Thiol Agents and Bcl-2 Identify an Alphavirus-induced Apoptotic Pathway That Requires Activation of the Transcription Factor NF-kappa B

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Abstract. Oxidative stress has been proposed as a common mediator of apoptotic death. To investigate further the role of oxidants in this process we have studied the effects of antioxidants on Sindbis virus (SV)-induced apoptosis in two cell lines, AT-3 (a prostate carcinoma line) and N18 (a neuroblastoma line). The thiol antioxidant, *N*-acetylcysteine (NAC), at concentrations above 30 mM, completely abrogates SV-induced apoptosis in AT-3 and N18 cells. The effects of NAC cannot be attributed to inhibition of viral entry or viral replication, changes in extracellular osmolarity or to increases in cellular glutathione levels, nor can they be mimicked by chelators of trace metals, inhibitors of lipid peroxidation or peroxide scavengers. In contrast, other thiol agents including pyrrolidine dithiocarbamate (PDTC, 75 μM) are protective. Because NAC and PDTC are among the most effective inhibitors of the transcription factor NF-kappa B, we examined SV's ability to activate NF-kappa B before the onset of morphologic or biochemical evidence of apoptosis. Within hours of infection, SV induced a robust increase in nuclear NF-kappa B activity in AT-3 and N18 cells; this activation was suppressible by NAC and PDTC. Overexpression of *bcl-2* in AT-3 cells, which has been shown to inhibit SV-induced apoptosis, also inhibits SV-induced NF-kappa B activation. To determine if NF-kappa B activation is necessary for SV-induced apoptosis in these cells, we used double stranded oligonucleotides with consensus NF-kappa B sequences as transcription factor decoys (TFDs) to inhibit NF-kappa B binding to native DNA sites. Wild-type, but not mutant, TFDs inhibit SV-induced apoptosis in AT-3 cells. In contrast, TFD inhibition of NF-kappa B nuclear activity in N18 cells did not prevent SV-induced apoptosis. Taken together, these observations define a cell type-specific, transcription factor signaling pathway necessary for SV-induced apoptosis. Understanding the precise mechanism by which Bcl-2 and thiol agents inhibit SV-induced nuclear NF-kappa B activity in AT-3 cells may provide insights into the pluripotent anti-apoptotic actions of these agents.

INFECTION of mice with Sindbis virus (SV)[1] results in encephalitis and has thus been used to model human encephalitides due to alphaviruses (Strauss and Strauss, 1994). Encephalitis results primarily from infection of neurons of the central nervous system. Studies of SV infection in vitro in cell lines and primary cultured neurons reveal two distinct outcomes of infection: (*a*) lytic infection in which intracellular replication of the virus leads to apoptotic cell death, or (*b*) persistent infection in which the virus produces progeny without killing the host cell (Levine et al., 1993). Under circumstances that lead to lytic infection, SV induces all the morphologic characteristics of apoptosis: chromatin condensation, DNA fragmentation and membrane blebbing (Levine et al., 1993). These morphologic changes reflect the events that lead to compartmentalization of the cell. Fragments of apoptotic cells can then be phagocytized by macrophages or neighboring cells before there is leakage of cytoplasmic contents (Wyllie et al., 1980; Kerr and Harmon, 1991; Barr and Tomei, 1994).

Neuronal outcome in the presence of SV infection appears to be regulated by developmental factors. Persistent, non-lytic infection by alphaviruses has been observed in weanling and adult mice as well as adult humans (Griffin

1. *Abbreviations used in this paper:* 2-ME, 2-mercaptoethanol; BHA, butylated hydroxyanisole; BSO, buthionine sulfoximine; CK-1, cytokine 1; DDTC, diethyl dithiocarbamate; DFO, deferoxamine mesylate; EMSA, electrophoretic mobility shift assays; ICE, interleukin 1-β converting enzyme; LDH, lactate dehydrogenase; MIM, mimosine; NAC, *N*-acetylcysteine; NAS, *N*-acetylserine; PDTC, pyrrolidine dithiocarbamate; PFU, plaque-forming units; SV, Sindbis virus; TFD, transcription factor decoys; WT, wild-type.

et al., 1994; Levine et al., 1994). Under these circumstances, virus is continuously produced by neurons, but viability of neurons and the organism is maintained. Newborn mice infected with SV, on the other hand, experience lytic neuronal infection and death. Primary neurons cultured in vitro (in the absence of immune cells) display a similar developmental susceptibility to lytic SV infection. Thus, while the distinct outcomes of SV infection appear to depend in part, on developmentally regulated virus–host interactions, the precise signals which govern lytic (apoptotic) infection versus persistent infection remain unclear.

An important clue to the mechanism by which SV induces apoptosis comes from the observation that overexpression of the proto-oncogene bcl-2 (Cleary et al., 1986; Tsujimoto and Croce, 1986) in cultured cell lines prevents SV-induced apoptosis and allows a persistent infection (Levine et al., 1993). In addition to preventing SV-induced apoptosis, Bcl-2 can prevent cell death induced by growth factor deprivation, the calcium ionophore A23187, chemotherapeutic agents, oxidative stress (Zhong et al., 1993) and influenza virus (Hinshaw et al., 1994). Although Bcl-2 is not protective in all apoptotic paradigms, its broad spectrum of action suggests that the death program triggered by multiple stimuli converge at, or before, a step that is blocked by Bcl-2.

One attractive but controversial hypothesis of Bcl-2 action contends that oxidative stress induced by diverse apoptotic stimuli is a critical step in triggering cell death and that Bcl-2 acts to neutralize the damaging effects of oxidants (Hockenbery et al., 1993) or to directly scavenge oxidants (Kane et al., 1993). This hypothesis has been challenged by recent studies which demonstrate that apoptosis induced by certain stimuli proceeds in the absence of oxygen radicals and that Bcl-2 can protect under these hypoxic conditions (Jacobson and Raff, 1995; Shimizu et al., 1995). However, support for a role for oxidative stress as one type of mediator of apoptosis comes from observations from several laboratories demonstrating that in cultured cells addition of oxidants or inhibition of antioxidant defenses leads to apoptosis (McConkey et al., 1988; Lennon et al., 1991; Hockenbery et al., 1993; Ratan et al., 1994a, b; Rothstein et al., 1994; Troy and Shelanski, 1994). Additionally, overexpression of antioxidant enzymes (Hockenbery et al., 1993; Greenlund et al., 1995), or treatment of cells with antioxidants (Mayer and Noble, 1994a; Ferrari et al., 1995) abrogates apoptosis induced either by growth factor deprivation, by endotoxin (Abello et al., 1994) or in thymocytes, by dexamethasone or etoposide (Wolfe et al., 1994). Finally, serum deprivation-induced apoptosis in cultured neurons has been shown to be linked to an increase in radical production (Zhong et al., 1993; Greenlund et al., 1995). These findings have focused attention on defining whether apoptosis induced by divergent stimuli, including viruses, is triggered by an oxidative signal and, if so, identifying the molecular targets of that signal.

In a previous study, we demonstrated that neuronal apoptosis can be induced by cystine deprivation, glutathione depletion and oxidative stress in cortical neuronal cultures (Ratan et al., 1994a). In this paradigm, protein synthesis inhibitors abrogate oxidative stress-induced apoptosis by shunting cysteine from global protein synthesis to the thiol antioxidant, glutathione rather than by inhibiting specific

death proteins (Ratan et al., 1994b). N-acetylcysteine (NAC), an agent which increases glutathione levels in cortical neurons by 50%, also prevents cystine deprivation-induced apoptosis (Ratan et al., 1994b). We thus sought in the present study to determine whether SV-induced apoptosis is associated with a depletion of glutathione and whether NAC could inhibit SV-induced death.

Herein, we demonstrate that NAC (30 mM) and other thiol agents, pyrrolidine dithiocarbamate (PDTC), DTT, and 2-mercaptoethanol (2-ME) block SV-induced apoptosis. However, in this paradigm, NAC's protective effects do not depend on its ability to increase glutathione levels. Rather, the protective effects of NAC (and other thiol agents) correlate with their ability to inhibit SV-induced activation of the transcription factor NF-kappa B. Furthermore, we report that suppression of NF-kappa B activation, either by Bcl-2 or by synthetic, double stranded NF-kappa B oligonucleotides employed as transcription factor decoys, inhibits SV-induced death in AT-3 prostate carcinoma cells. These results define NF-kappa B activation as a necessary component of SV-induced apoptosis in AT-3 cells and suggest that thiol agents such as NAC and antiapoptotic proteins such as Bcl-2 may inhibit apoptosis, in part, by acting on an NF-kappa B signaling pathway.

Materials and Methods

Cell Culture and Viability Studies

Mouse N18 neuroblastoma cells were grown as described (Levine et al., 1993) in MEM media containing 10% fetal calf serum, 2 mM L-glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml). For viability studies, the cells were plated in 24-well dishes at a density of 80,000 cells/ml. One day after plating, the media was changed and fresh media containing the following drugs alone or in combination (as designated in the results) were added: N-acetylcysteine (0.1–80 mM), N-acetylserine (30–80 mM), buthionine sulfoximine (20 μM), deferoxamine mesylate (10–500 μM), mimosine (50–500 μM), penicillamine (10 μM–1 mM), butylated hydroxyanisole (10–100 μM), trolox (10 μM–1 mM), catalase (purified from erythyrocytes or Aspergillus Niger;10–10,000 units/ml), dithiothreitol (0.1–10 mM), 2-mercaptoethanol (0.15–15 mM), pyrrolidine dithiocarbamate (10–100 μM), or diethyl dithiocarbamate (10 μM–10 mM). 2 h later, the media was changed to MEM containing low serum (2% fetal calf serum) and Sindbis virus (strain AR339) was added at multiplicity of infection of 1–5 plaque-forming units (PFU)/cell. After a 1-h infection period, the low serum media was replaced with normal media. In cases where the effects of pharmacologic agents were being evaluated, the drugs were maintained in the medium during and after infection. Viability was then assessed 48 h later using trypan blue exclusion (Levine et al., 1993) LDH release, ethidium homodimer (Ethid-1; Molecular Probes, Eugene, OR) staining or DNA fragmentation as previously described (Ratan et al., 1994a, b). Parallel studies were performed using the same drug treatments with AT-3 cells stably expressing pZip containing a neomycin resistance gene (Levine et al., 1993). AT-3 Neo cells were grown as previously described and plated in 24-well dishes at a density of 10,000 cells/well.

Replication In Vitro

N18 and AT-3 cells were grown to near confluency in 12-well plates and infected as described above. Supernatant fluid was removed from 3 wells at 4, 24, and 48 h after infection, virus from each well was quantitated by plaque formation in BHK-21 cells (PFU/ml), and the geometric mean was determined for each time point.

Glutathione Measurements

Total glutathione levels (GSH + GSSG) were measured using the method of Tietze (1969) as previously described (Ratan et al., 1994b). Briefly, at various intervals after infection, control and infected cells plated in 6-well dishes

were washed once with cold PBS. 1 ml of cold 3% perchloric acid was added per well and cells were scraped off the bottom of the dish using a rubber po-liceman. The lysates were collected in eppendorf tubes and spun at 4°C at 7,400 g. The supernatants were diluted with nine volumes of 0.1 M Na_2HPO_4 and the remaining steps of the glutathione assay were performed as previ-ously described (Ratan et al., 1994b). Glutathione levels were normalized to total protein. Protein levels were determined using the bicinchoninic acid reagent (Pierce Chem. Co., Rockford, IL) method (Smith et al., 1985).

EMSA Assays and Supershift Analysis

To investigate whether SV infection triggers NF-kappa B activation, we performed electrophoretic mobility shift assays (EMSAs) on nuclear ex-tracts from infected and uninfected mouse N18 neuroblastoma, AT-3 Neo, or AT-3 Bcl-2 cells using a ^{32}P-labeled oligonucleotide with a con-sensus NF-kappa B binding site. We chose the palindromic NF-kappa B sequence that lies upstream of the MHC Class I promoter (5'-GGG GAT TCC CC-3'). Parallel EMSAs were performed in infected and uninfected cells using radiolabeled AP-1 (5'-CGC TTG ATG ACT CAG CCG GAA-3') consensus binding sites. Briefly, 10^7 N18 cells or 4×10^6 AT-3 cells were infected with SV as described. At various times after infection, cells were lightly trypsinized, pelleted, and resuspended in PBS. All subse-quent steps were performed at 4°C. The cells were incubated for 17 min in 400 µl of a hypoosmotic buffer (10 mM Hepes, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 µg/ml aprotinin, leupeptin 0.5 µg/ml, 3 mM PMSF, and 3 mM DTT). 25 µl of 10% NP-40 was added and the nuclei were pelleted by centrifugation for 5 min in a microfuge. The supernatants containing the cytoplasmic proteins were removed and stored at −70°C; the pelleted nuclei were resuspended in a high salt buffer (50 mM Hepes, pH 7.4, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% (vol/vol) glyc-erol, 3 mM DTT, and 3 mM PMSF) to solubilize DNA binding proteins. The resuspended nuclei were gently shaken for 30 min at 4°C. The ex-tracts were spun in a microcentrifuge for 10 min and the clear superna-tants containing nuclear protein were aliquoted and stored at −70°C. PCA precipitated protein was measured from a representative aliquot of each sample and equal amounts were used for binding. Binding reactions were performed at room temperature for 15 min using 4–8 µg of nuclear protein (NF-kappa B or AP-1) and 0.25 ng (25,000 cpm) of labeled oligo-nucleotide in 30 µl of binding buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5% glycerol, and 2 µg poly dIdC. DNA-protein complexes were separated from unbound probe on native 4.5% polyacrylamide gels at 200–250 V for 2–3 h. The resultant gel was vacuum dried and exposed to Kodak film for 8–15 h at −80°C. Visual inspection of the free probe band at the bottom of the gel confirmed that equivalent amounts of radiolabeled probe were employed for each sample (data not shown). Cold competition was per-formed using consensus (5'-GGGGACTTTCCC-3') or mutant NF-kappa B binding sites (5'-GGCGACTTTCCC-3') (Santa Cruz Biotechnology, Santa Cruz, CA). Supershifts were performed with antibodies to NF-kappa B subunits Rel A, p50, c-rel, or rel-B or antibodies to C-fos, C-jun, or Egr-1 (Santa Cruz Biotechnology). The antibody (1 or 2 µl) was added to the binding mixture immediately after the addition of the radiolabeled NF-kappa B probe. The reaction mixture was incubated for 20 min and the complexes resolved as described above. Specificity of the antibodies for the corresponding subunit were confirmed by adding 200 ng of control peptide (Santa Cruz Biotechnology) prior to addition of the antibody to the reaction mixture.

NF-kappa B Transcription Factor Decoy Experiments

Phosphorothioated oligonucleotides were made as previously described (Higgins et al., 1993). The double stranded thioate competitors used as de-coys were prepared by annealing complementary strands in vitro in 1× annealing buffer (20 µM Tris-HCl, pH 7.5; 20 µM $MgCl_2$, and 50 µM NaCl). The mixture was heated to 100°C for 12 min and allowed to cool to room temperature slowly over 2 h. The kB-1 wild-type (WT) sequence was 5' CCA G**GA GAT TCC ACC**AG **GAG ATT CCA C**CC AG**G AGA TTC CACC** 3', and the mutant kB-1 sequence was 5'-GTC TGA GAT TCC ACG TCT GAG ATT CCA CGT CTG AGA TTC CACG 3'. The kB-2 WT sequence was 5' G**GGG ACT TTC C**GC TG**G GGA CTT TCC** AGG **GGG ACT TTC C** 3', and the mutant kB-2 sequence was 5'GTC TAC TTT CCG CTG TCT ACT TTC CAC GGT CTA CTT TCC 3'. A previous study employed EMSAs and demonstrated that the WT phosphorothioated decoy oligonucleotides used in the present study are capable of binding NF-kappa B, but mutant decoy oligonucleotides are not (Sharma et al., 1995).

For viability studies N18 cells and AT-3 cells were grown as described in 24-well or 6-well tissue culture plates. Kappa B-1 or kappa B-2 TFDs (0.125–30 µM) and the corresponding mutant TFDs were added directly to the bathing medium. After 18–24 h, the medium was changed, fresh TFD was added and the cells were infected with SV (AR339) as described above. 48 h later, the viability was measured by the percentage of lactate dehydrogenase (LDH) released into the medium or ethidium homodimer staining as previously described. DNA laddering was also assessed 18–24 h after infection as described (Ratan et al., 1994a, b). In parallel, cells grown in 100-mm dishes (4 × 10^6 cells/dish) were treated with individual TFDs or their corresponding mutants for 18–24 h and then infected as described above. At various intervals up to 4 h after infection, cells were harvested for preparation of nuclear extracts as described above. Subsequently, EMSAs were performed to evaluate the effects of WT TFDs and mutant TFDs on NF-kappa B activation. Nuclear extracts used for these EMSAs were also utilized to examine the effects of NF-kappa B TFDs on AP-1 nuclear binding.
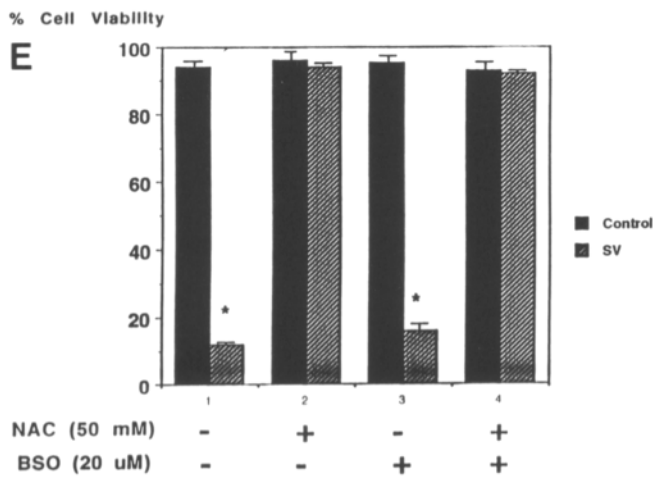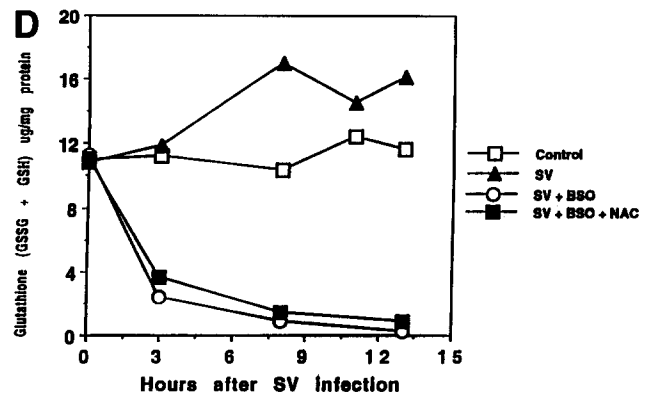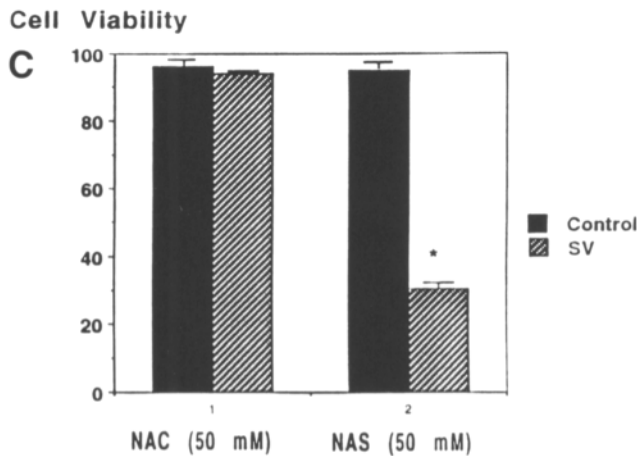
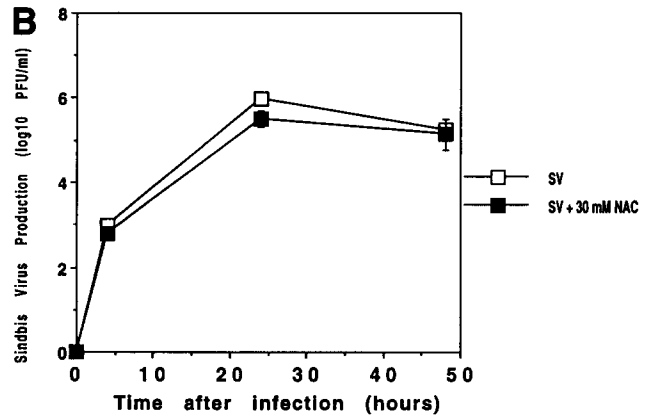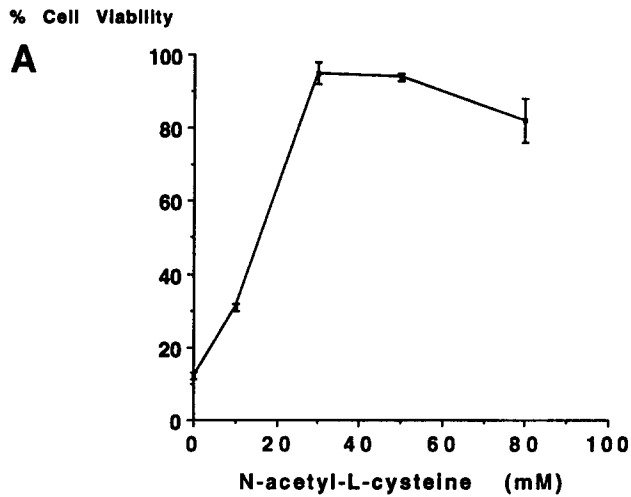## Results

NAC Inhibits SV-Induced Apoptosis

Infection of Mouse N18 neuroblastoma cells or rat AT-3 prostate carcinoma cells with SV (strain AR339, multiplic-ity of infection 1–5) has been shown to result in chromatin condensation and DNA fragmentation typical of apoptosis within 14–24 h (Levine et al., 1993). Associated with these morphologic and biochemical changes, viability of infected cells, as monitored by trypan blue exclusion or LDH re-lease, decreases to 5–15% of uninfected controls after 48 h (Fig. 1 A).

We have previously shown that 100 µM NAC prevents oxidative stress-induced apoptosis in cortical neurons (Ra-tan et al., 1994b). To investigate whether SV (AR339) in-duces apoptosis through a similar pathway, we examined the effects of NAC on SV(AR339)-induced death. While 100 µM NAC was not protective, concentrations of NAC above 30 mM completely inhibit SV(AR339)-induced ap-optosis in N18 neuroblastoma cells (Fig. 1 A) or AT-3 prostate carcinoma cells (not shown). To determine whether NAC protection was due to inhibition of viral entry or vi-ral replication we measured the cumulative amount of vi-rus produced by AT-3 (Fig. 1 B) and N18 cells (not shown) in the presence and absence of NAC at 4, 24, or 48 h after infection. At each time point examined, viral titers were sim-ilar in control and NAC-treated cells infected with SV (AR339) (Fig. 1 B).

To exclude the possibility that nonspecific effects related to the high concentrations of drug used were responsible for NAC protection, we treated cells with N-acetylserine (NAS) (Staal et al., 1993). NAS differs from NAC in that it has a hydroxyl group rather than a sulfhydryl group and therefore cannot act to increase glutathione or directly scav-enge radicals (Fig. 1 C). As shown in Fig. 1 C, NAC com-pletely suppressed SV (AR339)-induced apoptosis, whereas the same concentration of NAS revealed only a small de-gree of protection. Thus, the inhibitory effects of NAC de-pend almost completely on its reducing thiol group and not from nonspecific changes such as osmolarity associated with the presence of a high concentration of reagent.

NAC Inhibition of SV-induced Apoptosis Does Not Depend on Glutathione Synthesis

As mentioned previously, one of the proposed mechanisms

% Cell Viability

A

100

80

60

40

20

0

0   20   40   60   80   100

N-acetyl-L-cysteine   (mM)

B

8

6

4

2

0

Sindbis Virus Production (log10 PFU/ml)

0   10   20   30   40   50

Time after infection (hours)

□ SV
■ SV + 30 mM NAC

Cell Viability

C

100

80

60

40

20

0

NAC (50 mM)   NAS (50 mM)

■ Control
▨ SV

D

20

16

12

8

4

0

Glutathione (GSSG + GSH) ug/mg protein

0   3   6   9   12   15

Hours after SV infection

□ Control
▲ SV
○ SV + BSO
■ SV + BSO + NAC

% Cell Viability

E

100

80

60

40

20

0

NAC (50 mM)   −   +   −   +
BSO (20 uM)   −   −   +   +

■ Control
▨ SV

Figure 1. NAC promotes survival of N18 cells infected with Sindbis virus and this protection cannot be attributed to inhibition of viral entry or viral replication, changes in extracellular osmolarity or its ability to increase glutathione levels. (*A*) Concentration response of NAC protection 48 h after SV-infection. Cells were plated in 0.5 ml of MEM medium containing either no additive or the indicated concentrations of NAC. Viability was assessed by trypan blue exclusion and percent LDH release as described in the Materials and Methods. Error bars represent SEM ($n = 5$). (*B*) Cumulative Sindbis Virus production is unaltered by 30 mM NAC in N18 cells (not shown) and AT-3 cells. At 4, 24, or 48 h after infection, the media of infected cells (+/- NAC) were collected and viral titers were measured in plaque forming units per ml of media (PFU/ml) as described in the Materials and Methods. Error bars represent SEM ($n = 5$). (*C*) 50 mM NAC but not 50 mM *N*-acetyl-*L*-serine (*NAS*) completely abrogates SV-induced apoptosis in N18 cells suggesting that NAC protection is not dependent on non-specific effects such as changes in extracellular osmolarity. NAS differs from NAC in that it has a hydroxyl group rather than a sulfhydryl group and therefore cannot act to increase glutathione or directly scavenge radicals. Error bars represent SEM ($n = 3$). Asterisk denotes $p < 0.05$. (*D*) 20 μM BSO depletes total glutathione to less than 30% of control in the presence or absence of NAC. Total glutathione (GSSG + GSH) μg (normalized to total protein) was measured 3, 8, 11, and 13 h after SV infection as described in the Materials and Methods. After 13–14 h of SV infection in N18 cells, morphologic evidence of apoptosis as monitored by phase contrast microscopy is observed. SE bars for glutathione levels are not shown but represent less than 5% the mean value for each treatment group. *Open squares*, control, no drug; *closed triangles*, SV-infection, no drug; *open circles*, SV infection plus 20 μM BSO; *closed squares*, SV infection plus 20 μM BSO plus 50 mM *N*-acetyl-*L*-cysteine. Note that SV infection alone does not cause a decrease in total glutathione levels. Rather SV infection increases glutathione levels. (*E*) NAC protects from SV-induced apoptosis in N18 cells despite the depletion of glutathione induced by 20 μM BSO suggesting that glutathione is not necessary for NAC protection. Error bars represent SEM ($n = 3$). Asterisk denotes $p < 0.05$.

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of NAC's protective action relates to its ability to increase (or prevent a decrease in) intracellular glutathione. NAC has been shown to deliver cysteine, the rate-limiting amino acid precursor in glutathione synthesis, intracellularly (Meister and Anderson, 1983; Kudo and Kosower, 1990), and thus to increase glutathione levels (Issels et al., 1985). To test whether glutathione synthesis is required for NAC's prevention of SV-induced apoptosis, we used buthionine sulfoximine (BSO). BSO is an irreversible inhibitor of the enzyme γ-glutamyl cysteine synthetase, and acts to inhibit cysteine (or cysteine derived from deacylated NAC) incorporation into glutathione (Griffith and Meister, 1979). BSO (20 μM) lowered total cellular glutathione (GSSG + GSH) to less than 20% of control levels by 3 h after infection (Fig. 1 *D*), but did not cause cell death in uninfected cells nor did it affect SV's ability to induce apoptosis (Fig. 1 *E*). Additionally, when de novo glutathione synthesis was blocked with BSO, NAC still prevented SV-induced cell death (Fig. 1 *E*). Thus, glutathione synthesis is not required for NAC protection.

Metal Chelators and Inhibitors of Lipid Peroxidation Do Not Mimic NAC's Ability to Block SV-Induced Apoptosis

An additional well documented action of NAC is its ability to act as a chelator of trace metals. The chelating properties of NAC may be related to its resemblance to cysteine, which has a high affinity for copper, iron, and zinc. In high intravenous doses in vivo, NAC has been shown to have heavy metal-chelating effects (Lorber et al., 1973; Hjortso et al., 1990). By preventing several metal-catalyzed reactions that generate oxygen radicals, metal chelators can act as antioxidants. Indeed, iron chelators have been shown to inhibit oxidative stress and cell death in a number of paradigms (Sakaida et al., 1989; Connor and Benkovic, 1992; Gille et al., 1992; Hartley et al., 1993).

To test the possibility that these chelating properties of NAC are responsible for its protective actions, we exposed infected cells to the iron chelators, mimosine (MIM) and deferoxamine mesylate (DFO). DFO (50–500 μM) and MIM (50–300 μM) each failed to prevent SV (AR339)-induced apoptosis (Table I). At the highest concentrations utilized, control cells began to manifest toxic effects from the drugs. To ensure that the drugs were biologically active in our hands, we performed parallel experiments in which cortical neurons were subjected to glutathione depletion and oxidative stress. Treatment of cortical cultures with DFO (50 μM) or MIM (100 μM) completely inhibited oxidative stress-induced apoptosis (R. R. Ratan, unpublished observations).

In addition to DFO and MIM, the Fe/Cu/Zn chelator penicillamine also failed to prevent SV (AR339)-induced apoptosis. These results suggest that the protective effects of NAC do not relate solely to its ability to bind metals. Furthermore the absence of protection by metal chelators suggests that the metal-catalyzed formation of free radicals, in particular hydroxyl radical, is not likely to be an important mediator of SV-induced apoptosis. Additionally, we found that inhibitors of lipid peroxidation, butylated hydroxyanisole (BHA, 0.1 μM–100 μM), and the vitamin E analogue, Trolox (10 μM–1 mM), did not inhibit

Table I. Effects of Antioxidants on SV-induced Apoptosis

	% Viability ± SEM	
	CON	SV
Metal chelators		
Mimosine (0.05–0.5 mM)	77 ± 2	17 ± 2
Deferoximine Nesyiate (0.1–0.5 mM)	69 ± 6	17 ± 4
Penicillamine (0.1–1.0 mM)	93 ± 1	22 ± 12
Inhibitors of Lipid Peroxidation		
BHA (0.01–0.5 mM)	98 ± 1	11 ± 7
Trolox (0.1–1.0 mM)	94 ± 1	21 ± 1
Scavengers of Peroxide		
Catalase (10–3,000 units/ml)	96 ± 1	5 ± 1
Pyruvate (2–10 mM)	100 ± 1	12 ± 3
Thiol Agents		
Pyrrolidine dithiocarbamate (20–75 μM)	79 ± 4	76 ± 3**
Diethyl dithiocarbamate (0.05–10 mM)	80 ± 4	78 ± 3**
Dithiothreitol (0.1–10 mM)	65 ± 6	74 ± 2**
2-Mercaptoethanol (0.15–15.0 mM)	81 ± 2	83 ± 1**

Cells were infected with SV (MOI-1-5) ± potential inhibitors and percent viability assessed after 48 h as described in the Materials and Methods. Values (mean ± SEM for three to five different experiments) represent the highest concentration used for each drug treatment. Control viability refers to percent viability in drug treated, uninfected cells after 48 h.
** Statistical difference from control, infected cells. ($p < 0.05$).

SV (AR339)-induced apoptosis (Table I) nor did enzymic (catalase, 10–3,000 units/ml) or nonenzymic scavengers (pyruvate, 2–10 mM) of peroxide. These observations do not exclude the possibility that other oxygen-derived species such as superoxide or nitric oxide are operative. It is also important to note that we cannot rule out the possibility that NAC acts as metal chelator, peroxide scavenger, and/or an inhibitor of lipid peroxidation in a subcellular local unaffected by DFO, MIM, penicillamine, BHA, Trolox, catalase, or pyruvate.

Thiol Agents Such As PDTC, 2-Mercaptoethanol and DTT Inhibit SV (AR339)-induced Apoptosis

Since an isolated role for the glutathione enhancing (sparing) effects of NAC as well as its metal chelating properties seemed unlikely, we examined whether agents with only thiol reducing properties could mimic NAC's protective action. 2-ME and other thiols have been demonstrated to increase viability and proliferation in both neuronal and nonneuronal cells (Grill and Pixley, 1993; Sandstrom et al., 1993). The beneficial effects of 2-ME have been attributed to its ability to deliver cysteine intracellularly and/or act as a reducing agent and thereby inhibit oxidative stress or alter the redox state of protein sulfhydryls. 2-ME prevented SV-induced apoptosis in a dose-dependent manner with maximal protection at 14 mM (Table I). Other thiol agents such as DTT (10 mM) were also protective (Table I), while the vicinal dithiol, PDTC, was much more potent, significantly protecting SV (AR339)-infected N18 neuroblastoma cells or AT-3 prostate carcinoma cells at concentrations of 75 μM (Table I). Diethyl dithiocarbamate (DDTC), a structural analogue of PDTC, was also protective, but was less potent than PDTC. These findings suggest that it is the thiocarbamate moiety that is critical for PDTC's protective action. The differences in potency between PDTC and DDTC have been observed in other paradigms

and may relate to differences in membrane permeability between the two agents (Schreck et al., 1992). With both PDTC and DDTC, we noted a small and reproducible amount of cell death in uninfected cells (Table I).

In summary, we found that several thiol containing agents, including NAC (30 mM) and PDTC (75 μM) are effective suppressors of SV (AR339)-induced apoptosis. These results, added to observations from other laboratories that NAC delays apoptosis induced by depriving T cell hybridomas of growth factors (Hockenbery et al., 1993), prevents apoptosis induced by exposing sensitized T cells to myelin basic protein (Sandstrom et al., 1994), and prevents apoptosis induced by depriving PC12 cells of serum (Ferrari et al., 1995), are consonant with a common biochemical target for NAC (applied at millimolar concentrations) in diverse apoptotic paradigms. That other antioxidants such as metal chelators, BHA, Trolox, catalase, and pyruvate (Table I) are ineffective in preventing apoptosis may suggest that SV (AR339)-induced apoptosis can be redox modulated but is not oxidant initiated.

NAC and PDTC: Effective Inhibitors of NF-kappa B Activation

The ability of NAC and PDTC and other thiol agents to inhibit SV(AR339)-induced apoptosis led us to look for a known molecular target that is affected by these drugs. We were intrigued to find that NAC (30 mM) and PDTC (75 μM) are among the most effective known inhibitors of the nuclear translocation of the transcription factor NF-kappa B (Schreck and Baeuerle, 1991). The striking pharmacologic parallel between the ability of NAC and PDTC to inhibit SV (AR339)-induced apoptosis and the ability of these drugs at similar concentrations to inhibit NF-kappa B stimulation by diverse, often pro-apoptotic, stimuli, led us to examine whether SV (AR339) induces NF-kappa B activation.
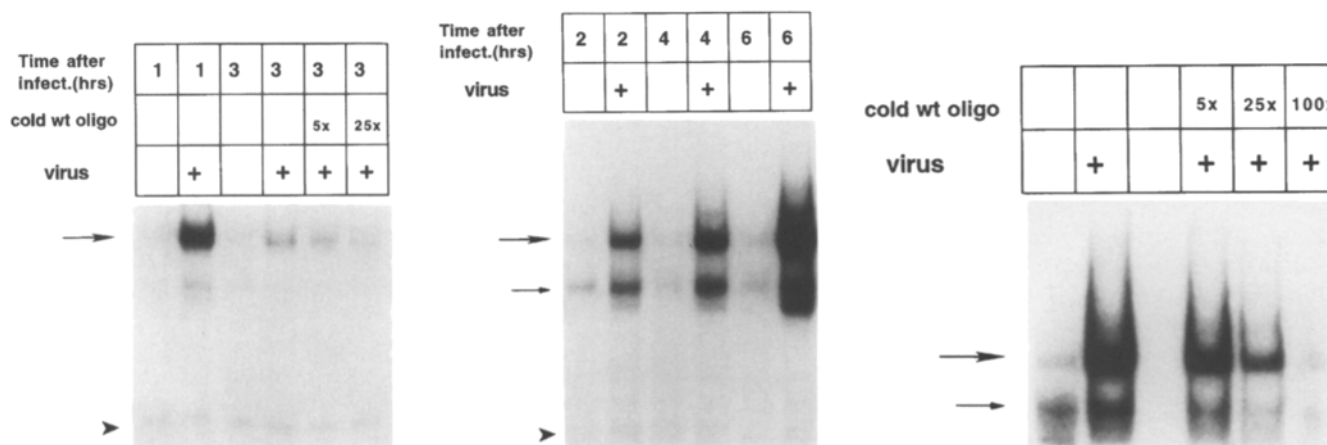
Sindbis Virus Induces NF-kappa B Activation

To investigate whether SV (AR339) infection triggers NF-kappa B activation, we performed EMSAs on nuclear extracts from infected and uninfected cells using a ^{32}P-labeled oligonucleotide with a consensus NF-kappa B binding site (Staal et al., 1993). We chose the palindromic NF-kappa B sequence that lies upstream of the MHC Class I promoter (5'-GGGGATTCCCC-3'). This binding site has been shown to bind a wide variety of heterodimers and homodimers of the NF-kappa B/rel family (Baldwin and Sharp, 1987; Baeuerle, 1991; Potter et al., 1993).

SV (AR339) infection strongly increased NF-kappa B like DNA binding in mouse N18 neuroblastoma cells and rat AT-3 prostate carcinoma cells (Fig. 2). Kinetic studies with a multiplicity of infection of 1-5 PFU per cell revealed that induction was maximal by 1 h and decreased by 3 h in the N18 cell line (Fig. 2 A). In the AT-3 prostate carcinoma cell line, activation of NF-kappa B was observed by 2 h and continued to increase up to 6 h after infection (Fig. 2 B). In both cell lines, the NF-kappa B binding activity was inhibited in a concentration-dependent manner by cold oligonucleotide with a similar NF-kappa B sequence (Fig. 2, A and C), but not oligos containing mutations in the NF-kappa B site (not shown).

Identification of NF-kappa B Subunits in Nuclear Extracts Following SV Infection

To identify the NF-kappa B subunits detected by EMSA in infected N18 and AT-3 cell lines, we utilized subunit-specific antibodies to p50, p65, c-rel, and rel-B. In AT-3 cells addition of individual, subunit-specific antibodies during in vitro DNA binding revealed that the slower migrating, major complex included p50/p65 while the faster migrating, minor complex was p50/p50 (Fig. 3). In N18 cells the identity of the major complex was composed of a het-

Figure 2. SV induces NF-kappa B activation in N18 neuroblastoma cells and AT-3 cells. (A) SV induces transient activation of NF-kappa B in N18 neuroblastoma cells. EMSAs were performed with 4-8 μg of nuclear extracts using a ^{32}P-labeled oligonucleotide with a consensus NF-kappa B sequence from the MHC Class I promoter. An unlabeled oligonucleotide containing a distinct NF-kappa B binding sequence (see Materials and Methods) was used for competition studies. Arrow points to predominant complex induced by SV. Arrowhead points to nonspecific complex. Note similar amounts of nonspecific nuclear activity among different treatment groups. (B) SV induces activation of NF-kappa B in AT-3 prostate carcinoma cells. EMSAs were performed as described in A. Large arrow points to predominant complex induced by SV, small arrow to minor complex. Arrowhead points to nonspecific complex. (C) SV-induced NF-kappa B activity in AT-3 cells can be competitively inhibited by excess unlabeled NF-kappa B oligonucleotide.
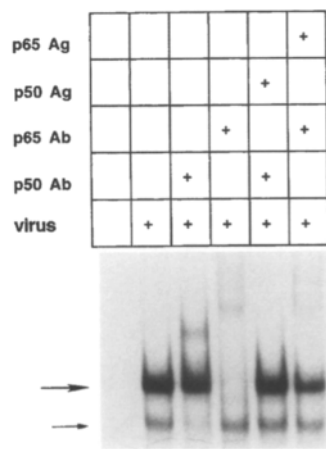
Figure 3. Identification of the major complex induced by SV in AT-3 cells as p50/p65. COOH-terminal subunit specific antibodies to p50 or p65 were added after the addition of radiolabeled NF-kappa B probe to the nuclear binding mixture as described in the Materials and Methods. Note that the p50 antibody decreases the intensity of the major complex (*large arrow*) and completely inhibits the intensity of the minor complex (*small arrow*). The p65 antibody completely inhibits the major complex (*large arrow*) and has no effect on the minor complex (*small arrow*). The major complex is thus composed of p50/p65 heterodimers and the minor complex p50/p50 homodimers. Addition of purified peptide (*Ag*) confirms the specificity of antibodies.

erodimer of rel-B/p50 while a faster migrating, but minor band was composed of p50/p50 (not shown). Antibodies to p65, c-rel, c-fos, Jun, and Egr-1 had no effect on any of the induced complexes.

NAC and PDTC Block SV (AR339)-induced NF-kappa B Activation as well as SV (AR339)-induced Apoptosis

Parallel experiments assessing the effects of NAC and PDTC on NF-kappa B binding were performed by pretreating cells with PDTC and NAC 2 h before SV (AR339) infection as in the viability studies. As observed previously

with other activators of NF-kappa B (Schreck et al., 1992), PDTC and NAC diminished the SV-induced increase in NF-kappa B activity in N18 cells (Fig. 4 *A*) and AT-3 cells (Fig. 4 *B*; PDTC not shown). Antioxidants such as BHA (100 μM; not shown) and DFO (500 μM, Fig. 4 *B*), which did not suppress SV-induced apoptosis, did not inhibit SV-induced NF-kappa B activation. We confirmed previous studies that have demonstrated that NAC and PDTC, when added in vitro with nuclear extracts, do not affect the DNA binding of NF-kappa B. Rather, these agents appear to act by inhibiting translocation of NF-kappa B to the nucleus (Schreck et al., 1991, 1992; Meyer et al., 1994).

Bcl-2 Overexpression in AT-3 Cells Inhibits SV-induced Apoptosis and SV-induced NF-kappa B activation

Bcl-2 has been postulated to act in an antioxidant pathway in preventing apoptosis (Hockenbery et al., 1993; Kane et al., 1993), but the nature of the signaling pathway affected by bcl-2 remains unclear. The AT-3 prostate carcinoma cells used in this study overexpress the neomycin resistance gene alone (AT-3 Neo) and have previously been demonstrated to undergo SV-induced apoptosis (Levine et al., 1993). However, AT-3 Neo cells overexpressing Bcl-2 (AT-3 Bcl-2) do not undergo SV-induced apoptosis, despite their ability to produce progeny virus for many months (Levine et al., 1993). Bcl-2 is thus able to convert SV infection from a lytic (apoptotic) to a persistent form. To determine whether Bcl-2's protective effects, like NAC and PDTC, are correlated with inhibition of NF-kappa B signaling, we compared SV-induced NF-kappa B activation in AT-3 Neo and AT-3 Bcl-2 cells. As shown in Fig. 4 *C*, up to 6 h after infection, SV-induced NF-kappa B activation was almost completely suppressed in the presence of high lev-
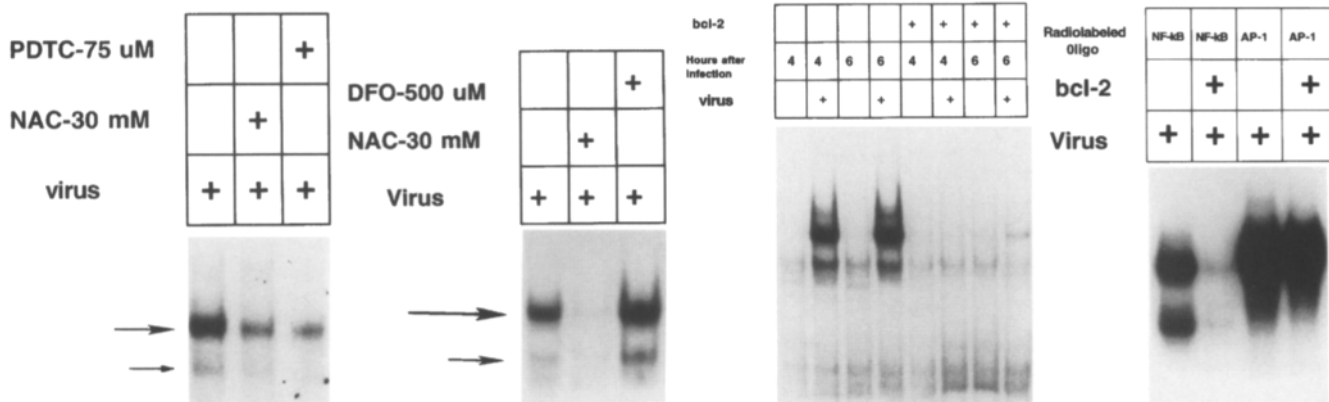

Figure 4. NAC, PDTC and Bcl-2, agents that block SV-induced apoptosis, also inhibit SV-induced NF-kappa B activation. The iron chelator and antioxidant, deferoxamine mesylate (*DFO*, 500 uM) does not inhibit SV-induced apoptosis and does not inhibit SV-induced NF-kappa B activation. (*A*) Effects of NAC (30 mM) and PDTC (75 μM) on SV-induced NF-kappa B activation in N18 cells. Cells were pretreated for 2 h with NAC or PDTC before infection and nuclear extracts were harvested 1 h after the end of infection. Large arrow points to major complex, p50/rel B, whereas small arrow points to minor complex p50/p50. (*B*) Effects of NAC (30 mM) and DFO (500 μM) on SV-induced NF-kappa B activation in AT-3 cells. Drugs were added as in *A* and nuclear extracts were harvested 4 h after end of the 1-h infection period. Large arrow points to major complex p50/p65, whereas small arrow points to minor complex p50/p50. (*C*) Effects of bcl-2 overexpression on SV-induced NF-kappa B activation in AT-3 cells. 4 and 6 h after SV infection, nuclear extracts were prepared from AT-3 Neo and AT-3 Bcl-2 cells. In parallel, Western blots using a bcl-2 antibody confirmed that the AT-3 Bcl-2 levels have high levels of Bcl-2 protein whereas none is detectable in the AT-3 Neo cells (not shown). Additionally, AT-3 Bcl-2 but not AT-3 Neo cells are resistant to SV-induced apoptosis (not shown). (*D*) Effects of overexpression of Bcl-2 in AT-3 cells on nuclear NF-kappa B binding activity and nuclear AP-1 binding activity. Nuclear extracts were obtained from AT-3 Neo and AT-3 Bcl-2 cells as in *C* and EMSAs were performed using 4 μg of protein and radiolabeled NF-kappa B or AP-1 probe. Excess unlabeled wild-type and mutant AP-1 oligonucleotides were used to confirm that the AP-1 binding activity observed in AT-3 Neo and AT-3 Bcl-2 cells is specific (not shown).

els of Bcl-2. Deoxycholate treatment of cytoplasmic extracts from AT-3 Neo and AT-3 Bcl-2 cells revealed similar amounts of NF-kappa B activity (not shown), suggesting the absence of nuclear NF-kappa B DNA binding activity in AT-3 Bcl-2 cells cannot be attributed to loss of NF-kappa B expression. Additionally, differences in nuclear NF-kappa B activity between SV-infected AT-3 Neo and AT-3 Bcl-2 cell lines could not be attributed to global differences in nuclear proteins, as levels of the transcription factor AP-1 were similar in these two groups (Fig. 4 D). In data not shown, we confirmed previous studies that have shown that the AT-3 Bcl-2 cell line expresses high levels of Bcl-2 protein and that these cells are resistant to SV-induced apoptosis (Levine et al., 1993).

NF-kappa B Activation Is Necessary for SV (AR339)-induced Apoptosis in Rat AT-3 Prostate Carcinoma Cells, But Not in Mouse N18 Neuroblastoma Cells

The ability of thiol agents and Bcl-2 to block NF-kappa B activation led us to determine whether NF-kappa B induction is necessary for SV-induced apoptosis. To inhibit SV-induced NF-kappa B activation we employed double stranded phosphorothioate oligonucleotides containing three NF-kappa B binding sites. Several previous studies have shown that these oligonucleotides, also known as transcription factor decoys (TFDs) are efficiently taken up by cells, bind activated NF-kappa B, and thus prevent NF-kappa B from binding to authentic target sites within the nucleus (Bielinska et al., 1990; Eck et al., 1993; Goldring et al., 1995). TFDs with an NF-kappa B binding sequence or mutant TFDs (incapable of binding NF-kappa B with high affinity) were added to AT-3 cells 18–24 h before infection. Two distinct TFDs and their mutant pairs were utilized: (a) Kappa B-1 TFD, a 42-nucleotide oligomer with three NF-kappa B binding sequences from the cytokine 1 (CK-1) region of the GM-CSF promoter capable of binding p65 homodimers or c-rel/p65 heterodimers (Dunn et al., 1994), and (b) kappa B-2 TFD, a 42-nucleotide oligomer with three consensus sequences capable of binding NF-kappa B heterodimers (e.g., p50/p65 or p50/rel B). After the 18–24-h pretreatment period, the medium was changed, virus and TFDs were added, and then viability was measured 48 h later.
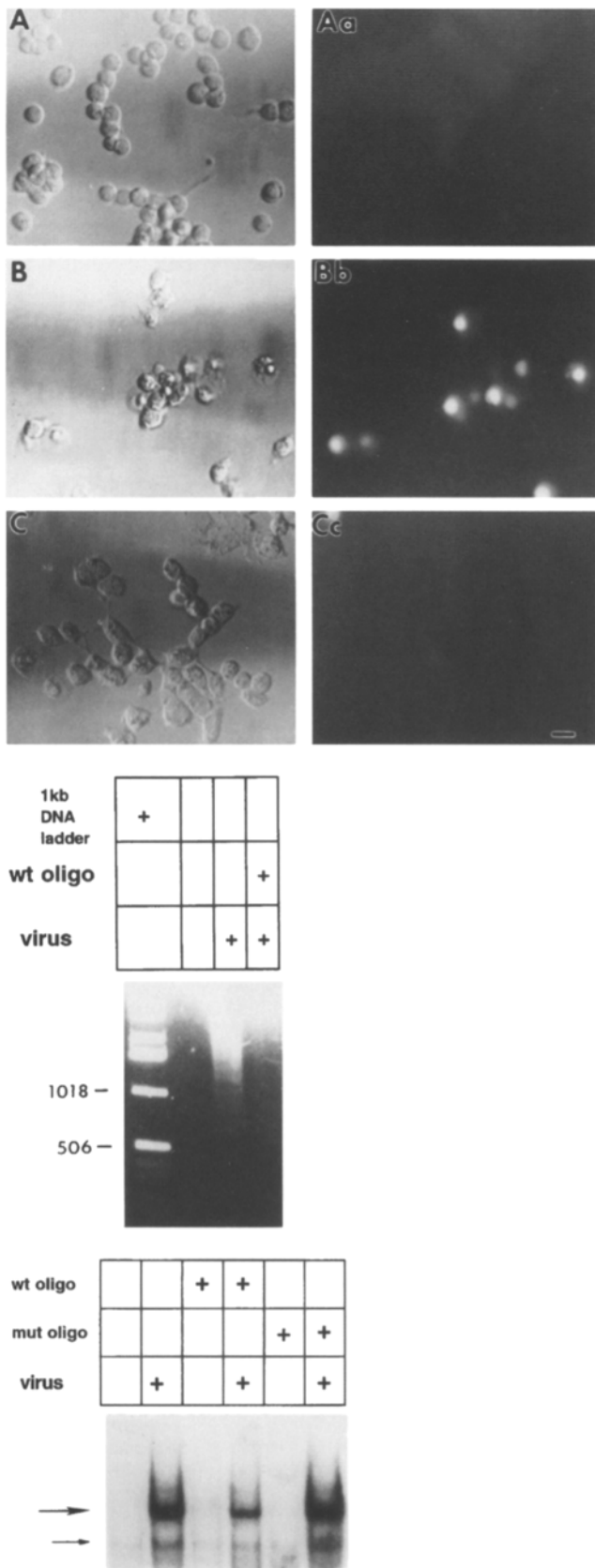
Concentrations of Kappa B-1 TFD as low as 500 nM showed nearly complete protection of AT-3 cells from SV (AR339)-induced apoptosis as measured by LDH release (Fig. 5, A and B), ethidium homodimer staining (a dead cell stain) (Fig. 6, A–C) and DNA fragmentation (Fig. 6 D). The protection was concentration dependent (Fig. 5 A) and was not observed with 500 nM of the mutant Kappa B-1 TFD (Fig. 5 B). The ability of wild-type kappa B-1 TFD but not the mutant kappa B-1 TFD to inhibit NF-kappa B translocation was verified by measurements of nuclear NF-kappa B binding as assayed by EMSA (Fig. 6 E). To confirm that inhibition of NF-kappa B binding to ^{32}P-labeled oligonucleotide was not due to the presence of residual cold TFDs copurified with nuclear extracts, we performed a mixing experiment. If the inhibition of NF-kappa B binding seen in nuclear extracts from virus-infected, TFD-treated cells were due to residual cold TFDs, then
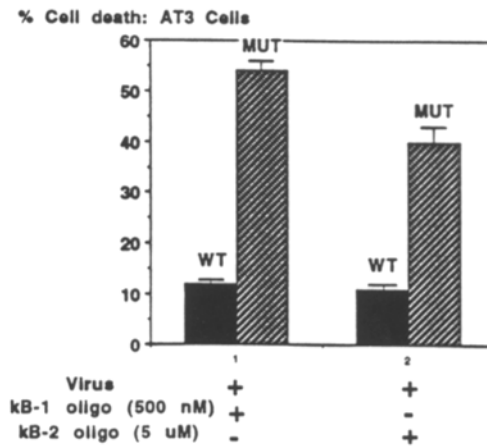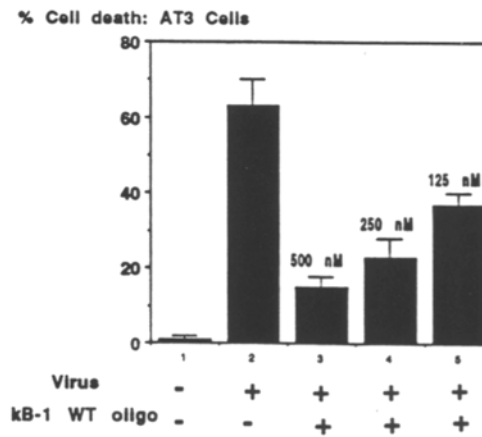
Figure 5. Inhibition of NF-kappa B activation using NF-kappa B–specific TFDs blocks SV-induced apoptosis in AT-3 cells. (A) Concentration response of protection by kB-1 WT TFDs 48 h after SV infection in AT-3 cells. 125–500 nM WT kB-1 was added to AT-3 cells 18–24 h before infection. The cells were infected and maintained after infection in the presence of the TFD until the percent viability was measured by determining the percent LDH released into the medium as described in the Materials and Methods. The kB-1 TFD binds p65 homodimers or p65/c-rel heterodimers. Error bars represent SEM (n = 3). (B) Effects of wild-type TFDs and mutant TFDs on SV-induced apoptosis. The binding specificity of kappa B-1 is described in A. Kappa B-2 binds p50/p65 and other NF-kappa B heterodimers. Mutant TFDs (kappa B-1 or kappa B-2) did not alter viability in uninfected cultures (not shown). Error bars represent SEM (n = 3).

they should be able to inhibit the NF-kappa B binding activity detected in nuclear extracts from control, virus-infected cells. We found that the intensity of NF-kappa B binding in extracts prepared from virus-infected, TFD untreated cells was not diminished by incubation with virus-infected, TFD treated extract suggesting that cold TFD was not copurified with the nuclear extract. We also verified that incubation of AT-3 cells with wild-type NF-kappa B TFDs does not alter the nuclear activity of another re-

dox-sensitive transcription factor, AP-1, in control or virus infected cells (data not shown). We also verified that wild-type and mutant NF-kappa B TFDs do not affect viral production at 4, 24, or 48 h after infection (data not shown).

Kappa B-2 TFD (5 μM), but not the mutant kappa B-2 TFD (5 μM) was equally as effective as kappa B-1 in preventing SV-induced apoptosis (Fig. 5 B). The ability of two TFDs with distinct NF-kappa B binding site sequences to inhibit SV (AR339)-induced apoptosis suggests that NF-kappa B activation is necessary for SV (AR339)-induced apoptosis in AT-3 cells.

Accumulating evidence suggests the existence of multiple stimulus-specific and cell type-specific signaling paths to apoptosis. For example, while thymocyte apoptosis induced by radiation or etoposide is dependent on p53, thymocyte apoptosis induced by glucocorticoids or calcium is p53-independent (Clarke et al., 1993). Additionally, recent observations indicate that Bcl-2 overexpression prevents SV-induced apoptosis in AT-3 prostate carcinoma cells, while overexpression of Bcl-2 in BHK cells has no effect on SV-induced apoptosis (J. M. Hardwick, unpublished observations). To determine whether kB TFDs prevent SV-induced apoptosis in a cell type-specific manner, we examined the effects of these agents on N18 neuroblastoma cells. Treatment of mouse N18 neuroblastoma cells with 500 nM–30 μM kappa B-1 TFD or 500 nM–30 μM kappa B-2 TFD had no effect on SV-induced apoptosis and did not affect cell survival in uninfected cultures (Fig. 7 A). Despite its failure to inhibit SV-induced apoptosis, the kappa B-2 TFD was capable of nearly completely inhibiting SV-induced nuclear NF-kappa B activity as shown by EMSA (Fig. 7 B). These results suggest that while NF-kappa B activation is necessary for SV-induced apoptosis in AT-3 cells, it is not required for SV-induced apoptosis in N18 cells.

Discussion

An Alphavirus-Induced Apoptotic Pathway That Requires NF-kappa B Activation

Using double stranded phosphorothioated oligodeoxynucleotides as decoys to inhibit competitively binding of NF-

Figure 6. NF-kappa B TFDs block SV-induced increases in dead cell staining and DNA fragmentation characteristic of apoptosis. (*A*) Uninfected AT-3 cells visualized by Hoffman microscopy. (*Aa*) AT-3 cells depicted in *A* treated with Ethid-1. (*B*) AT-3 cells, 48 h after SV infection, visualized by Hoffman microscopy. (*Bb*)

AT-3 cells depicted in *B* treated with Ethid-1. (*C*) AT-3 cells, 48 h after SV infection in the presence of 500 nM kappa B-1 WT oligonucleotide visualized by Hoffman microsopy. (*Cc*) AT-3 cells depicted in *C* treated with Ethid-1. (*D*) Kappa B-1 WT TFD 500 nM blocks SV-induced DNA fragmentation. DNA was harvested from 10^5 uninfected or infected cells 18 h after SV infection and subjected to agarose gel electrophoresis and ethidium bromide staining. In parallel, 4×10^5 cells treated with 500 nM kappa B-1 WT TFD (*wt oligo*) were infected and the DNA was harvested. The bands from the standard 1-kb DNA ladder (GIBCO BRL) contain from 1 to 12 repeats of a 1,018-bp DNA fragment. In addition to these 12 bands, the ladder contains vector DNA fragments that range from 75 to 1636 bp. (*E*) Kappa B-1 wt TFD (*wt oligo*) but not kappa B-1 mutant TFD (*mutant oligo*) block SV-induced nuclear NF-kappa B activity. Wt or mutant TFDs (500 nM) were added to AT-3 cells 18–24 h before infection and maintained in the medium after infection as in Fig. 5 A. 4 h after infection, nuclear extracts were obtained from cells treated with no TFD, wt TFD (500 nM) and mutant TFD (500 nM) and then EMSAs were performed. Bar, 25 μm.

A

% Cell death: N18 cells

Virus	-	+	-	+	-	+
kB-1 (30 uM)	-		+		-	
kB-2 (30 uM)	-		-		+	

wt oligo		+
virus	+	+
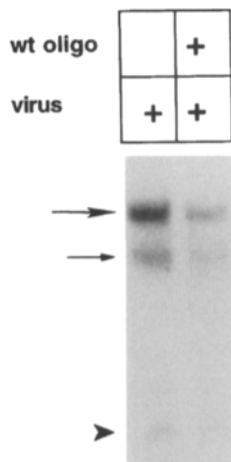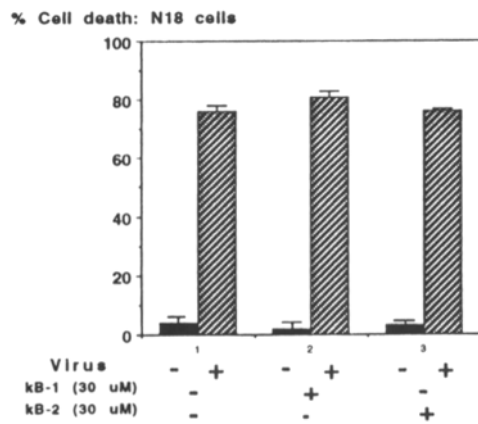
Figure 7. NF-kappa B TFDs do not block SV-induced apoptosis in N18 cells. (*A*) N18 neuroblastoma cells were treated with 30 μM WT or mutant TFD (kappa B-1 and kappa B-2) as described for AT-3 cells in Fig. 5. Percent viability was assessed 48 h after infection by the percent of LDH released into the bathing medium. Error bars represent SEM ($n = 4$). (*B*) Kappa B-2 WT TFD (*wt oligo*) blocks SV-induced NF-kappa B activation in N18 cells. 5 μM kB-2 WT TFD was added to N18 cells 18–24 h before SV-infection. Cells were infected and nuclear extracts were harvested one hour after the end of the infection period. Large arrow points to the p50/relB complex and the small arrow points to the minor, p50/p50 complex. The arrowhead points to nonspecific binding activity.

kappa B to native DNA binding sites, we have shown that NF-kappa B activation is necessary for SV-induced apoptosis in AT-3 prostate carcinoma cells. Single-stranded antisense oligodeoxynucleotides designed to inhibit translation of mRNAs have been shown to possess antiviral activity through nonspecific mechanisms including inhibition of viral polymerase, inhibition of viral absorption to the cell surface, and finally, disruption of proper intracellular vesicle trafficking essential for viral release into the cytoplasm (Milligan et al., 1993; Fennewald et al., 1995). Several observations suggest that the double stranded phosphorothioated oligodeoxynucleotides (TFDs) used in the present study are acting specifically to inhibit NF-kappa B activation.

First, two wild-type NF-kappa B TFDs (kappa B-1 and kappa B-2) with distinct consensus NF-kappa B binding sequences, but not their corresponding mutant TFDs, block SV-induced nuclear NF-kappa B activity (Fig. 6 *E*) and SV-induced apoptosis (Figs. 5 and 6, *A–D*). The kappa B-1 TFD binds p65/c-rel complexes and probably inhibits p50/p65 nuclear translocation (Fig. 6 *E*) indirectly by binding p65 (Dunn et al., 1994) or by inhibiting the expression of p65 (R. Narayanan, unpublished observations). The kappa B-2 TFD directly binds p50/p65 and other NF-kappa B

heterodimers and thereby competitively inhibits the nuclear translocation of these complexes (Bielinska et al., 1990; Eck et al., 1993).

Second, the wild-type TFDs inhibit SV-induced nuclear NF-kappa B activity but not nuclear AP-1 activity. Recent studies have identified c-jun, a member of the jun family and a subunit of the transcription factor AP-1, as a necessary component of growth factor deprivation-induced apoptosis in sympathetic neurons (Estus et al., 1994; Ham et al., 1995). Our observations exclude the possibility that NF-kappa B TFDs are altering levels of AP-1 nuclear activity and thereby inhibiting apoptosis. Finally, the failure of NF-kappa B TFDs at concentrations up to 30 μM to inhibit SV-induced apoptosis in N18 neuroblastoma cells (Fig. 7 *A*) suggests that nonspecific effects of oligodeoxynucleotides on viral adsorption, viral replication or viral entry are unlikely. Indeed, we found no effect of wild-type or mutant NF-kappa B TFDs on virus production at 4, 24, or 48 h after infection (data not shown).

Our results suggest that identification of gene targets for NF-kappa B in AT-3 cells may shed light on regulators or effectors of SV-induced apoptosis. Several genes implicated in apoptosis such as p53 (Wu and Lozano, 1994), c-myc (Evan et al., 1992), TGF-β (Kyprianou and Isaacs, 1989; Perez et al., 1994) murine interleukin 1-β converting enzyme (ICE) (Casano et al., 1994), have consensus NF-kappa B sites in their promoters. Expression of one or more of these known proteins and/or as yet undefined proteins may thus be inhibited by NF-kappa B TFDs. However, our results do not allow us to establish whether gene(s) transactivated by NF-kappa B and required for SV-induced apoptosis are constitutively expressed or whether they are upregulated in response to viral infection. NF-kappa B TFDs were added to cells 18–24 h before infection allowing sufficient time not only for accumulation of TFDs intracellularly, but potentially, also for inhibition of constitutively expressed NF-kappa B–dependent genes before viral infection. Available evidence suggests that effectors of apoptosis are constitutively expressed in a variety of cell types, while as yet undefined regulators may require de novo protein synthesis after the onset of an apoptotic stimulus (Jacobson et al., 1994). Future studies will clarify whether NF-kappa B regulates constitutively expressed and/or virus-induced proteins necessary for apoptosis.

Several independent observations suggest a role for NF-kappa B in regulating apoptosis by other stimuli in addition to SV. First, almost all activators of NF-kappa B, including TNF-α (Polunovsky et al., 1994), IL-1 β (Ankarcrona et al., 1994), peroxide (Hockenbery et al., 1993), endotoxin (Abello et al., 1994), and HIV-1 (Malorni et al., 1993), have been shown to induce apoptosis in cultured cell lines. Second, all members of the NF-kappa B family share homology in a 300 NH_2-terminal amino acid stretch called the rel domain. Recent studies have implicated one member of this family c-rel, in mediating programmed cell death during avian development (Abbadie et al., 1993). The mRNA for this transcription factor is selectively increased in those cells undergoing programmed cell death in the developing chick embryo. Additional in vitro studies revealed that overexpression of c-rel in bone marrow cells triggers apoptosis, consistent with a role for c-rel in this process. Third, increased expression of v-rel, a truncated

form of c-rel that lacks the transactivating domain, is associated with resistance to apoptosis induced by radiation, calcium ionophores or growth factor withdrawal (Neiman et al., 1991). It has been postulated that in this paradigm, v-rel acts as a dominant negative and blocks gene transactivation by c-rel. Finally, NF-kappa B nuclear activity is increased in lymphocytes undergoing dexamethasone or heat shock-induced apoptosis (Sikora et al., 1993). While these converging lines of inquiry make the idea of NF-kappa B as a general mediator of apoptosis compelling, the observation that NF-kappa B TFDs block SV-induced apoptosis in AT-3 cells but not N18 cells suggests the existence of apoptotic signaling pathways that are NF-kappa B independent. These results also suggest that the same stimulus may initiate different apoptosis pathways in a cell type-specific manner. The stimulus and cell type specificity of NF-kappa B's role in apoptosis is further underscored by a recent study that demonstrated that p65 knockout mice undergo extensive programmed cell death in the liver during embryogenesis (Beg et al., 1995).

Cell Type-specific Apoptotic Pathways

In this study, several differences in the SV-induced NF-kappa B response between AT-3 and N18 cells may explain the apparent lack of dependence of SV-induced apoptosis in N18 cells on NF-kappa B. First, in N18 cells, NF-kappa B is activated by 1 h after infection and decreases to near basal levels by 3 h after infection (Fig. 2 A). In contrast, in AT-3 cells, NF-kappa B activation persists and increases up to 6 h after infection (Fig. 2 B). Thus, persistent NF-kappa B activation by a stimulus may result in activation of an apoptotic program, whereas those cells where NF-kappa B is transiently activated may use other transcriptional or posttranscriptional paths to apoptosis. Ghosh and coworkers have recently shown that NF-kappa B activation can be transient or persistent depending on whether the stimulus induces the activation of Ikappa B-α complexes (transient response) or the activation of Ikappa B-α and Ikappa B-β complexes (persistent response) (Thompson et al., 1995). These observations predict that in N18 cells, SV induces the activation of Ikappa B-α complexes, whereas in AT-3 cells, SV-induces Ikappa B-α and Ikappa B-β complexes. They also raise the intriguing possibility that Ikappa B-β regulates NF-kappa B complexes that can activate the apoptotic program.

Second, the SV-induced heterodimer in N18 cells is rel B/p50 (not shown), whereas the induced heterodimer in AT-3 cells is p50/p65 (Fig. 3). The recent characterization of phenotypic differences in mutant mice deficient in rel B or p50 provides support for the view that individual NF-kappa B/rel family members play distinctive roles that cannot be mimicked by other family members (Sha et al., 1995; Weih et al., 1995). Thus, the p50/p65 heterodimer induced by SV in AT-3 cells may transactivate genes that are not activated by rel B/p50 in N18 cells. Finally, it is possible that N18 cells possess compensatory and/or redundant pathways that allow activation of the apoptotic program despite inhibition of nuclear NF-kappa B activity.

Mechanism of Action of Thiol Agents and Bcl-2

In addition to implicating NF-kappa B in SV infection and apoptosis, our results identify SV-induced death as another apoptotic paradigm where drugs with antioxidant capability such as NAC and PDTC are protective. NAC and PDTC have also been shown to inhibit peroxide induced NF-kappa B activation (Schreck et al., 1992). These observations have led to a model in which oxygen radicals, possibly peroxides, are a common second messenger utilized by multiple stimuli to activate NF-kappa B (Schreck and Baeuerle, 1991) and under some circumstances, to activate apoptosis (Buttke and Sandstrom, 1994). Several observations herein suggest that NAC and PDTC may not be acting to buffer increases in free radical production or augment antioxidant defenses during SV infection: (a) millimolar concentrations of NAC are required to prevent SV-induced NF-kappa B activation (Fig. 4, A and B) and apoptosis (Fig. 1 A) in N18 and AT-3 cells compared to μM concentrations of NAC required to inhibit apoptosis in known paradigms of oxidative stress (Ratan et al., 1994b; Rothstein et al., 1994), (b) the failure of well established inhibitors of oxidative stress such as metal chelators, inhibitors of lipid peroxidation, and peroxide scavengers to abrogate SV-induced apoptosis (Table I) or SV-induced NF-kappa B activation (Fig. 4 B), and (c) the absence of a decrease in total glutathione, a marker of oxidative stress (Rehncrona et al., 1980; Kuo et al., 1993; Ratan et al., 1994b), in SV-infected cells before morphologic evidence of cell death (Fig. 1 C). Indeed, the recent observation that apoptosis induced by growth factor deprivation can occur under conditions of hypoxia argues against oxygen radicals as common mediators of apoptosis (Jacobson and Raff, 1995).

If NAC, PDTC and other thiol agents are not inhibiting SV-induced oxidative stress, by what other mechanisms could these agents exert their protective actions? Accumulating evidence suggests that antioxidants can alter protein function by modulating the redox state of critical cysteine residues or metals bound to proteins (Stamler, 1994). Among the proteins identified as targets for oxidants and antioxidants are protein kinases and protein phosphatases (Bauskin et al., 1991; Fischer et al., 1991). Indeed, PDTC has been demonstrated to inhibit phosphorylation of Ikappa B, an event considered critical for NF-kappa B activation (Traenckner et al., 1994). Additionally, recent studies have shown that induction of NF-kappa B by a wide variety of stimuli including peroxide can be inhibited by tyrosine kinase inhibitors (Anderson et al., 1994). Furthermore, tyrosine kinase inhibitors have been shown to abrogate apoptosis due to ionizing radiation (Ucklun et al., 1992). Iden tification of SV-induced signaling pathways necessary for NF-kappa B activation may thus identify putative molecular targets for NAC and other thiol agents that are critical to the anti-apoptotic actions of these agents.

In this study, we also demonstrate that Bcl-2 overexpression almost completely suppresses SV-induced NF-kappa B activation up to 6 h after infection in AT-3 cells (Fig. 4, C and D). Bcl-2 is thus able to inhibit NF-kappa B activation as well as apoptosis induced by SV. These results are consistent with previous observations that suggest that bcl-2 abrogates NF-kappa B activation induced by some, but not all cytotoxic stimuli (Albrecht et al., 1994). They are also consistent with a causal role of NF-kappa B in SV-induced apoptosis in AT-3 cells. Future studies will define the basis for bcl-2 inhibition of NF-kappa B signaling and

may provide insights into a molecular mechanism by which this pluripotent inhibitor of apoptosis exerts its protective actions.

Sindbis virus is one of a growing list of viruses capable of activating NF-kappa B and inducing apoptosis (Pahl and Baeuerle, 1995). We have used thiol agents such as NAC and PDTC, the protooncogene Bcl-2 and NF-kappa B transcription factor decoys to demonstrate that SV-induced NF-kappa B signaling is necessary for SV-induced apoptosis in AT-3 prostate carcinoma cells. These results add apoptosis to the extensive list of cell functions that can be regulated by NF-kappa B (Thanos and Maniatis, 1995). It will be important to determine whether this transcription factor signaling pathway is necessary for apoptosis induced by other viruses and other, nonviral apoptotic stimuli. However, even if NF-kappa B is not a general mediator of apoptotic cell death, the ability of pluripotent inhibitors of apoptosis such as thiol agents and bcl-2 to abrogate SV-induced NF-kappa B signaling and SV-induced apoptosis suggest that SV is a good model system in which to investigate the mechanism of action of these agents. Such investigations may elucidate novel approaches to diseases such as AIDS where NF-kappa B and apoptosis are believed to play a pathogenic role (Gougeon and Montagnier, 1993; Staal et al., 1993) as well as to neurological diseases such as Spinal Muscular Atrophy (SMA) where apoptosis has been implicated (Roy et al., 1995).

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