

Suppression of Steady-state, but not Stimulus-induced NF- κ B Activity Inhibits Alphavirus-induced Apoptosis

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Abstract. Recent studies have established cell type-specific, proapoptotic, or antiapoptotic functions for the transcription factor NF- κ B. In each of these studies, inhibitors of NF- κ B activity have been present before the apoptotic stimulus, and so the role of stimulus-induced NF- κ B activation in enhancing or inhibiting survival could not be directly assessed. Sindbis virus, an alphavirus, induces NF- κ B activation and apoptosis in cultured cell lines. To address whether Sindbis virus-induced NF- κ B activation is required for apoptosis, we used a chimeric Sindbis virus that expresses a superrepressor of NF- κ B activity. Complete suppression of virus-induced NF- κ B activity neither prevents nor poten-

tiates Sindbis virus-induced apoptosis. In contrast, inhibition of NF- κ B activity before infection inhibits Sindbis virus-induced apoptosis. Our results demonstrate that suppression of steady-state, but not stimulus-induced NF- κ B activity, regulates expression of gene products required for Sindbis virus-induced death. Furthermore, we show that in the same cell line, NF- κ B can be proapoptotic or antiapoptotic depending on the death stimulus. We propose that the role of NF- κ B in regulating apoptosis is determined by the death stimulus and by the timing of modulating NF- κ B activity relative to the death stimulus.

MULTICELLULAR organisms use a programmed process known as apoptosis to eliminate damaged or unwanted cells during development, in adulthood, or in disease states. In some instances, cells destined to die by apoptosis must synthesize new mRNAs encoding putative death proteins for ordered self-destruction to occur. Indeed, general inhibitors of transcription and translation inhibit apoptotic death in response to a host of stimuli, even when added many hours after the onset of the death stimulus (Martin et al., 1988; Oppenheim et al., 1990; Ratan et al., 1994; Jacobson et al., 1997). A requirement for transcription in the proper execution of some types of apoptosis has stimulated a search for DNA-binding proteins known as transcription factors that are activated by apoptotic stimuli, and that govern expression of putative death proteins (Jehn and Osborne, 1997). Several apoptosis-associated transcription factors have been identified, including p53 (Lowe et al., 1993), c-jun (Estus et al., 1994; Ham et al., 1995), Nurr-77/Nor-1 (Liu et al.,

1994; Woronicz et al., 1994), and interferon regulatory factor-1 (Tanaka et al., 1994).

Recent data suggests that another transcription factor, NF- κ B, though classically associated with immune and inflammatory responses (Baeuerle and Henkel, 1994), can also be added to the list of apoptosis-associated transcription factors. The NF- κ B family of transcription factors is composed of five different subunits, including p50, p52, p65/Rel A, c-Rel, and Rel-B, which can form either homodimers or heterodimers (Baeuerle and Baltimore, 1996). Overexpression of one of these NF- κ B subunits, c-rel, in chick bone marrow cells leads to apoptosis (Abbadie et al., 1993). These observations suggest that in some cell types, NF- κ B activation is sufficient to activate the apoptotic process. Additionally, inhibition of NF- κ B activity using κ B-specific transcription factor decoys inhibits Sindbis virus-induced cell death in AT-3 prostate carcinoma cells (Lin et al., 1995). Moreover, overexpression of a truncated dominant-negative form of p65/rel A inhibits serum deprivation-induced NF- κ B-dependent gene activation and apoptosis in human embryonic kidney cells (Grimm et al., 1996). These results identify apoptotic paradigms where NF- κ B activation is necessary for cell death initiation, and confirm that NF- κ B can act as a proapop-

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totic transcription factor. Indeed, several proapoptotic genes, including c-Myc (La Rosa et al., 1994), p53 (Wu and Lozano, 1994), Fas ligand (Takahashi et al., 1994), and interleukin-1 β -converting enzyme (caspase-1; Casano et al., 1994) have NF- κ B-binding sequences in their promoter regions.

In contrast to the aforementioned observations, NF- κ B activity appears to regulate genes that suppress some types of apoptosis. For example, suppression of NF- κ B activity in immature B cells after adding anti-IgM (Wu et al., 1996); in cells stimulated with TNF- α , radiation, or daunorubicin (Liu et al., 1996; Beg and Baltimore, 1996; Wang et al., 1996; van Antwerp et al., 1996); or in liver during development (Beg et al., 1995); dramatically enhances cell death. Putative NF- κ B-regulated antiapoptotic genes include manganese superoxide dismutase (Wong et al., 1989) and the zinc finger protein A20 (Opipari et al., 1992). Thus, NF- κ B activity can play a pivotal role in triggering antiapoptotic or proapoptotic pathways; however, the precise factors that determine NF- κ B's ability to regulate these divergent biological outcomes remain unclear.

Under basal nonstress conditions, NF- κ B is sequestered in most cells in the cytoplasm by a member of the I κ B family of proteins: I κ B α , I κ B β , or I κ B ϵ (Baeuerle and Baltimore, 1988; Baeuerle and Baltimore, 1996). Binding of I κ B to NF- κ B masks nuclear localization signals on NF- κ B and prevents its translocation to the nucleus (Beg et al., 1992). In response to a host of stimuli, including TNF- α , virus infection, lipopolysaccharide, and UV light, I κ B α is phosphorylated, followed by its degradation via the ubiquitin-proteasome pathway (Henkel et al., 1993; Palombella et al., 1994; Thanos and Maniatis, 1995; Chen et al., 1996; DiDonato et al., 1995; DiDonato et al., 1996; DiDonato et al., 1997; Régnier et al., 1997). Degradation of I κ B α allows free NF- κ B to translocate to the nucleus to influence expression of its target genes. Degradation of I κ B α can be inhibited by mutating either serine 32 or 36 in the NH₂-terminal region of I κ B α to alanines. (Brown et al., 1995; Traenckner et al., 1995; DiDonato et al., 1996). Overexpression of the 32A/36A NH₂-terminal mutant (I κ B M) is an effective molecular strategy for suppressing NF- κ B activation in cultured cells (Wang et al., 1996). Indeed, stable cell lines containing the superrepressor of NF- κ B, I κ B M have been generated and used to demonstrate an antiapoptotic role for NF- κ B activity in TNF α , daunorubicin, or UV irradiation-induced apoptosis (Wang et al., 1996; van Antwerp et al., 1996). However, this approach does not distinguish whether suppression of NF- κ B activity must be initiated before or after the apoptotic stimulus to regulate apoptosis.

To begin to address this question, we used the alphavirus Sindbis virus, which induces apoptosis in cultured cells (Levine et al., 1993) and in the brains of newborn mice (Lewis et al., 1996). Like many other inducers of apoptosis, the precise mechanisms by which Sindbis virus (SV)¹ induces apoptosis is unknown, but many general antagonists of apoptosis, including Bcl-2 (Levine et al., 1993; Le-

vine et al., 1996), Bcl-X_L (Cheng et al., 1996), or the antioxidant *N*-acetylcysteine (NAC; Lin et al., 1995) can block SV-induced death. Moreover, SV induces activation of NF- κ B via a classical ubiquitin-proteasome pathway many hours in advance of morphological evidence of apoptosis (Lin et al., 1998). Previous studies from our laboratory demonstrated that NF- κ B activity is required for cell death in AT-3 prostate carcinoma cells (Lin et al., 1995), but as with other studies, the NF- κ B inhibitors were present before the apoptotic stimulus was applied, and so the role of SV-induced NF- κ B activation in regulating apoptosis could not be directly assessed.

Recently, a recombinant form of SV has been developed as a vector to express heterologous proteins after infection, allowing their roles in SV-induced death to be examined (Cheng et al., 1996). We used this vector to generate a recombinant SV that carries the superrepressor form of I κ B α . Such a vector can not only elicit apoptosis, but can also inhibit nuclear NF- κ B activity after, but not before infection. We report here that complete suppression of NF- κ B activation after SV infection neither suppresses nor potentiates cell death. In contrast, stable expression of a superrepressor form of I κ B α in cell lines resulting in constitutive suppression of NF- κ B activation suppresses SV-induced death. These data suggest that NF- κ B activity induced by an apoptotic stimulus such as SV infection does not influence cell viability, while NF- κ B activity before SV infection is critical in regulating a proapoptotic response.

Materials and Methods

Culture and Viability Studies

AT-3 rat prostate carcinoma cells were cultured as previously described (Lin et al., 1995) in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 μ g/ml). Wild-type, p65 knockout (p65^{-/-}), and p50 knockout (p50^{-/-}) 3T3 fibroblasts or EFs (mouse embryonic fibroblasts) were cultured as previously described (Beg and Baltimore, 1996). For viability studies, 10³ AT-3 cell/well or 10⁴ mouse embryo fibroblasts/well were plated in 96-well dishes. One day after plating, SV (strain AR339) or recombinant SV (wild-type, M, or R) were added at a multiplicity of infection of 10 plaque-forming units per cell. Viability was assessed using a viability index generated by measuring the cell-associated LDH activity (Promega Corp., Madison, WI) in SV-infected cells and dividing this value by the cell-associated LDH activity in mock-infected cells. In parallel, viability was also assessed by using the metabolic label alamarBlue (Accu Med International, Chicago, IL) and by making morphological observations of cell death using phase contrast microscopy. Apoptotic cells were identified by the presence of phase bright apoptotic bodies, or by pyknosis associated with membrane blebbing. Previous studies have verified that these morphological changes induced by SV in AT-3 cells are correlated with DNA laddering in multimers of 185 bp, and hypercondensation and fragmentation of chromatin characteristic of apoptosis (Lin et al., 1995).

For the cytotoxicity experiments involving TNF α (mouse; Boehringer Mannheim Corp., Indianapolis, IN), 10⁴ AT-3 Zeo or AT-3 I κ B Zeo cells were seeded/well in a 96-well plate; for cytotoxicity experiments involving H₂O₂, staurosporine 10³ AT-3 Zeo or AT-3 I κ B Zeo cells were seeded/well in a 96-well plate. In each of these death paradigms, viability was assessed as described above.

Generation of Recombinant Sindbis Viruses Containing I κ B wild-type (wt), I κ B M, and I κ B R

A fragment of hemagglutinin (HA; 3 \times)-tagged I κ B α wt or an HA (3 \times) tagged-I κ B α with serines 32 and 36 mutated to alanines (M) was cut from the HindIII/NotI sites of the corresponding Bluescript II Ks+ plasmids (DiDonato et al., 1996). Each fragment containing I κ B wt or I κ B M was

1. *Abbreviations in this paper:* EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; M, mutant; NAC, *N*-acetylcysteine; R, reverse; SV, Sindbis virus; STS, staurosporine; wt, wild-type.

blunt end-ligated into the BstEII site of a recombinant SV vector (ds TE12Q) in forward and reverse orientations. The SV vectors containing wild-type and mutated forms of HA-I κ B α were linearized with PvuII and transcribed in vitro with SP6 RNA polymerase (GIBCO BRL, Gaithersburg, MD). The resultant RNA transcripts were then transfected into BHK cells using 10 μ g of lipofectamine (GIBCO BRL) according to the manufacturer's instructions. 24 h after transfection, recombinant viruses were harvested from the supernatant of the culture media of the transfected BHK cells. Viral titers were determined by standard plaque assays as previously described.

Electrophoretic Mobility Shift Assays (EMSAs)

Cytoplasmic and nuclear extracts were generated as previously described (Lin et al., 1995). The protein concentration was determined by BCA assay (Pierce Chemical Co., Rockford, IL). Binding reactions for EMSA were performed at room temperature for 15 min using 6–8 μ g of nuclear protein and 40,000 cpm (NF- κ B) or 25,000 cpm (AP-1) of radiolabeled oligonucleotide. DNA-protein complexes were separated from unbound probe on native 5.3% polyacrylamide gels at 200–250 V for 2–3 h. The resultant gel was vacuum-dried and exposed on film (Eastman Kodak Co., Rochester, NY) overnight at –80°C.

Immunoblot Analysis

20 μ g of protein from cytoplasmic extracts were boiled in Laemmli buffer and electrophoresed under reducing conditions on 12% polyacrylamide gels. Proteins were then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Nonspecific binding was inhibited by incubation in Tris-buffered saline (TBST: 50 mM Tris-HCl, pH 8.0, 0.9% NaCl, 0.1% Tween 20) containing 5% nonfat dried milk for 2 h. Rabbit anti-I κ B α antibody (C-21; Santa Cruz Biotechnology, Santa Cruz, CA) and a polyclonal antibody recognizing Sindbis virus structural proteins (a gift from Dr. Diane Griffin, The Johns Hopkins University School of Hygiene and Public Health) were diluted in 1% milk-TBST at 1:1,000. Mouse anti-HA (12 CA5, Boehringer Mannheim) monoclonal antibody was diluted 1:1,000 in 1% milk TBST. After exposure of membranes to HRP-conjugated anti-rabbit secondary antibody or HRP-linked anti-mouse secondary antibody for 1.5 h, immunoreactive proteins were detected according to the enhanced chemiluminescent protocol (Amersham Corp., Arlington Heights, IL). For quantitative analysis of I κ B α degradation after SV infection, secondary antibody incubation and immunocomplexes detection were performed according to the enhanced chemiluminescent fluorescence protocol (Amersham Corp.).

Generation of AT-3 Cell Lines with Enforced Expression of I κ Bs

A HindIII/Not I fragment containing HA-tagged I κ B M was blunt-ended and ligated into the NheI site of an expression vector, pcDNA 3.1/Zeo (Invitrogen, San Diego, CA). 2 μ g of pcDNA 3.1/HA-I κ B Zeo or empty vector pcDNA 3.1/Zeo was transfected into AT-3 cells (2×10^5) using lipofectamine as described above. Resistant pools of clones containing the transfected plasmids were selected with Zeocin (Invitrogen Corp.) at 350 μ g/ml. Approximately 30 pools of clones (AT-3 I κ B Zeo or AT-3 Zeo) were isolated and expanded. Expression of I κ B M in two independent pools of clones was verified by immunoblotting using an anti-I κ B α antibody as described above.

Luciferase Assays

10^5 AT-3 Zeo or AT-3 I κ B Zeo cells were transfected using the lipofectamine method, with 1.5 μ g of SV40 κ B-luc reporter constructs (Ting et al., 1996) along with 500 ng of pCMV- β gal plasmids. Luciferase activity was determined 30 h after transfection, and was normalized on the basis of β -galactosidase expression level. Both the luciferase assay system and β -galactosidase assay system were purchased from Promega Corp.

Results

Inhibition of SV-induced NF- κ B Activity in AT-3 Cells by Expression of I κ B α in a Sindbis Virus Vector

To test whether SV-induced NF- κ B activity is required for

SV-induced apoptosis, we generated a recombinant SV that carries the wild-type I κ B α gene (SV-I κ B wt), or a more stable mutant form of I κ B α (SV-I κ B M), each containing three influenza virus HA epitope tags. As a control, we inserted the HA-tagged I κ B wt in the reverse orientation into the SV vector. Infection of AT-3 cells with SV-I κ B wt or SV-I κ B M revealed the expression of HA-tagged I κ B 8 h after infection (Fig. 1, A and B). Using an anti-HA antibody, we detected two bands reflecting HA-I κ Bs that likely are translated from different initiation sites within the HA-tagged region on the virus vector (Fig. 1 B). As expected, quantitative analysis of immunoblots using ECF and phosphorimaging showed 20% greater levels of HA-I κ B in extracts from SV-I κ B M-infected cells as compared with extracts from SV-I κ B wt-infected cells.

Additionally, overexpression of I κ B by the vector SV appeared to slow degradation of endogenous I κ B α because we were able to detect modified, likely phosphorylated higher molecular weight forms of endogenous I κ B α in cytoplasmic extracts from cells infected with SV-I κ B wt and SV-I κ B M, but not SV-I κ B R-infected cells (Fig. 1 A). Moreover 39, 25, and 37% of endogenous I κ B α from AT-3 cells infected with SV-I κ B wt, SV-I κ B M, and SV-I κ B R, respectively, was degraded or converted to a modified form 16 h after infection as compared with I κ B α in mock-infected cells (corresponding band is labeled by a star in Fig. 1 A). Altogether, these data demonstrate that we are able to express heterologous I κ B wt and I κ B M in AT-3 cells using the SV vector, and that, like the wild-type SV (Lin et al., 1998), the recombinant SVs result in degradation of endogenous I κ B α .

To determine whether the expressed I κ Bs are able to suppress SV-induced NF- κ B activity, we performed EMSAs on nuclear extracts from infected and mock-infected cells using a 32 P-labeled probe with a consensus NF- κ B binding site. As shown in Fig. 1 D, 8 h after infection, SV-I κ B R elicited a dramatic increase in two NF- κ B complexes, previously defined as p50–p65 and p50–p50 (Lin et al., 1995); SV-I κ B M completely suppressed p50–p65 activity and diminished p50–p50 activity; and SV-I κ B wt induced a level of p50–p65 activity intermediate between the SV-I κ B M and the SV-I κ B R. The differences in NF- κ B activity among recombinant SV-I κ Bs was not due to their different replication rates in AT-3 cells, because levels of SV structural proteins (envelope glycoproteins, E1/E2, and capsid proteins) were similar in cells infected with each recombinant virus (Fig. 1 C).

Complete Suppression of SV-induced NF- κ B Activity Does Not Inhibit SV-induced Apoptosis in AT-3 Cells

We demonstrated that SV-induced NF- κ B activity could be suppressed using a recombinant SV containing a wt or mutant form of I κ B α . To determine the effects of suppressing SV-induced NF- κ B activity on apoptosis, we measured viability of cells infected with the different recombinant viruses at several time points after infection. As shown in Fig. 2, we found that SV-I κ B wt, SV-I κ B M, and SV-I κ B R triggered cell death with similar kinetics in AT-3 cells. Similar results were observed in BHK cells (data not shown). These results suggest that SV-induced NF- κ B activity is not required for SV-induced apoptosis. However,

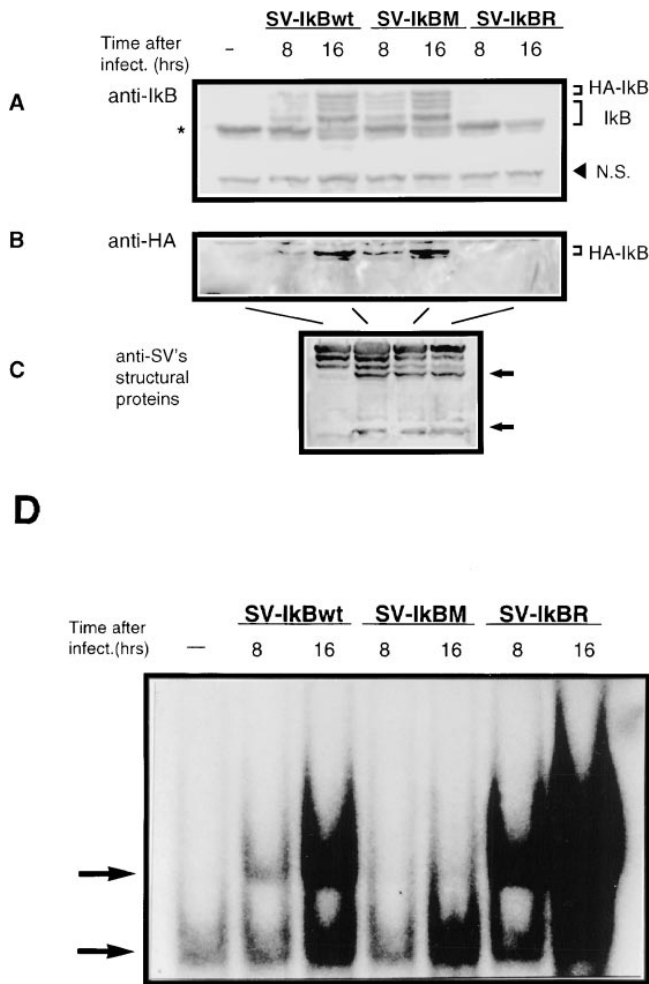


Figure 1. Inhibition of SV-induced NF- κ B activity in AT-3 cells by heterologous expression of the inhibitory subunit of NF- κ B, I κ B α in a SV vector. (A–C) Cytoplasmic extracts were prepared from AT-3 cells infected with SV-I κ B wt, SV-I κ B M, or SV-I κ B R at the time points indicated. A shows a Western blot analysis against I κ B α . HA-I κ B corresponds to the heterologous HA-tagged human I κ B α ; I κ B corresponds to the endogenous I κ B α ; endogenous nonphosphorylated I κ B α and nonspecific bands are denoted by an asterisk and N.S., respectively. (B) The blot shown in A was stripped, and anti-HA immunoreactive proteins are shown. C shows the Western blot analysis against the sindbis virus structural proteins. The upper arrow points to the viral envelope glycoproteins E1 and E2; the lower arrow points to the viral capsid protein. (D) EMSA performed with 6–8 μ g of nuclear extracts corresponding to cytoplasmic extracts used in A–C using a 32 P-labeled oligonucleotide with a consensus NF- κ B sequence from the MHC class I promoter. Upper arrow points to the predominant complex induced by SV, p50-p65 heterodimer; the lower arrow points to p50-p50 homodimers. The composition of the induced NF- κ B complexes were identified in a previous study (Lin et al., 1995).

in a previous study we established that NF- κ B is required for SV-induced death by pretreating AT-3 cells with NF- κ B-specific transcription factor decoys that competitively inhibit binding of NF- κ B to its target DNA sites. To reconcile these apparently conflicting observations, we hypothesized that NF- κ B activity before infection is necessary for SV-induced apoptosis; that is, steady-state levels of NF- κ B may regulate expression of latent death genes before SV infection.

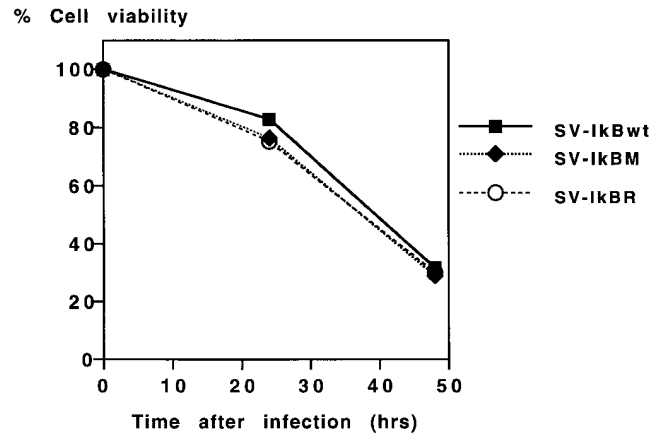


Figure 2. Cell viability of AT-3 cells infected with SV-I κ B wt, SV-I κ B M, and SV-I κ B R. Percent viability was determined 24 and 48 h after infection by the alamar blue assay. Results are expressed as the mean \pm SEM for experiments performed in triplicate ($P > 0.05$). Error bars that did not show up are hidden by the symbols.

Stable Overexpression of I κ B M in AT-3 Cells Delays SV-induced Apoptosis

AT-3 cells that overexpress I κ B M were established to examine whether inhibition of NF- κ B activity beginning before SV infection inhibits SV-induced death. Two pools of cell clones stably expressing HA-tagged I κ B M (AT-3 I κ B Zeo) were generated along with a pool of control clones expressing a zeocin resistance gene product alone (AT-3 Zeo; Fig. 3 A). A pool of cell clones represents a population of one or more individual clones. EMSA indicated that I κ B M overexpression in AT-3 cells functions as a superrepressor because there was significant suppression of NF- κ B binding activity after SV infection in AT-3 I κ B Zeo clones compared with the AT-3 Zeo cells (Fig. 3 B). A slight induction of NF- κ B activity 6 h after infection in the AT-3 I κ B cells was observed, likely due to the heterogeneous levels of HA-I κ B M expression in our pool of AT-3 I κ B clones.

AT-3 I κ B Zeo cells that constitutively suppress NF- κ B activity are more resistant to SV-induced death than are AT-3 Zeo cells (Fig. 3 C). The protection conferred by stable overexpression of I κ B M could not be attributed to decreased viral entry or viral replication in these cells, because viral production measured 4 and 8 h after infection was similar ($P > 0.5$) in AT-3 I κ B Zeo and AT-3 Zeo cells (data not shown). These results suggest that inhibition of NF- κ B activity beginning before, but not after SV infection prevents SV-induced death. Indeed, using a luciferase reporter regulated by eight NF- κ B binding sites, we found nearly complete suppression of constitutive NF- κ B activity in AT-3 I κ B Zeo cells compared with AT-3 Zeo cells (Fig. 3 D) or parental AT-3 cells (not shown).

Short-term Treatment of AT-3 Cells with N-acetyl-L-cysteine Combined with Infection of AT-3 Cells with SV-I κ B M, but not SV-I κ B R, Prevents Cell Death

We next used a different approach to confirm that inhibi-

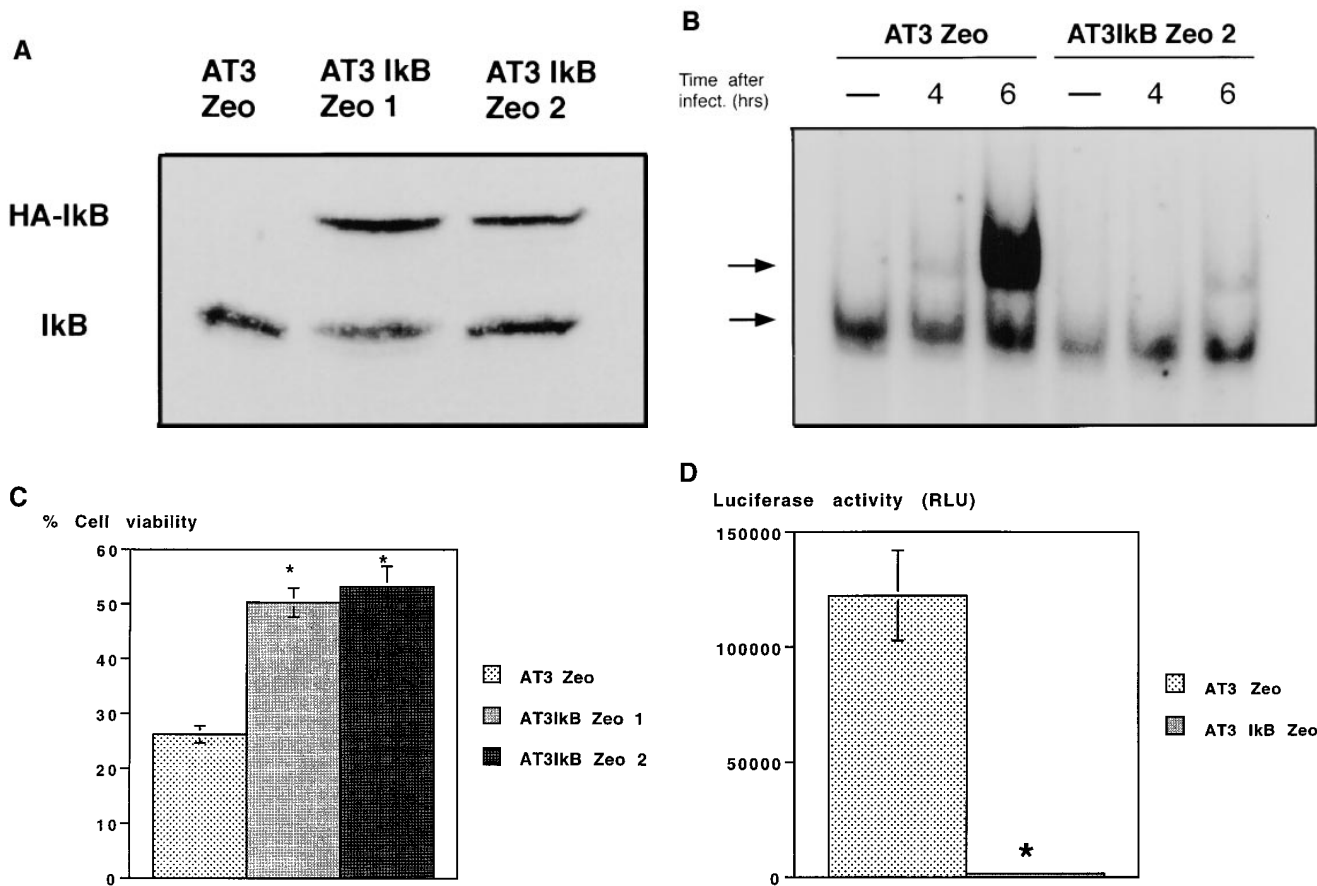


Figure 3. The effect of stable overexpression of IκB α M on SV-induced apoptosis in AT-3 cells. (A) Western blot analysis demonstrating expression of HA-tagged IκB M in two pools of AT-3 IκB Zeo cell clones, but not in the corresponding AT-3 cells containing the zeocin resistance gene alone. (B) EMSA was performed using nuclear extracts obtained in parallel from SV-infected AT-3 Zeo or AT-3 IκB Zeo cells 0, 4, and 6 h after infection. The upper arrow points to the p50-p65 complex, and the lower arrow points to p50-p50 homodimers. (C) Cell viability of AT-3 Zeo and AT-3 IκB Zeo cells 40 h after SV infection. Viability was determined by LDH assay as described in Materials and Methods, and is expressed as mean \pm SEM for three separate experiments ($P < 0.005$). (D) Basal level of NF- κ B transcriptional activity was nearly completely suppressed in AT-3 IκB Zeo cells compared with AT-3 Zeo cells. Basal level of NF- κ B activity was determined by κ B luciferase reporter system ($P < 0.005$).

tion of NF- κ B activity must be initiated in advance of the apoptotic stimulus to prevent SV-induced death. In previous studies, we demonstrated that NAC at 30 mM prevents SV-induced apoptosis without interfering with viral replication (Lin et al., 1995). Therefore, we infected AT-3 cells with recombinant SV-IκBs in the presence of NAC. In this way, we could suppress activation of apoptosis induced by SV, but allow the recombinant SV to replicate and concomitantly express the exogenous HA-tagged IκB M. As expected, nearly complete survival was maintained in SV-IκB M and SV-IκB R-infected cells in the presence of 30 mM NAC (Fig. 4 A), and expression of HA-tagged IκB M in the SV-IκB M-infected cells, but not the SV-IκB R-infected cells, was verified by immunoblotting (data not shown). After 19 h, the NAC was removed. The cells infected with SV-IκB M were significantly protected from cell death as compared with cells infected with SV-IκB R 24 and 48 h after NAC withdrawal (Fig. 4 A). AT-3 cells that have been treated with NAC for 19 h appear to have no defects in NF- κ B activation, because 4 h after NAC withdrawal, cells infected with SV-IκB R, but not the SV-IκB M, elicited increased nuclear NF- κ B activity (Fig. 4 B).

Embryonic Fibroblasts or 3T3 Cells Derived from p65^{-/-} Mice, but Not Embryonic Fibroblasts Derived from p50^{-/-} Mice, are Resistant to SV-induced Apoptosis

We next investigated whether inhibition of NF- κ B activity must be initiated before the apoptotic stimulus to observe protection from SV-induced apoptosis in other cell types. Cell survival was examined in primary embryonic fibroblasts (EFs) or immortalized fibroblasts (3T3) from p65 knockout (p65^{-/-}), p50 knockout (p50^{-/-}), and wt mice infected with SV. Similar to our observations in AT-3 cells, SV-IκB wt, SV-IκB M, and SV-IκB R induced death in 3T3 cells with identical kinetics (Fig. 5 A). In contrast, p65^{-/-} 3T3 cells, but not p50^{-/-} 3T3 cells, were more resistant to SV-induced apoptosis than the wt 3T3 cells. These effects are unlikely to be due to secondary changes resulting from immortalization, because EFs from p65^{-/-} mice, but not p50^{-/-} mice, showed significant protection from SV-induced cell death as well (Fig. 5 B). Viral titers determined from the infected cell supernatant of wt 3T3 cells and p65^{-/-} 3T3 cells 4, 8, and 12 h after infection indicated that SV was replicating equally well in both cells ($P > .05$; data not shown).

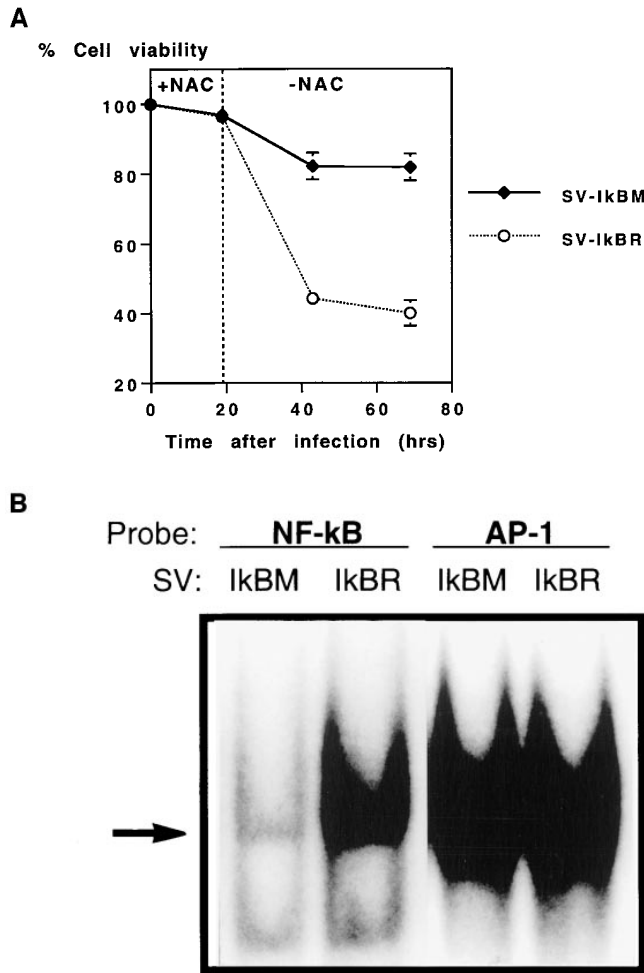


Figure 4. Short-term treatment of AT-3 cells with *N*-acetyl-L-cysteine (NAC) combined with infection with SV-IκB M, but not SV-IκB R, prevents cell death. (A) 2.5×10^3 cells/well in 96-well plates were infected with SV-IκB M or SV-IκB R, and were treated with 30 mM NAC for 19 h. The cells were washed twice with PBS, and fresh media without NAC was added. Cell viability was determined by the alamar blue assay 0, 24, and 48 h after NAC withdrawal. Results are expressed as the mean \pm SEM for three experiments ($P < 0.005$) 24 and 48 hours after NAC withdrawal. (B) EMSAs were performed on nuclear extracts from SV-IκB M and SV-IκB R-infected cells 4 h after NAC withdrawal using 6–8 μ g of protein and radiolabeled NF- κ B or AP-1 probes. The quality of the nuclear extracts from the treated and infected cells was confirmed by analysis of AP-1 DNA activity, indicating that NAC does not interfere with global nuclear protein activity. The arrow points to the NF- κ B p50–p65 complex; the lower band is the NF- κ B p50–p50 complex.

Altogether, data from AT-3 prostate carcinoma cells and mouse embryo fibroblasts experiments argue that inhibition of NF- κ B activity must be initiated before SV infection to suppress cell death, while inhibition of SV-induced NF- κ B activity alone neither potentiates nor inhibits SV-induced death.

The Proapoptotic and Antiapoptotic Roles of NF- κ B are Determined by the Death Stimulus

Our observations that fibroblasts from p65^{-/-} mice are

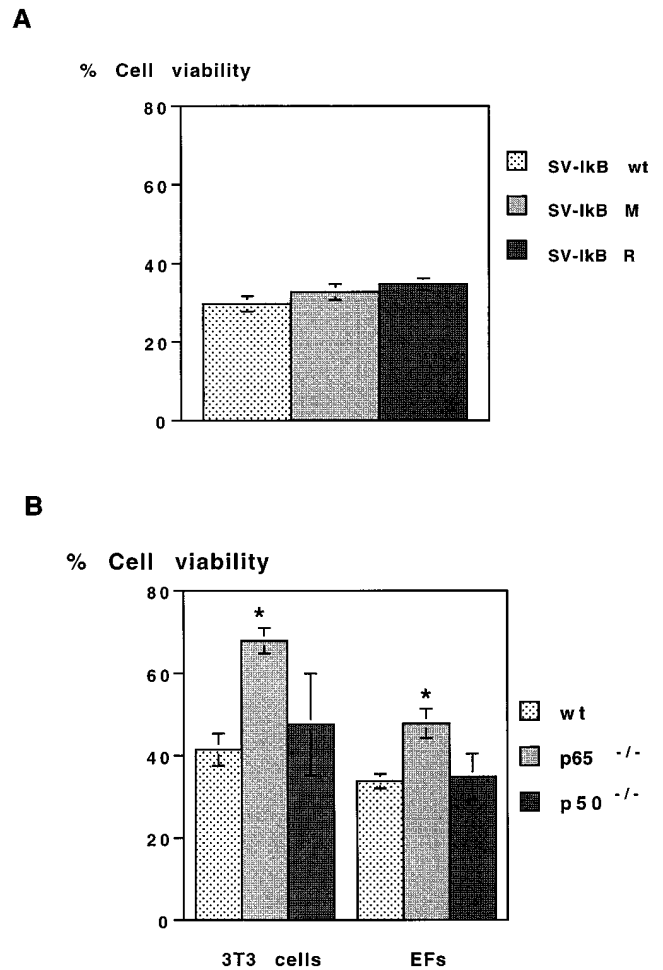


Figure 5. Cell viability of wt, p65 knockout, and p50 knockout embryo fibroblasts. (A) Viability of wt 3T3 cells infected with SV-IκB wt, SV-IκB M, and SV-IκB R 24 h after infection ($P > 0.05$). (B) Viability of wt, p65^{-/-}, and p50^{-/-} 3T3 cells and EFs infected with wt SV 24 h after infection. 3T3 refers to immortalized fibroblasts, while EFs refers to primary fibroblasts. Viability was determined by LDH assay as described in Materials and Methods and normalized to identical cell densities among the three cell types. Results are expressed as mean \pm SEM for 3–5 experiments ($P < 0.05$).

more resistant to SV-induced apoptosis are in contrast to previous observations that TNF α -induced apoptosis is potentiated in fibroblasts from p65^{-/-} mice (Beg and Baltimore, 1996). These results suggest that the proapoptotic or antiapoptotic roles of NF- κ B are determined by the death stimulus, and are not necessarily dependent on the cell type. To examine this possibility further, we compared viabilities in populations of AT-3 Zeo and AT-3 IκB M clones in response to a broad range of cytotoxic stimuli, including hydrogen peroxide (H₂O₂), TNF α , the general protein kinase inhibitor staurosporine (STS), and serum deprivation. Similar to SV infection, we found that cell death induced by 100 μ M (not shown) or 500 μ M peroxide was delayed in two populations of IκB M overexpressing cell clones as compared with control cells (Fig. 6 A); thus, in addition to SV infection, NF- κ B also appears to exhibit prodeath activity after peroxide treatment. In contrast

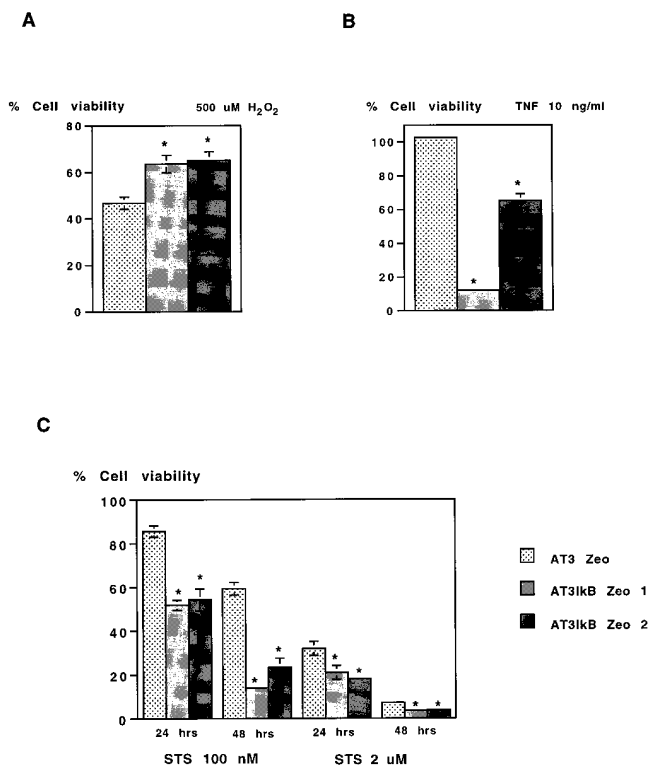


Figure 6. The effect of overexpression of IκB M on H₂O₂, TNF α-, and STS-induced death in AT-3 cells. Cell viability was determined by LDH assay as described in Materials and Methods, and results are expressed as mean ± SEM for three separate experiments. AT-3 Zeo or AT-3 IκB Zeo cells exposed to: (A) 500 μM peroxide for 4 h ($P < 0.005$), (B) TNF-α (10 ng/ml) for 24 h ($P < 0.005$), and (C) 100 nM or 2 μM STS for 24 or 48 h ($P < 0.005$).

(and in agreement with observations from others; Liu et al., 1996; Beg and Baltimore, 1996; Wang et al., 1996; van Antwerp et al., 1996), exposure of AT3-IκB M cells to TNF α (10 ng/ml) or staurosporine (STS at 100 nM or 2 μM) results in significantly greater levels of cell death than AT3-Zeo cells exposed to each of these stimuli (Fig. 6, B and C). Finally, AT-3 Zeo or AT-3 IκB cells died at similar rates ($P > 0.1$) in response to 48 h of serum deprivation (Table I). These observations support the notion that the death stimulus is critically important in determining whether NF-κB regulates prodeath, antideath proteins, or is an innocent bystander.

Discussion

Elucidation of the signaling pathways leading to activation of NF-κB, the precise identity of NF-κB family members, and definition of consensus DNA-binding sequences of this family of DNA-binding proteins have provided powerful molecular tools for suppressing nuclear NF-κB activity in intact cells, and in evaluating the role of NF-κB in apoptosis (Abbadie et al., 1993; Barger et al., 1995; Lin et al., 1995; Beg and Baltimore, 1996; Wang et al., 1996; van Antwerp et al., 1996). In each of these studies, deletion of individual NF-κB subunits or expression of inhibitors of NF-κB nuclear activity were initiated before the apoptotic stimulus, so it could not be determined if the critical cell

Table I. The Role of NF-κB in Regulating Apoptosis in AT-3 Cells Depends on the Death Stimulus

Stimulus	Proapoptotic	Antiapoptotic
SV	+	-
H ₂ O ₂ (100 μM and 500 μM)	+	-
TNF (10 ng/ml)	-	+
STS (100 nM and 2 μM)	-	+
Serum deprivation (48 h)	-	-

death regulatory function(s) of NF-κB activity occur before or after the death stimulus. Indeed, in previous studies we showed that suppression of SV-induced NF-κB activity by pretreatment with NF-κB-specific transcription factor decoys inhibits SV-induced apoptosis (Lin et al., 1995). Our previous results could not distinguish between two likely models (Fig. 7). In the first model (see Fig. 7 A), SV induces degradation of IκB, leading to translocation of NF-κB to the nucleus and expression of one or more κB-dependent death proteins. In the second model (see Fig. 7 B), nuclear NF-κB activity occurring at some time before the SV infection is required for expression of a protein involved in apoptosis that is directly or indirectly activated by SV infection. In the second scheme, SV-induced NF-κB activity could be involved in regulating responses not directly related to cell death, such as the inflammatory response. Herein, we show that a recombinant SV carrying a superrepressor form of IκB α suppresses SV-induced NF-κB nuclear activity (Fig. 1), but does not inhibit or potentiate apoptosis in AT-3 prostate carcinoma cells (Fig. 2) or 3T3 embryo fibroblasts (Fig. 5 A). These results, combined with our observations that AT-3 cells stably overexpressing the superrepressor of NF-κB (Fig. 3) or mouse embryo fibroblasts deleted in the p65 subunit of NF-κB (Fig. 4) are more resistant to SV-induced death, are consistent with the second model. Furthermore, these data imply a requirement for a p65-containing NF-κB complex to drive expression of an essential gene for SV-induced cell death.

If Sindbis-virus-induced NF-κB activation is not an important determinant in cell viability, what is the stimulus before infection leading to NF-κB-dependent synthesis of prodeath proteins? Several lines of evidence have demonstrated that NF-κB activity can be modulated during cell cycle progression (Baldwin et al., 1991; Li et al., 1993; Evans et al., 1993; Duckett et al., 1995; Bash et al., 1997). In quiescent 3T3 fibroblasts, NF-κB is quickly induced by stimulation with serum or growth factors as cells transition from G₀ to G₁ stages of the cell cycle (Duckett et al., 1995). Moreover, specific cyclin-dependent kinases were shown to regulate transcriptional activation by NF-κB through the interaction with the coactivator p300 (Perkins et al., 1997). Thus, a cell cycle-dependent NF-κB activity could be activated before infection. Such an activity may be undetectable by EMSA (Fig. 1 D and Fig. 3 B), but detectable by NF-κB reporter gene assay (Fig. 3 D), which is more sensitive. The inability to detect NF-κB DNA binding may be due to the unsynchronized nature of the population of cells we study, or because cell cycle-dependent activation of NF-κB-dependent genes occurs as a result of phosphorylation of nuclear p65-containing complexes without enhanced nuclear translocation of NF-κB (Finco et al., 1997). We speculate that the cell cycle-dependent

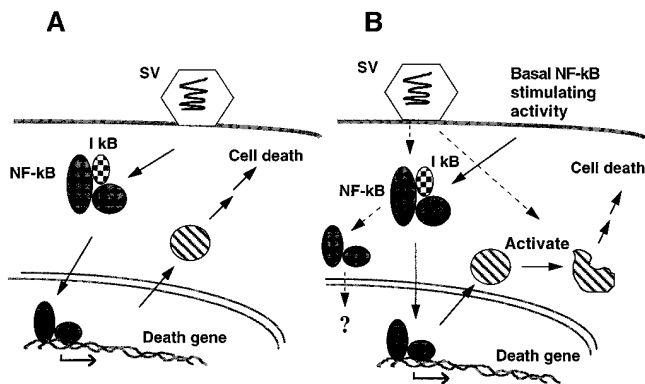


Figure 7. Proposed models by which NF- κ B regulates SV-induced apoptosis. (A) SV induces degradation of I κ B, allowing translocation of an NF- κ B heterodimer from the cytoplasm to the nucleus. NF- κ B activates transcription of one or more unknown death genes required for cell death. (B) Before infection, steady state NF- κ B activity regulates expression of an inactive form of a death protein that is posttranscriptionally or posttranslationally activated by SV, leading to apoptosis. In this scheme, SV-induced NF- κ B activity is not involved in regulating SV-induced apoptosis (dashed line).

activation of NF- κ B may regulate expression of inactive forms of one or more apoptotic effectors or regulators before the apoptotic stimulus. Of note, previous studies have shown that inducible expression of a dominant negative Ras inhibits cell cycle progression and SV-induced apoptosis, while infection of PC12 cells with recombinant SV carrying a dominant negative Ras has no effect on viability (Joe et al., 1996). These findings, along with data presented herein and recent observations demonstrating that oncogenic Ras activates NF- κ B-dependent gene expression, are consistent with the following model: a cell cycle-dependent Ras-induced signal transduction pathway activates NF- κ B that regulates the expression of proapoptotic genes before SV infection.

How can the proapoptotic role of NF- κ B in the setting of SV infection be reconciled with the established antiapoptotic role of NF- κ B in response to other apoptotic stimuli? The stimulus, the cell type, and the subunit of NF- κ B activated have all been proposed as critical factors that determine whether this transcription factor promotes or suppresses apoptosis. We provide evidence suggesting that the stimulus may be the main factor in determining NF- κ B's ability to regulate divergent biological outcomes. First, overexpression of I κ B M in AT-3 cells protects from SV and H₂O₂-induced cell death, but potentiates TNF- α and staurosporine-induced cell death, indicating that suppression of NF- κ B activity can be proapoptotic or antiapoptotic in the same cell type, depending on the stimulus. Second, fibroblasts from p65^{-/-} mice, but not p50^{-/-} mice, are resistant to SV-induced death, but more sensitive to TNF- α -induced death. These observations show that the deleted subunit of NF- κ B is required for specific target gene activation leading to either a pro- or antiapoptotic response, but is not a necessary determinant in the proapoptotic or antiapoptotic actions of NF- κ B. Finally, our observation that serum deprivation-induced death occurs with similar kinetics in AT-3 Zeo and AT-3 I κ B Zeo cells sug-

gests that NF- κ B is not part of this common pathway of apoptotic death, and that other stimulus-specific pathways may need to be simultaneously activated to determine NF- κ B's role in determining cell survival or death.

Our results are congruent with the emerging concept of private cell death pathways (Smith and Osborne, 1997). This notion acknowledges the existence of a common effector phase to apoptotic cell death, but states that within a given cell, many stimuli are capable of inducing apoptosis, and that each stimulus may do so by inducing a unique set of genes. These genes in turn may positively or negatively regulate activation of the common effector pathway. Heretofore, the best-studied examples of stimulus-specific cell death pathways have been in lymphocytes, where ionizing radiation (Lowe et al., 1993), glucocorticoids (Liu et al., 1994), and T cell activation (Woroniec et al., 1994) each activate unique transcription factor responses that converge on a common final effector pathway to apoptosis. Our studies carry this concept a step further, and suggest that a single transcription factor, NF- κ B, can mediate activation or suppression of cell death signals in the same cell type depending on the stimulus (Fig. 6; Table I). Furthermore, we identify that timing of NF- κ B activation is another important variable in determining whether this transcription factor regulates expression of prodeath proteins, or is an innocent bystander (Figs. 2, 3 C, and 5). The demonstration herein that steady-state NF- κ B activity is required to promote SV-induced cell death in AT-3 prostate carcinoma cells and 3T3 fibroblasts will focus future studies aimed at defining one or more NF- κ B-regulated genes required for SV-induced apoptosis.

Accumulating evidence indicates that apoptosis results from activation of a constitutively expressed suicide program that is conserved in evolution from nematodes to humans (Steller, 1995; Martin and Green, 1995). Interestingly, in prokaryotes such as *Escherichia coli*, a protease whose specific substrate is the translational elongation factor EF-Tu, is constitutively expressed. During infection with bacteria phage, this protease is activated and cleaves EF-Tu, leading to death of the cells (Snyder, 1995; Jacobson et al., 1997). Our findings suggest a parallel example in mammalian cells where SV infection posttranscriptionally or posttranslationally activates a constitutively expressed suicide program that is regulated in part, by NF- κ B before infection.

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