Apoptosis Promotes a Caspase-induced Amino-terminal Truncation of IκBα That Functions as a Stable Inhibitor of NF-κB*

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Caspases are cell death cysteine proteases that are activated upon the induction of the apoptotic program and cleave target proteins in a sequence-specific manner to promote cell death. Recently, Barkett et al. (Barkett, M., Xue, D., Horvitz, H. R., and Gilmore, T. D. (1997) J. Biol. Chem. 272, 29419–29422) have shown that IκBα, the inhibitory subunit of the transcription factor NF-κB, can be cleaved by caspase-3 in vitro at a site that potentially produces a dominant inhibitory form of IκBα. The involvement of NF-κB in the inhibition of cell death led us to ask whether apoptotic stimuli would induce the caspase-mediated cleavage of IκBα in vivo. In this study, we show that apoptosis leads to the caspase-mediated amino-terminal truncation of IκBα (ΔN-IκBα). Our data show that ΔN-IκBα can bind NF-κB, suppress NF-κB activation, and sensitize cells to death. Since activated NF-κB plays a role in the inhibition of cell death, these data suggest that caspase-mediated cleavage of IκBα may be a mechanism to suppress NF-κB and its associated antiapoptotic activity.

The proper development and homeostasis of multicellular organisms requires a defined process of autonomous cell death known as apoptosis (1). Apoptosis involves the activation of cysteine proteases, known as caspases (2), that regulate the selective proteolysis of internal cellular proteins. Cleavage of these proteins promotes a cellular death process that is characterized by DNA condensation, blebbing of the plasma membrane, and cytoplasmic shrinkage that ultimately leads to the formation of apoptotic bodies that are destroyed by neighboring cells (3).

The mammalian caspases comprise a family of proteins that were first implicated in apoptosis based on their homology to the cell death proteins in Caenorhabditis elegans (4–7). Caspases are activated in response to cellular death signals such as cytokine withdrawal of cytokine-dependent cells (8). Caspases are synthesized as inactive precursor molecules (pro-caspases) (2) that are cleaved in response to apoptotic stimuli and associate into tetramers to produce mature enzymes. The result is the activation of a caspase cascade that leads to the sequence-specific cleavage of additional caspases (9) as well as specific internal cellular proteins. Recent work has identified a number of caspase substrates including DNA fragmentation factor (10), p21-activated kinase 2 (11), and mitogen-activated protein kinase/Erk kinase-1 (12), which are activated upon cleavage, and DNA-dependent protein kinase (13) and the retinoblastoma protein (14), which are inactivated following caspase cleavage. Cleavage of these proteins contributes to cell death by activating cell death-promoting proteins and inactivating proteins involved in cell survival or DNA repair.

NF-κB is a transcription factor that regulates genes involved in the inhibition of apoptosis and in the activation of immune and inflammatory responses (15–20). NF-κB family members share homology in their Rel homology domain, a region important for DNA binding and dimerization between family members (15, 16). The regulation of NF-κB family members is achieved through a post-translational mechanism that involves interactions with a family of inhibitory proteins known as IκB, which bind and sequester NF-κB in the cytoplasm (15, 21). Stimulation of cells with NF-κB inducers, such as tumor necrosis factor α (TNFα) and interleukin (IL)-1, initiates a signal transduction cascade that leads to the activation of the IκB-kinase complex that specifically phosphorlates IκB (22–26). This phosphorylation occurs on Ser-32 and Ser-36, the same sites that have been previously shown to be the signal-induced phosphorylation sites on IκB (27–31). Phosphorylation of these sites targets IκB for ubiquitination on lysines 21 and 22 (32, 33) by the ubiquitin-conjugating enzyme system and subsequent degradation by the 26 S proteasome (34–37). Degradation of IκB exposes the nuclear localization signal of NF-κB, allowing NF-κB to enter the nucleus to direct the transcription of target genes (15, 16).

The role for NF-κB in apoptosis emerged when it was determined that the embryonic lethality observed in mice lacking the p65 subunit of NF-κB was the result of massive liver destruction due to apoptosis (38, 39). Research from our laboratory and others have since shown that inhibition of NF-κB sensitizes cells to killing by various stimuli including TNFα and cancer therapy drugs (17–20). In addition, inhibition of constitutive NF-κB activity in B cells induces apoptosis (40). More recent evidence has revealed that NF-κB plays a role in apoptosis by controlling the expression of genes involved in the inhibition of cell death (41–43).

It has been previously shown that IκBα can be cleaved in vitro within its amino terminus by caspase-3 (44). This cleavage has been proposed to occur immediately 5′ of Ser-32, one of the inducible phosphorylation sites in human IκBα, and produces an amino-terminal truncation of IκBα. Due to the involvement of NF-κB in apoptosis, we reasoned that IκBα was a likely candidate for caspase cleavage in vivo. Since NF-κB

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1 The abbreviations used are: TNFα, tumor necrosis factor α; IL, interleukin; FCS, fetal calf serum; EMSA, electrophoretic mobility shift assay; Z-VAD, benzoylcarbonyl-VAD-CH2F; DEVD, DEVD-CH2F; YVAD, YVAD-CH2F.

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regulates the induction of genes involved in cell survival, such as cIAP-1, cIAP-2, XIAP, and IEX-1L (41–43, 45), we reasoned that IxBα may be cleaved by caspases during apoptosis to produce an inhibitor of NF-κB activation. Therefore, we were interested in determining whether δN-IxBα is generated in vivo in response to apoptosis and whether δN-IxBα can bind NF-κB and suppress its activity.

In this study, we found that IxBα is cleaved in cells undergoing TNFα-induced cell death as well as in cells induced to undergo apoptosis in response to IL-3 withdrawal. Caspase-3 or a related caspase leads to the amino-terminal truncation of IxBα (δN-IxBα) in vivo because a mutation in the caspase target sequence of IxBα as well as a caspase-3 inhibitor prevents the generation of δN-IxBα upon apoptosis. The generation of δN-IxBα during apoptosis is a potentially significant event, since δN-IxBα can bind the p65 subunit of NF-κB in vivo. Importantly, this interaction suppresses NF-κB activation because δN-IxBα is resistant to degradation in response to inducers of NF-κB. In addition, the expression of δN-IxBα sensitizes cells to apoptosis. Together, these data suggest that caspase-directed cleavage of IxBα generates a dominant inhibitory molecule that suppresses the activity of NF-κB during apoptosis. This could be a potential mechanism whereby genes involved in cell survival cannot be activated once the apoptotic machinery has been turned on.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—32D cells stably expressing the IxBα-SR have been previously described (46). 32D myeloid cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; BioWhittaker, Walkersville, MD), 10% Wehi conditioned medium as a source of IL-3, and 100 μg/ml each penicillin and streptomycin (Sigma). 32D/IxBα-SR cells were maintained in RPMI-1640 medium supplemented with 10% FCS, 10% Wehi conditioned medium, 100 μg/ml each penicillin/streptomycin, and 0.5 mg/ml Geneticin (Life Technologies, Inc.). The primers used to incorporate the mutation at amino acid 31 were 1) 3′-GAGGAGCAGCAAGTCCGCTCGGCG-5′ and 2) 5′-GTTGCA-GCGCTAAGCGCTGCCTGCCTGCTC-3′. Underlined sequences are the nucleotides for mutagenesis. WT p3xMIC WTH-4F2 (WT + B luc) and 3xMIC mutant luc (- mutant + B luc) were gifts from B. Sugden (University of Wisconsin, Madison, WI) (47).

**Cell Extract Preparation and Reagents**—As shown in Fig. 1A, 2 × 10^6 32D and 32D/IxBα-SR cells were plated in 5 ml of medium 15 min prior to treatment with 10 ng/ml mouse TNFα (Roche Molecular Biochemicals) for 0, 2, 4, or 6 h. Cells were collected and lysed in sample buffer (125 mM Tris, pH 6.8, 20% glycerol, 4% SDS, 1.4 mM β-mercaptoethanol, bromphenol blue), and the cell lysate was analyzed by Western blotting. As shown in Fig. 1B, p65−/− cells were plated in a 15-cm dish in medium containing 5% FCS and incubated at 37 °C overnight. The following day, cells at approximately 55% confluence were plated in fresh medium containing 5% FCS and treated with 37 °C overnight. The following day, cells at approximately 55% confluence were plated in fresh medium containing 5% FCS and treated with 37°C overnight. The following day, cells were collected and washed three times in medium lacking IL-3 and incubated at 37 °C for 0, 12, 16, 20, or 24 h. The cells were collected and lysed in sample buffer for Western blotting. As shown in Fig. 2A, 1 × 10^6 32D/IxBα-SR cells were plated in 2 ml 15 min prior to the addition of 10 ng/ml mouse TNFα for 0 or 6 h. The cells were collected and washed three times with medium lacking IL-3 and incubated at 37 °C for 4 h. The cells were treated as in Fig. 1B (see above); however, 60 μM Z-DEVD-FMK was added 30 min prior to TNFα treatment. In Fig. 5, 6 × 10^6 32D cells were plated in the absence of IL-3, or 2 × 10^6 cells were plated in the presence of IL-3 in 2 ml of medium/well of a 12-well dish and incubated at 37 °C for 16 h. These cells were then treated with 10 ng/ml mouse TNFα for 0, 5, 15, 30, or 60 min and lysed in sample buffer for Western blotting. In samples 10–15, 20 μg/ml cycloheximide was added 1 h prior to treatment with TNFα. In Fig. 6A, the NF-κB reporter assay (see above); however, nuclear extracts were prepared from these cells. In Fig. 6, A and B, nuclear extracts were prepared as described previously (48).

**Western Analysis**—The protein from 2–5 × 10^6 32D/IxBα-SR cells were plated in 15 ml of fresh media 15 min prior to TNFα treatment. The cells were lysed in 800 μl of ELB buffer (50 mM Hepes, pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaN3, 1 mM Na3VO4, and 5 μg/ml each aprotinin, leupeptin, pepstatin, and soybean trypsin inhibitor) for 15 min at 4 °C. The lysate was precleared with Protein A-Sepharose beads (Amersham Pharmacia Biotech) for 10 min at 4 °C. The beads were collected by pulse centrifugation, and the lysate was separated in 5 ml of medium/well of a 12-well dish and incubated at 4 °C. Protein A-Sepharose beads were added for 1 h at 4 °C. The beads were collected by pulse centrifugation at 4 °C, and the cytoplasmic supernatant was discarded. The immuno precipitated proteins on the beads were washed three times with 500 μl of ELB buffer. Immuno precipitated proteins were analyzed by 12% SDS-PAGE and Western blotting.

**Transient Transfection**—2–5 × 10^6 293T cells were plated per well of a six-well dish 48 h prior to transfection. Transfections were performed by the calcium phosphate method by diluting 3 μg of pCDNA3 or pCDNA3-IxBα-SR and 1 μg of luciferase reporter (if applicable) into 250 μl of Hepes-buffered saline, pH 7.05 (137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM dextrose, and 20 mM Hepes). To the DNA/Hepes-buffered saline mix, 25 μl of 1.25 mM CaCl2 was added and immediately vortexed for 10 s. The transfection mix was incubated at room temperature for 15–20 min, added dropwise to the cells, and incubated at 37 °C for 4–5 h. The media was removed and replaced with fresh, complete medium, and the cells were incubated at 37 °C overnight. For luciferase reporter assays, the medium was replaced with fresh medium containing 0.5% FCS and incubated at 37 °C overnight.

**Electrophoretic Mobility Shift Assay (EMSA)**—Cell extracts were prepared as described above. EMSAs were performed as described previously (46).

**Luciferase Reporter Assays**—The cells were collected 24 h post-transfection, and 50 μg of cell lysate were assayed as described previously (46).

**RESULTS**

**Apoptotic Conditions Induce the Generation of δN-IxBα**—It has been previously shown that inhibition of NF-κB activity sensitizes cells to killing by TNFα (17–20). Inhibition of NF-κB activity can be achieved through expression of an IxBα molecule with serine to alanine mutations at its sites of inducible gene expression, which is regulated by NF-κB activity. Therefore, we were interested in determining the induction of apoptosis, we were interested in determining...
Apoptosis-induced Cleavage of IκBα

A, TNFα-induced apoptosis leads to cleavage of IκBα in 32D/IκBα-SR cells. Cell extracts were prepared from 32D and 32D/IκBα-SR cells treated with TNFα for 2, 4, or 6 h by lysing an equal number of cells in sample buffer. Extracts were analyzed by Western blotting with a carboxyl-terminal IκBα antibody. Mobilities of IκBα-SR, endogenous IκBα, and ΔN-IκBα-SR are indicated with arrows. B, TNFα-induced apoptosis leads to cleavage of IκBα in p65−/− cells. Adherent cells (A) were collected from p65−/− cells treated with TNFα for 0 or 24 h. In addition, the detached cells (D) were collected from the p65−/− cells treated with TNFα. Cell extracts were prepared and analyzed as in A. Mobilities of endogenous IκBα and ΔN-IκBα are indicated with arrows. C, IL-3 withdrawal leads to apoptosis and cleavage of IκBα in 32D cells. Cell extracts were prepared from 32D cells grown in the absence of IL-3 for 0, 12, 16, 20, or 24 h and were analyzed as in A. Mobilities of endogenous IκBα and ΔN-IκBα are indicated with arrows.

Whether IκBα was cleaved under apoptotic conditions

Cell extracts were made from 32D or 32D/IκBα-SR cells treated with TNFα for various times and analyzed by Western blotting with a carboxyl-terminal IκBα-specific antibody. TNFα treatment of 32D/IκBα-SR cells led to apoptosis, and Western blot analysis revealed that an amino-terminal truncated IκBα-SR (ΔN-IκBα-SR) accumulates at 4 h and more impressively at 6 h post-treatment (Fig. 1A). In contrast, TNFα treatment of 32D cells did not lead to the accumulation of ΔN-IκBα (Fig. 1A), because NF-κB is activated in these cells and the caspase cascade is subsequently suppressed (42). In addition, Western blot analysis with a carboxy-specific IκBβ antibody showed that IκBβ is not cleaved in response to apoptosis in these cells (data not shown).

It has been reported that cells that lack expression of the RelA/p65 subunit of NF-κB (p65−/−) have increased sensitivity toward apoptosis induced by TNFα than wild-type cells (17). Therefore, immortalized p65−/− cells were treated with TNFα for 24 h. This treatment resulted in the production of two cell populations. The first population consists of cells that have begun the apoptotic process in response to TNFα and have rounded up and detached from the surface of the plate. The second population consists of cells that have not detached from the surface because they have not begun the process of apoptosis or are in the early stages of apoptosis. Western analysis revealed that untreated p65−/− cells express full-length IκBα (Fig. 1B). Western analysis of TNFα-treated p65−/− cells revealed that the adherent (A) cells express full-length IκBα, whereas the detached (D) cells exclusively contain ΔN-IκBα (Fig. 1B).

In order to determine if ΔN-IκBα is produced by apoptotic conditions other than that induced by TNFα treatment, we monitored the accumulation of ΔN-IκBα during IL-3 withdrawal-induced cell death. 32D myeloid cells require IL-3 for growth and viability, and in its absence cells undergo apoptosis (49). 32D cells were deprived of IL-3 for varying times, and total cell extract was collected and analyzed by Western blotting with a carboxy-terminal IκBα-specific antibody. Accumulation of ΔN-IκBα began to occur 16 h post-IL-3 withdrawal, and a significant amount of ΔN-IκBα was detected 24 h after IL-3 withdrawal (Fig. 1C).

Truncation of IκBα Induced by TNFα Requires Caspase-3 Activity—Since IκBα is cleaved by caspase-3 in vitro (44), we were interested in determining whether caspase-3 (or a similarly directed caspase) controlled the amino-terminal truncation of IκBα during either TNFα- or IL-3-induced apoptosis. Caspase activity can be inhibited through the use of specific peptide inhibitors that compete with substrate for enzyme binding in a caspase-specific manner. Therefore, 32D/IκBα-SR cells were treated with TNFα for 6 h in the presence or absence of a peptide caspase-3 inhibitor (DEVD), a caspase-1 inhibitor (YVAD), or a broadly specific inhibitor of the caspases (Z-VAD). Western analysis revealed that YVAD treatment of 32D/IκBα-SR cells undergoing TNFα-induced cell death has no effect on the generation of ΔN-IκBα-SR (Fig. 2A). However, treatment of the 32D/IκBα-SR cells with TNFα in the presence of the caspase inhibitor DEVD or Z-VAD suppressed the generation of ΔN-IκBα-SR (Fig. 2A). These data suggest that the generation of ΔN-IκBα-SR in response to TNFα requires caspase-3 activity or a DEVD-directed caspase.
We also wanted to determine whether the caspase-3 inhibitor had any effect on the generation of ΔN-IkBα in p65−/− cells treated with TNFa. As described previously, treatment of p65−/− cells with TNFa for 24 h generates two populations of cells, adherent cells that express full-length IkBα and detached cells that contain ΔN-IkBα (Figs. 1B and 2B). Exposure of TNFa-treated p65−/− cells to DEVD has no effect on the detachment of cells from the plate; however, the number of viable cells is significantly increased (data not shown). Interestingly, Western analysis of these cells revealed that IkBα had not been cleaved to ΔN-IkBα (Fig. 2B). These data suggest that caspase-3 or a DEVD-sensitive caspase cleaves IkBα in p65−/− cells treated with TNFa.

Barkett et al. (44) have previously shown that caspase-3 cleaves IkBα NH2-terminal to Ser-32 in human IkBα in vitro. In order to provide direct evidence that caspases cleave IkBα in vivo, it was important to mutate the caspase target sequence within IkBα. Since caspases cleave carboxy-terminal to Asp within consensus target sequences (50), we reasoned that mutation of Asp-31 to Ala in IkBα (IkBα-D31A) would result in an IkBα that is resistant to degradation in response to apoptosis. Therefore, p65−/− cells were transiently transfected with WT IkBα or IkBα-D31A and induced to undergo apoptosis with TNFa for 24 h. Western blot analysis of IkBα in the detached cells (Fig. 3) revealed that although WT IkBα was cleaved to ΔN-IkBα, cleavage of IkBα-D31A was significantly reduced. It is important to note that the protein from the detached cells in Fig. 3 was overloaded in order to detect the presence of any cleaved ΔN-IkBα-D31A. These data indicate that cleavage of IkBα indeed occurs within the proposed caspase target sequence and most likely by caspase-3. Lastly, we were interested in determining whether IkBα is cleaved with similar kinetics to poly(ADP-ribose), another caspase-3 substrate (51). 32D cells were cultured in the absence of IL-3 for varying times in order to induce apoptosis. Western blot analysis with antibodies specific for poly(ADP-ribose) and IkBα revealed that both are cleaved 16 h after the induction of apoptosis (data not shown). These data indicate that IkBα is cleaved with kinetics similar to the activation of caspase-3 and that IkBα is not cleaved as a consequence of but as a component of the apoptotic process.

ΔIkBα Retains the Ability to Bind NF-κB—Since we were interested in determining whether ΔN-IkBα can act as a constitutive inhibitor of NF-κB, we tested whether ΔN-IkBα is resistant to degradation by inducers of NF-κB, TNFa treatment of parental cells leads to the activation of NF-κB through the phosphorylation and degradation of IkBα. Activation of NF-κB leads to the transcription of IkBα, resulting in an auto-regulatory loop that culminates in the reactivation of NF-κB in the cytoplasm by newly synthesized IkBα (52, 53). The degradation and resynthesis of IkBα can be easily monitored through Western blotting as shown in Fig. 5. This analysis reveals that treatment of parental 32D cells with TNFa over time leads to the degradation and reaccumulation of IkBα (Fig. 5, lanes 1–4, upper panel). However, treatment of 32D cells with TNFa in the presence of the protein synthesis inhibitor cycloheximide (CHX) results in degradation of IkBα but does not allow the resynthesis of IkBα (Fig. 5, lanes 10–13, upper panel). When 32D cells are grown in the absence of IL-3, they undergo apoptosis and accumulate ΔN-IkBα (Fig. 1C and Fig. 5, lanes 5 and 14, upper panel). Treatment of IL-3-deprived cells with TNFa leads to the degradation and resynthesis of endogenous IkBα, but ΔN-IkBα protein levels are unaffected (Fig. 5, lanes 5–9, upper panel). In addition, TNFa treatment of CHX pretreated cells results in the degradation of endogenous IkBα without resynthesis of protein. However, ΔN-IkBα protein levels remain constant, indicating that the presence of ΔN-IkBα is not a result of degradation of newly synthesized IkBα (Fig. 5, lanes 14–18, upper panel). In addition, EMSAs performed on nuclear extracts from the samples in Fig. 5 revealed that activation of NF-κB by TNFa was suppressed in cells that had accumulated ΔN-IkBα following IL-3 withdrawal when compared with control cells (described below; Fig. 6). These results indicate that ΔN-IkBα cannot be degraded by inducers of NF-κB.

Release of NF-κB from IkBα in the cytoplasm relies on the phosphorylation of IkBα on Ser-32 and Ser-36 (27–31), ubiquitination of lysines 21 and 22 (32, 33), and degradation by the 26 S proteasome (32, 35–37). ΔN-IkBα may be unable to be ubiquitinated on lysines 21 and 22 because these sequences are
FIG. 5. \(\Delta N-IkBa\) is resistant to degradation induced by TNF\(\alpha\). 32D cells were grown in the presence or absence of IL-3 for 15 h and subsequently treated with TNFs for 0, 5, 15, 30, or 60 min (lanes 1–9). Cell extracts were prepared by lysis in sample buffer and analyzed by Western blotting with a carboxyl-terminal IkBa antibody (upper panel) or a phosphospecific 32-specific IkBa antibody (lower panel). Cycloheximide was added 1 h prior to TNF\(\alpha\) treatment (lanes 10–18). Mobilities of endogenous IkBa, \(\Delta N-IkBa\), and phospho-IkBa are indicated with arrows.

probably lost during cleavage of IkBa. Presumably, \(\Delta N-IkBa\) is not degraded because recognition by the 26 S proteasome cannot occur. We were, however, interested in determining whether \(\Delta N-IkBa\) can be inducibly phosphorylated on Ser-32 and Ser-36 in vivo. Western analysis was performed on 32D cells extracts with a phosphospecific 32-specific polyclonal antibody. Endogenous IkBa was strongly phosphorylated within 5 min following exposure to TNF\(\alpha\) in the presence or absence of IL-3 (Fig. 5, lanes 2, 6, 11, and 15, lower panel). Phosphorylated IkBa was then degraded (Fig. 5, lanes 3, 7, 12, 16, lower panel) and subsequently resynthesized (Fig. 5, lanes 4, 8, 9, lower panel).

Interestingly, resynthesized IkBa was phosphorylated on Ser-32, indicating that IkBa-kinase complex is still active at this time point. However, phosphorylation of \(\Delta N-IkBa\) following TNF\(\alpha\) treatment was not evident (Fig. 5, lanes 2, 4, 6, 8, 9, 11, and 15, lower panel). These data suggest that the loss of sequences immediately 5′ of Ser-32 inhibits the ability of Ser-32 in \(\Delta N-IkBa\) to be phosphorylated by IkBa-kinase complex, suggesting that the absence of these sequences may lead to the loss of a potential recognition site for IkBa-kinase complex. It is also possible that the phosphospecific 32-specific antibody cannot recognize phosphospecific 32 in \(\Delta N-IkBa\) in the absence of the 5′-flanking sequences. Nevertheless, \(\Delta N-IkBa\) is not degraded following stimulation by an inducer of NF-\(\kappa\)B, a result that is due to the loss of lysines 21 and 22 and/or to the loss of phosphorylation at Ser-32 and potentially at Ser-36. Therefore, \(\Delta N-IkBa\) is resistant to phosphorylation-induced degradation by an inducer of NF-\(\kappa\)B.

\(\Delta N-IkBa\) Prevents NF-\(\kappa\)B Activation—The generation of \(\Delta N-IkBa\) during apoptotic conditions, its association with the p65 subunit of NF-\(\kappa\)B, and its resistance to degradation by inducers of NF-\(\kappa\)B imply that \(\Delta N-IkBa\) is an inhibitor of NF-\(\kappa\)B. To verify that the amino-terminal truncation of IkBa produces a dominant inhibitory molecule, we tested whether endogenous \(\Delta N-IkBa\) could suppress the activation of NF-\(\kappa\)B. NF-\(\kappa\)B activation is often measured by its increased nuclear translocation, an event that can be monitored through EMSA. Therefore, 32D cells were grown in the presence of IL-3 or in the absence of IL-3 to generate \(\Delta N-IkBa\) and were subsequently treated with TNF\(\alpha\) to induce the nuclear translocation of NF-\(\kappa\)B. EMSAs performed with these nuclear extracts revealed that the amount of TNF\(\alpha\)-induced NF-\(\kappa\)B DNA binding is decreased in cells grown in the absence of IL-3 (Fig. 6A, lanes 1–4) when compared with control cells grown in the presence of IL-3 (Fig. 6A, lanes 5–9). Since IL-3 withdrawal leads to the accumulation of \(\Delta N-IkBa\) (see Figs. 1C and 5), these data suggest that the presence of \(\Delta N-IkBa\) suppresses the activation of NF-\(\kappa\)B. Although TNF\(\alpha\)-induced NF-\(\kappa\)B DNA binding was decreased in the presence of \(\Delta N-IkBa\) (Fig. 6A, lanes 5–9), it was not completely abolished. This is probably due to the fact that not all of these cells contain \(\Delta N-IkBa\) (see Fig. 5) and that NF-\(\kappa\)B activation can only be suppressed in these cells.

In order to further confirm that \(\Delta N-IkBa\) is a dominant inhibitory molecule, we constructed a \(\Delta N-IkBa\) expression vector and tested its ability to block NF-\(\kappa\)B activation. To construct this vector we assumed that the site of caspase-directed cleavage of IkBa in vivo occurs at the same sequence in which it occurs in vitro (44). Therefore, we deleted the first 31 amino acids of IkBa and cloned \(\Delta N-IkBa\) downstream of the cytomegalovirus promoter in the pCDNA3 vector. EMSAs were performed on nuclear extracts of 293T cells transiently transfected with pCDNA3 or pCDNA3-\(\Delta N-IkBa\) and treated with TNF\(\alpha\) for various times. TNF\(\alpha\) treatment resulted in the enhanced nuclear accumulation and DNA binding activity of NF-\(\kappa\)B in 293T cells expressing the control vector (Fig. 6B). However, expression of \(\Delta N-IkBa\) resulted in a decrease in nuclear NF-\(\kappa\)B in untreated cells and suppressed NF-\(\kappa\)B nuclear translocation and DNA binding induced by TNF\(\alpha\) (Fig. 6B).

NF-\(\kappa\)B activation can also be monitored through the increase in transcription of NF-\(\kappa\)B-regulated genes. Therefore, luciferase reporter assays were performed to determine whether \(\Delta N-IkBa\) can block TNF\(\alpha\)-induced NF-\(\kappa\)B-dependent gene expression. These assays were performed utilizing a luciferase reporter construct fused to a promoter containing three NF-\(\kappa\)B binding sites (WT \(\kappa\)B luc). 293T cells were transiently co-transfected with WT \(\kappa\)B luc and pCDNA3 or pCDNA3-\(\Delta N-IkBa\) and subsequently treated with TNF\(\alpha\) for 8 h. TNF\(\alpha\) treatment of 293T cells led to a 9-fold increase in NF-\(\kappa\)B-dependent gene expression, while NF-\(\kappa\)B activation by TNF\(\alpha\) was blocked in 293T cells expressing \(\Delta N-IkBa\) (Fig. 6C). A reporter construct with three mutant NF-\(\kappa\)B binding sites (mutant \(\kappa\)B luc) was not affected by expression of pCDNA3-\(\Delta N-IkBa\), suggesting that the loss of NF-\(\kappa\)B-dependent gene expression by TNF\(\alpha\) in these cells is not due to a general inhibition of transcription (Fig. 6C). The EMSA and luciferase reporter assay data suggest that \(\Delta N-IkBa\) is, indeed, a dominant inhibitor of NF-\(\kappa\)B.

The presence of \(\Delta N-IkBa\) in cells may also cause increased sensitivity to cellular death through the inhibition of NF-\(\kappa\)B. Therefore, 293T cells were transiently transfected with pCDNA3 or pCDNA3-\(\Delta N-IkBa\) and subsequently treated with TNF\(\alpha\). Consistent with the known antiapoptotic function of NF-\(\kappa\)B, TNF\(\alpha\) treatment led to apoptosis in cells expressing \(\Delta N-IkBa\), whereas it had no affect on control cells (data not shown).

**DISCUSSION**

In this study, we provide in vivo evidence that commitment to apoptosis leads to the cleavage of IkBa to generate \(\Delta N-IkBa\). Specifically, we have shown that caspase-3 or a DEVD-directed caspase leads to the cleavage of IkBa under TNF\(\alpha\) and cytokine deprivation-induced apoptosis. The cleavage of IkBa does not appear to be absolutely essential for apoptosis, because p65−/−
promotes cell survival by positively regulating genes involved in the inhibition of cell death such as cIAP-1, XIAP, and IEX-1L (41–43). Inappropriate activation of NF-κB during apoptosis would lead to the transcription of genes involved in the suppression of apoptosis, an event that would result in the survival of unwanted, damaged cells. Apoptotic signals from stress factors and survival signals from growth factors can converge on a single cell within the body. Since signals emanating from growth factors can lead to the activation of NF-κB (15, 16) and the subsequent inhibition of apoptosis, it would be advantageous for the commitment to apoptosis to provide a mechanism to inhibit this activation. The generation of ΔN-IκBα may be a mechanism whereby the suppression of NF-κB is guaranteed.

The disruption of NF-κB activation may be a general theme during apoptosis because it has been reported that γ-irradiation-induced apoptosis also leads to the cleavage of IκBα (57) and that Fas-induced apoptosis leads to the caspase-3-mediated cleavage of the p50 and p65 subunits of NF-κB (58). Whether caspase substrates become inactivated (i.e. p55, DNA-dependent protein kinase, and retinoblastoma protein (13, 14, 58)) or become proapoptotic proteins (i.e. IκBα, DNA fragmentation factor, and mitogen-activated protein kinase/Erk kinase kinase kinase-1 (10, 12)) following cleavage, their role is to ensure that apoptosis is not disrupted once the cell is committed to undergo programmed cell death.

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