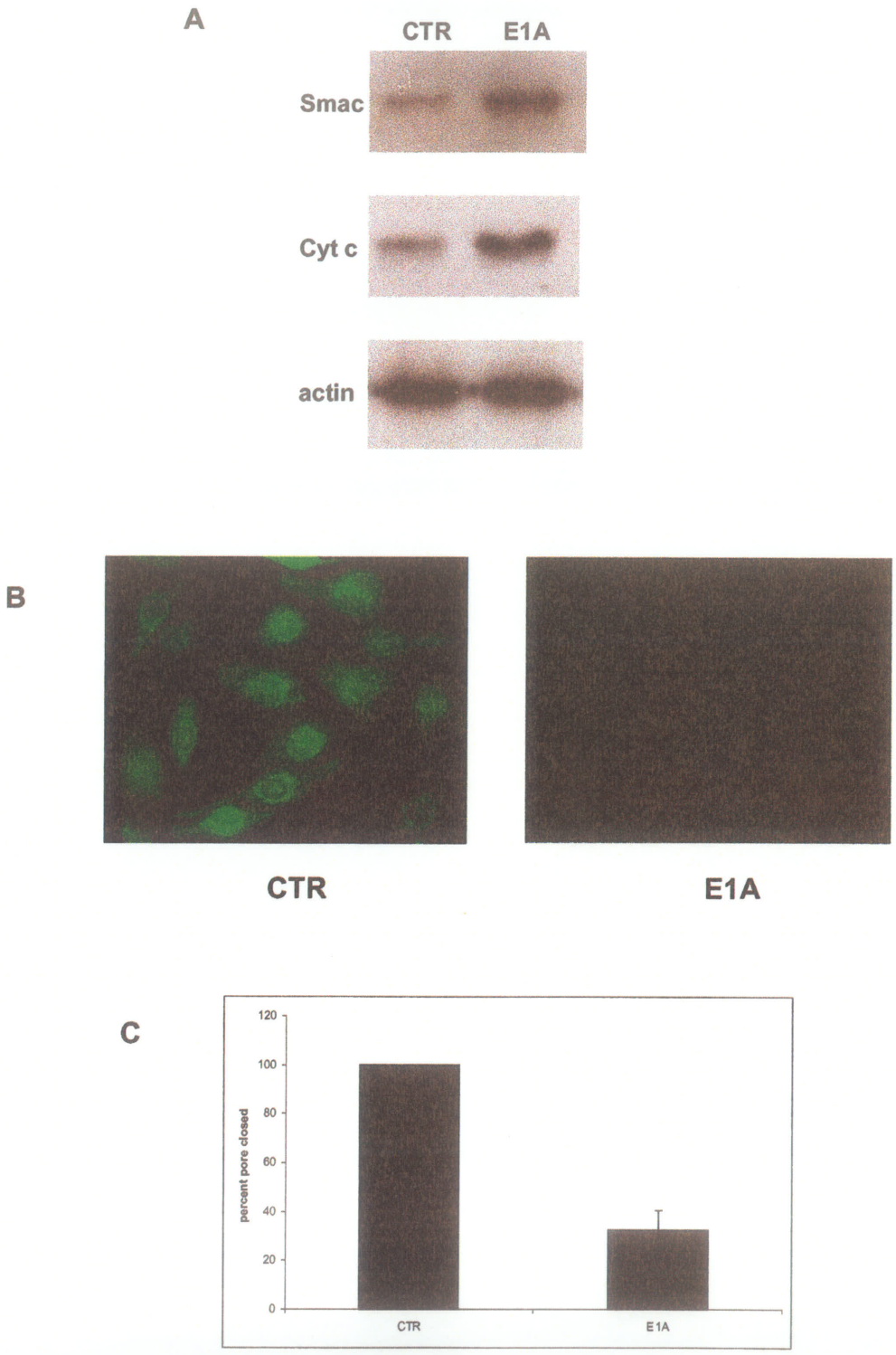

Figure 9. E1A disrupts mitochondrial function. Panel A, the cytosolic fraction of cardiac cell lysate from control and E1A infected myocytes was subjected to western blot analysis with an antibodies directed towards smac and cytochrome c. Panel B, Mitochondrial permeability transition (PT) pore was monitored in ventricular myocytes. Cells infected with E1A resulted in opening of the PT pore. Panel C, Histogram of data shown in panel B. Data were obtained from n=4 myocyte isolations, counting at least >200 cells per condition.

A role for pro-apoptotic Bcl-2 family member BNIP3

Given that E1B 19kDa and Bcl-2 proteins can suppress E1A-mediated apoptosis, together with the reported ability of these proteins to interact with BNIP3, a recently identified BH3 only member of the Bcl-2 gene family, raises the strong possibility that BNIP3 may be involved in E1A-mediated cell death of ventricular myocytes. Interestingly, our data has shown that BNIP3 levels were increased slightly (1.8x increase), or at least stabilized in cells expressing E1A. This would support the notion that BNIP3 may be involved in E1A-induced apoptosis (Figure 10).

Our earlier work identified that under basal conditions BNIP3 is found loosely associated with the mitochondrial outer membrane (KM Regula and LA Kirshenbaum, unpublished data) but readily integrates into the mitochondrial membrane following a death signal, such as hypoxia (150). To ascertain whether E1A provokes integration of BNIP3 into mitochondrial membranes, mitochondria from control and E1A expressing myocytes were subjected to alkali extraction, which will dissociate and solubilize unintegrated membrane proteins. After alkali extraction of mitochondria, BNIP3 was minimally detected in the mitochondrial membranes of control cells, indicating it was mostly alkali soluble and not membrane integrated. However, in myocytes expressing E1A, BNIP3 was readily detectable in the mitochondrial fraction after alkali extraction, indicating that it was tightly associated with mitochondrial membranes (Figure 11). Due to the difficulty of isolating mitochondrial protein, even loading was difficult, therefore

integrated mitochondrial protein; VDAC was used as a loading control. After normalizing the results with VDAC, BNIP3 levels were 3.58x higher in myocytes expressing E1A compared to control cells. These results indicate that E1A is sufficient to provoke the integration of BNIP3 into mitochondrial membranes.

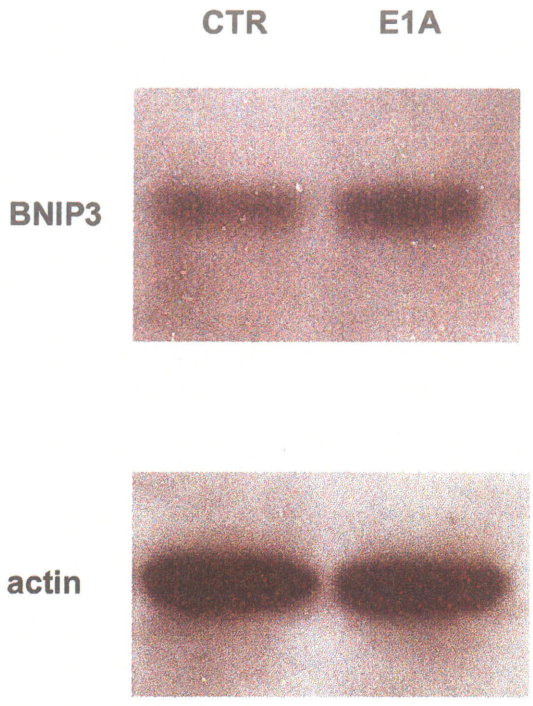

Figure 10. E1A induces expression of BNIP3 in ventricular myocytes. Western blot analysis of cardiac cell lysate derived from control cells and cells infected with E1A for 24 hours. E1A infected cells showed a 1.8x increase in BNIP3 expression levels. Actin protein was detected with mouse anti-actin antibody to demonstrate equivalent protein loading.

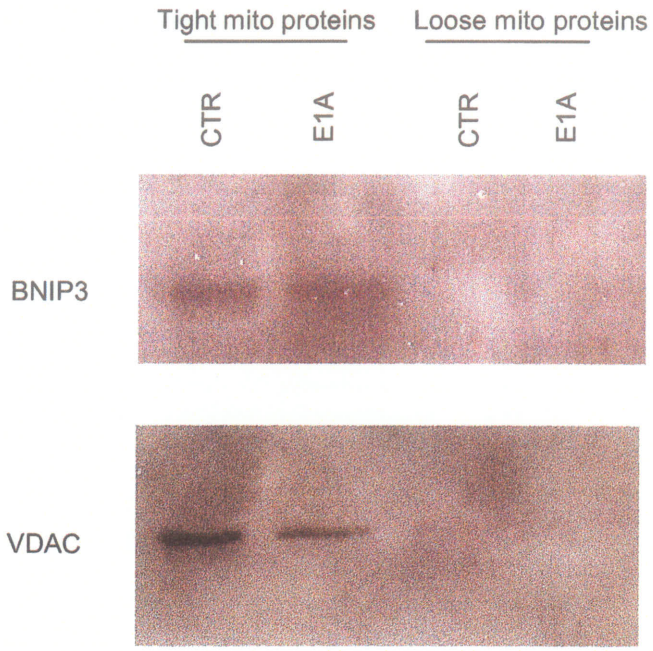

Figure 11. E1A-induced BNIP3 integration into the mitochondrial membrane. Alkaline extraction of mitochondria showed a 3.58x increase of BNIP3 in E1A infected cells in the tightly associated mitochondrial proteins versus control cells, indicating that BNIP3 integrated into mitochondrial membrane in response to E1A. Samples were normalized using integrated mitochondrial protein; VDAC, as a protein loading control.

E1A-induced mitochondrial defects can be rescued with BNIP3ΔTM

To substantiate the hypothesis that E1A-mediated integration of BNIP3 into mitochondria underlies E1A-induced mitochondrial defects of neonatal myocytes, we tested whether a carboxyl-terminal transmembrane deletion mutant of BNIP3 (BNIP3ΔTM), defective for mitochondrial integration could suppress E1A-mediated mitochondrial defects. As shown in Figure 12, panel A, E1A-induced mitochondrial cytochrome c and Smac release were suppressed in cells expressing BNIP3ΔTM. Furthermore, cells expressing BNIP3ΔTM were not different from control cells with respect to mitochondrial PT pore changes, indicating that BNIP3ΔTM was not toxic to the myocytes. Importantly, E1A-induced PT pore opening was markedly suppressed in cells expressing BNIP3ΔTM (Panels B,C).

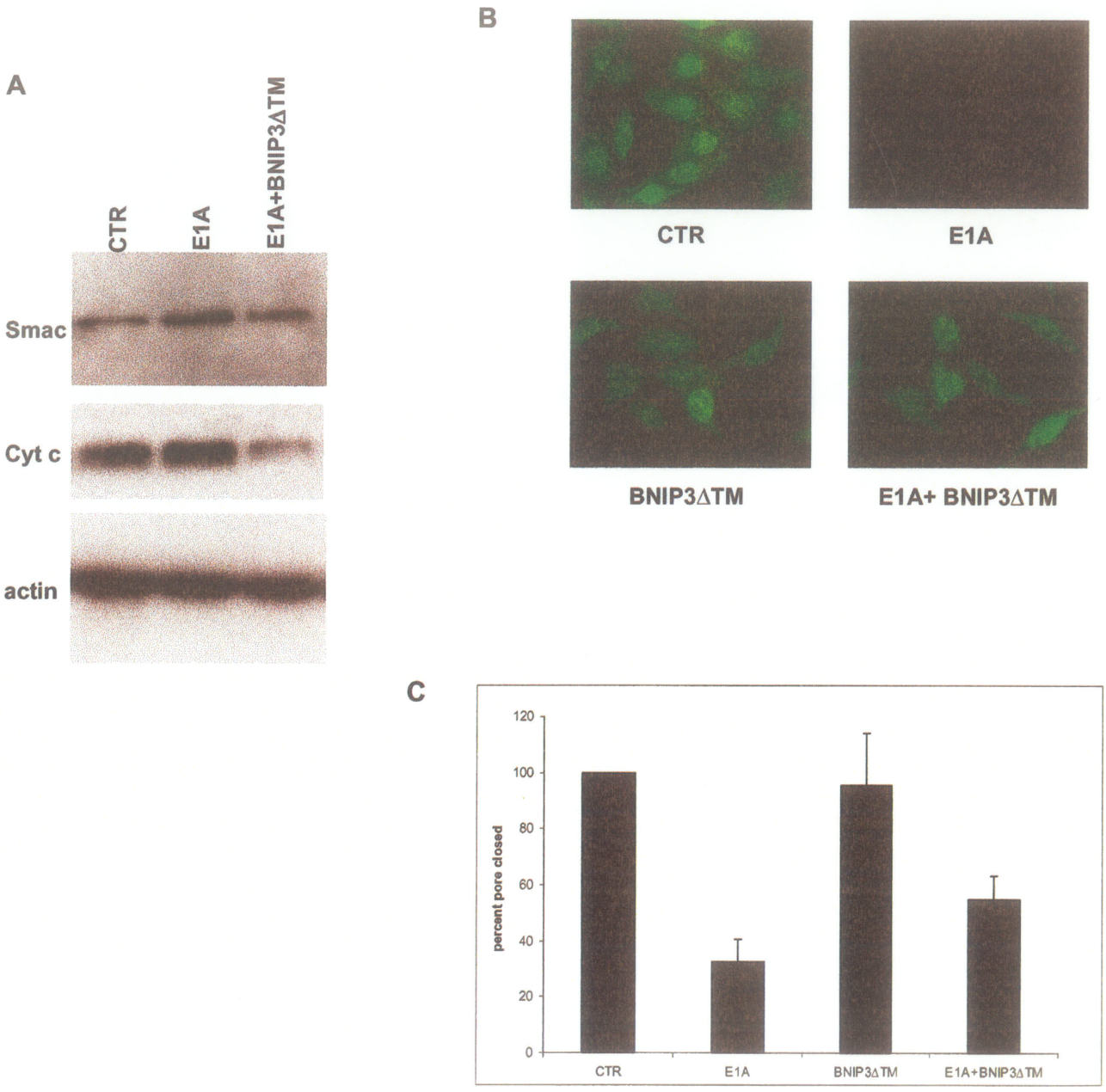

Figure 12. BNIP3ΔTM rescues mitochondrial perturbations provoked by E1A. Mitochondrial function was determined as described for figure 9.

BNIP3ΔTM rescues E1A-induced apoptosis of neonatal myocytes

As shown in Figure 13, apoptosis provoked by E1A can be rescued by co-expressing myocytes with BNIP3ΔTM. E1A alone resulted in a 5-fold increase in apoptosis, as opposed to a 1.3-fold increase in apoptosis when E1A was co-expressed with BNIP3ΔTM.

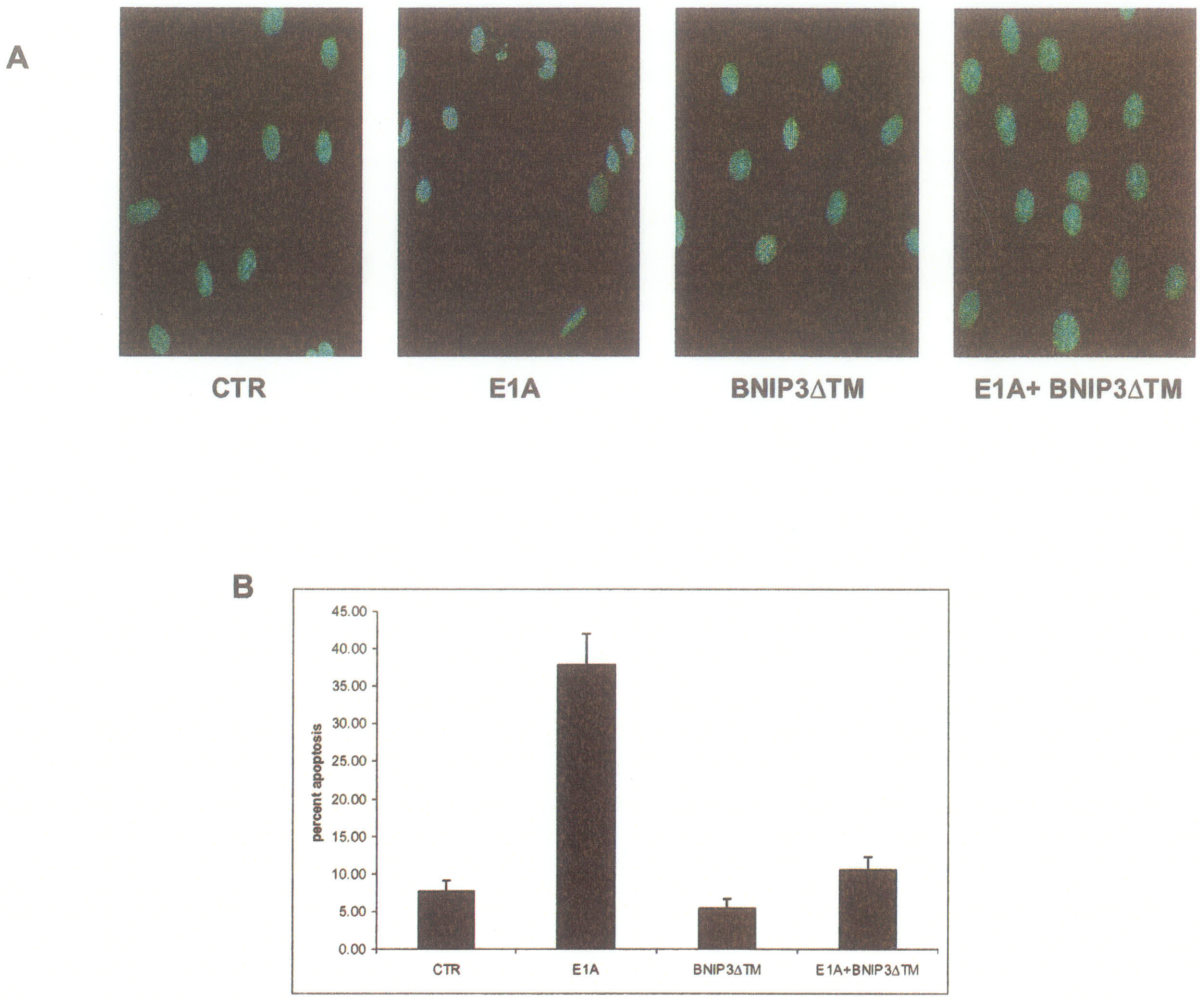

Figure 13. BNIP3ΔTM rescues E1A-induced apoptosis of ventricular myocytes. Panel A, myocytes stained for nuclear morphology with Hoechst 33258 dye and imaged with an epifluorescence microscope. Panel B, Histogram of data shown in panel A. Data were obtained from at least n=3 independent myocyte isolations, counting at least >200 cells per condition.

V. Discussion

Apoptosis is a normal physiological mode of cell death that can be initiated by the cell itself as a method of self-maintenance, or induced by pathologic stimuli. Since myocytes are terminally differentiated cells, fixed in a quiescent stage of the cell cycle, they don't divide, nor undergo apoptosis under normal conditions. However, loss of viable myocytes from the myocardium due to insult, predominantly occurs via an apoptotic pathway (9). Therefore, it is beneficial to study the molecular factors responsible for initiating and executing the cell death response, so that we may intercept the pathway and reverse the fate of the cell as a means of treating disease and preventing heart failure.

For these experiments, the 12S gene product of the Adenovirus early region one protein (E1A) was used as a tool for studying the underlying mechanisms that regulate cellular growth, i.e. as a way of jumping into the complex matrix of signaling pathways inside the cell in an attempt to dissect what regulates apoptosis. To date, a functional non-viral homologue to E1A has not been found. However, a functional homologue to the adenovirus 19 kDa protein E1B has been found, and it is known as Bcl-2 (192). Consistent with other reports (88, 89), we showed that E1A induced apoptosis of neonatal myocytes (Figure 2). A recent report by Cook *et al.* (95) stated that neither E1A alone, nor TNFα alone, induce cell death in NIH 3T3 fibroblasts, however, the combination does provoke cell death because E1A sensitizes the cells to TNFα, by repressing the NFKB-dependent defense against TNFα. NFKB plays a role in blocking apoptosis induced by TNFα (186-189). Similarly, Shao *et al.* (94) demonstrated

that E1A sensitizes cells to TNF-induced apoptosis in human ovarian cancer cell lines. In our findings, E1A alone was sufficient to provoke apoptosis of neonatal myocytes (Figure 2).

The mode by which E1A provokes apoptosis, however, remains poorly defined. Consistent with other reports (95), we showed that E1A represses NFKB-dependent gene transcription. Nuclear factor kappa beta (NFKB) is a ubiquitously expressed inducible transcription factor responsible for regulating the expression of a wide variety of anti-apoptotic genes, including cIAP, A20, and IEX-1L (75-78). However, what remained to be determined was the level that E1A affected NFKB-dependent gene transcription. Does E1A affect NFKB post-translationally in the cytosol, which would alter subsequent gene activation, as reported by Shao *et al.* (94), or pre-transcriptionally as reported by Cook *et al.* (95)? Shao *et al.* stated that E1A represses TNFα induced apoptosis through inhibition of IKKB activity, and thus NFKB activation. Our findings are in concordance with Cook *et al.* which stated that IKBα levels did not change due to E1A, and that E1A was not involved in NFKB activation, and subsequent translocation (Figures 5&6). The gel shift caused by TNFα (95) and by IKKB (Figure 6) was not affected, suggesting that E1A does not inhibit NFKB from physically binding to the promoter. The activity however, was not examined.

To study this possibility, we determined the capacity for E1A to affect the transactivation domain of NFKB, the p65 subunit. Our findings provide evidence that E1A represses NFKB-dependent gene transcription by indirectly or directly inhibiting the p65 subunit, independent of DNA binding (Figure 7).

Although evidence exists that E1A binding to either p300 or Rb is important, there is little evidence to support which is more important for E1A's role in apoptosis. Cook *et al.* (95) reported that E1A represses NFKB-dependent gene transcription via Rb interaction. However, our finding that p300 is more important than Rb binding is substantiated by other reports. p300 co-activates NFKB by interacting with p65 (84). E1A inhibits p65-dependent transactivation of Schneider SL-2 cells and COS cells, and when p300 was overexpressed, the repressive effect of E1A was rescued (190). Similarly, in neonatal myocytes, we showed that the E1A deletion mutant (Δ2-36) which prevents p300 binding, was not able to repress gene transcription, and when p300 was overexpressed in cells expressing E1A, rescue of gene transcription was observed (Figure 7).

Evidence suggests that cell death in the heart involves an apoptotic program (9, 38, 42-49). By entering the signaling pathway of an apoptotic cell, induced by E1A, we were able to determine that NFKB repression plays a role. By overexpressing IKKB, we were able to rescue the myocytes from apoptosis (Figure 8). Although, E1A is a viral gene, the apoptotic pathway it activates may be similar to pathways involved in heart failure. The ultimate goal would be to prevent apoptosis of myocytes due to heart failure.

The effect of E1A on mitochondrial function has never been examined, despite its importance in apoptosis. We discovered that mitochondrial release of Cytochrome c and Smac into the cytosol, occurs in neonatal myocytes expressing E1A (Figure 9). When these proteins are released into the cytosol, they activate the apoptotic pathway. To further elucidate E1A's role in

mitochondrial function, we examined the state of the mitochondrial membrane permeability transition pore (PT pore). Bradham *et al.* (191) demonstrated the significance of PT pore opening and depolarization for nuclear fragmentation, caspase-3 activation, and Cytochrome c release from the mitochondria in TNFα-induced apoptosis. In this study, we report that E1A-mediated apoptosis of neonatal ventricular myocytes involves PT pore opening (Figure 9). It remains unknown whether the open PT pore causes a change in membrane permeability, thus allowing the release of Cytochrome c and Smac, or if they directly leak out of the open pore.

Cytochrome c release has been shown to occur due to specific Bcl-2 family members inserting themselves into the mitochondrial membrane. Bax activation leads to its membrane insertion, facilitating cytochrome c release (155, 156). Although E1A has been shown to activate Bax and Bak, which is a novel mechanism rather than the pathway requiring tBid to cause insertion (197), we decided to examine the Bcl-2 pro-apoptotic family member: BNIP3 for three reasons. 1.) We recently identified that BNIP3 protein levels increased and it became inserted into mitochondrial membranes in hypoxia-mediated cell death. Furthermore, we demonstrated that the carboxyl-terminal transmembrane domain of BNIP3 is necessary for mitochondrial insertion, and BNIP3ΔTM is sufficient to rescue hypoxia-induced apoptosis of both adult and neonatal ventricular myocytes (150). 2.) BNIP3 was first identified as a E1B 19 kDa interacting protein. E1B 19 kDa functions as an apoptosis inhibitor in infected human cells (193). E1B 19 kDa also interacts with Bax, and inhibits apoptosis by

interacting with the BH3 domain of Bax (194). 3.) Rats that were subjected to left main coronary artery ligation, displayed no change in Bax protein in myocytes at the peak of apoptotic cell death in the myocardium (34). Our data reveals a slight increase in BNIP3 protein levels in neonatal myocytes expressing E1A. However, this is not surprising because there is always endogenous levels of BNIP3 in the cells, but Bcl-2 family members require activation before they convert to become more pro-apoptotic. In other words, it would be more significant if E1A caused BNIP3 activation. In our findings, E1A caused integration of BNIP3 into mitochondrial membranes.

To confirm this finding that E1A mediates integration of BNIP3 into mitochondrial membranes (which may be the trigger that initiates mitochondrial defects of neonatal myocytes), we tested whether a carboxyl-terminal transmembrane deletion mutant of BNIP3 (BNIP3ΔTM) that is defective for mitochondrial integration could suppress E1A-mediated mitochondrial defects. We discovered that both Cytochrome c and Smac release into the cytosol were prevented. In addition, the PT pore remained in the closed configuration when E1A was co-expressed with BNIP3ΔTM. By overexpressing BNIP3ΔTM in myocytes expressing E1A, apoptosis is prevented.

In this study, we have identified two modes that can suppress an apoptotic pathway. Overexpressing IKKB, activated the anti-apoptotic NFKB pathway. Alternatively, Kirshenbaum *et al.* (143) demonstrated that the NFKB pathway could be activated by overexpressing anti-apoptotic Bcl-2 family member protein; Bcl-2, which interacts with Raf-1/Mekk-1 complex via its BH4 domain, thereby

activating IKKB, causing IKBα degradation, and NFKB activation. Pro- and anti-apoptotic proteins can target multiple pathways. Bcl-2 also regulates an antioxidant pathway at sites of free radical generation, and protects cells from oxidative damage (145). In contrast, by overexpressing a defective BNIP3 protein (BNIP3ΔTM), we prevented the action of the pro-apoptotic protein BNIP3.

The very reason which makes viruses unwelcome to humans, makes them attractive delivery systems for gene therapy. They are highly efficient at nucleic acid delivery, they can be directed to specific cell types, and they can avoid the host's immune system. Already an insulin-like growth factor (IGF)-encoding gene therapy viral vector for treating heart disease is being considered (196). IGF-I is currently being used to treat heart disease, but because it circulates throughout the body, it causes substantial negative side effects (195). Thus, tissue targeted overexpression of IGF-I could have significant advantages. Perhaps we will develop an IKKB or BNIP3ΔTM encoding virus in the future that will treat and prevent heart failure.

VI. Conclusions

Adenovirus 12S E1A gene product (E1A) induces apoptosis of different cell types. Using neonatal ventricular myocytes, we examined the mode by which E1A provokes apoptosis. We verified that E1A alone is sufficient to induce apoptosis in neonatal ventricular myocytes, and determined two pathways involved in E1A-induced apoptosis.

In summary, the conclusions from this study are:

1) E1A triggers apoptosis of neonatal ventricular myocytes.
2) NFKB-dependent gene transcription is repressed by E1A.
3) E1A does not affect NFKB post-translationally by altering IKBα expression levels.
4) The N-terminal domain of E1A represses transcription of NFKB-dependent genes through the transactivation domain (p65) of NFKB, independent of DNA binding, and can be rescued with p300.
5) Overexpression of IKKB rescues E1A-induced apoptosis.
6) E1A induces mitochondrial defects including release of cytochrome c and smac, and opening of the permeability transition pore.
7) E1A induces BNIP3 protein integration into the mitochondrial membrane.
8) Overexpression of BNIP3ΔTM rescues mitochondrial defects induced by E1A, and rescues apoptosis.

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