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 (procaspases) (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 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)[1] and interleukin (IL)-1, initiates a signal transduction cascade that leads to the activation of the IκB-kinase complex that specifically phosphorylates IκBα (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 IκBα (27–31). Phosphorylation of these sites targets IκBα 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 IκBα 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

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

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 IκBα may be cleaved by caspases during apoptosis to produce an inhibitor of NF-κB activation. Therefore, we were interested in determining whether ΔN-IκBα is generated *in vivo* in response to apoptosis and whether ΔN-IκBα can bind NF-κB and suppress its activity.

In this study, we found that IκBα 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 IκBα (ΔN-IκBα) *in vivo* because a mutation in the caspase target sequence of IκBα as well as a caspase-3 inhibitor prevents the generation of ΔN-IκBα upon apoptosis. The generation of ΔN-IκBα during apoptosis is a potentially significant event, since ΔN-IκBα can bind the p65 subunit of NF-κB *in vivo*. Importantly, this interaction suppresses NF-κB activation because ΔN-IκBα is resistant to degradation in response to inducers of NF-κB. In addition, the expression of ΔN-IκBα sensitizes cells to apoptosis. Together, these data suggest that caspase-directed cleavage of IκBα 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 IκBα-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/IκBα-SR cells were maintained in RPM-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.). p65$^{-/-}$ mouse embryo fibroblast and 293T human embryonic kidney cells were maintained in DMEM-H supplemented with 10% FCS and 100 μg/ml each penicillin/streptomycin.

Plasmids—To construct the pCDNA3-ΔN-IκBα vector, an ATG start site was introduced immediately 5′ of Ser-32 in IκBα through the use of the polymerase chain reaction. The resulting polymerase chain reaction product was cloned into pCDNA3. pCMV-IκBα-D31A was generated through site-directed mutagenesis using pCMV-IκBα and the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used to incorporate the mutation at amino acid 31 were 1) 5′-GACGACCGCCAC<u>GCT</u>AGCGGCCTGGAC-3′ and 2) 5′-GTCCAG-GCCGCT<u>AGC</u>GTGGCGGTCGTC-3′. Underlined sequences are the nucleotides for mutation. 3XMHC WTluc (WT κB luc) and 3XMHC mutantluc (mutant κB luc) were gifts from B. Sugden (University of Wisconsin, Madison, WI) (47).

Cell Extract Preparation and Reagents—As shown in Fig. 1*A*, 2×10^6 32D and 32D/IκBα-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, 1.44 M β-mercaptoethanol, bromphenol blue), and the cell lysate was analyzed by Western blotting. As shown in Fig. 1*B*, 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 85% confluency were plated in fresh medium containing 5% FCS and treated with 50 ng/ml mouse TNFα for 0 or 24 h and subsequently lysed in sample buffer for Western blotting. As shown in Fig. 1*C*, 5×10^5 32D cells were plated in 3 ml in the presence of IL-3 and incubated at 37 °C overnight. The following day, the 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. 2*A*, 1×10^6 32D/IκBα-SR cells were plated in 2 ml 15 min prior to the addition of 10 ng/ml mouse TNFα for 0 or 6 h. Three hours after TNFα treatment, 25 μM benzyloxycarbonyl-VAD-CH$_2$F (Z-VAD), 25 μM benzyloxycarbonyl-DEVD-CH$_2$F, and 100 μM benzyloxycarbonyl-YVAD-CH$_2$F (Enzyme Systems Products, Livermore, CA) were added to the cells. Cells were lysed in sample buffer for Western blotting. In Fig. 2*B*, cells were treated as in Fig. 1*B* (see above); however, 60 μM DEVD was added 30 min prior to TNFα treatment. In Fig. 5, 6×10^5 32D cells were plated

in the absence of IL-3, or 2×10^5 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 10–18, 20 μg/ml cycloheximide was added 1 h prior to treatment with TNFα. In Fig. 6*A*, samples were treated as in Fig. 5 (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 $2–5 \times 10^5$ cells lysed in sample buffer were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose. For all Western experiments, equal protein loading was confirmed by staining the nitrocellulose membrane with Ponceau S. Western blotting was performed as described previously (46) with an anti-carboxyl-terminal IκBα polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Western blotting with the antiphosphoserine-32 IκBα polyclonal antibody (New England BioLabs, Inc., Beverly, MA) was performed as per the manufacturer's instructions.

Immunoprecipitation—9×10^6 32D/IκBα-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 NaF, 1 mM Na$_3$VO$_4$, and 5 μg/ml each aprotinin, leupeptin, pepstatin), and the lysate was collected by centrifugation at maximum speed 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 half and incubated with 1 μl of preimmune serum or 1 μl of anticarboxyl-terminal p65 polyclonal antibody (Rockland, Boyertown, PA) for 1 h 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 supernatant was discarded. The immunoprecipitated proteins on the beads were washed three times with 500 μl of ELB buffer. Immunoprecipitated proteins were analyzed by 12% SDS-PAGE and Western blotting.

Transient Transfection—2.5×10^5 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-IκBα-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 Na$_2$HPO$_4$·7H$_2$0, 6 mM dextrose, and 20 mM Hepes). To the DNA/Hepes-buffered saline mix, 25 μl of 1.25 M CaCl$_2$ 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-IκBα—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 IκBα molecule with serine to alanine mutations at its sites of inducible phosphorylation (Ser-32 and Ser-36) (29, 30). This modified IκBα molecule generates a "super-repressor" protein (IκBα-SR) that constitutively sequesters NF-κB in the cytoplasm. Treatment of cells stably expressing the IκBα-SR with TNFα causes the cells to undergo apoptosis (19, 20). Therefore, 32D myeloid cells were generated that stably express the IκBα-SR (46). Expression of the IκBα-SR in these cells causes a marked down-regulation in the levels of endogenous IκBα, which is most likely due to a decrease in free IκBα (because uncomplexed IκBα has a short half-life) and to a decrease in IκBα gene expression, which is regulated by NF-κB (15). Since the proteolytic activity of caspases plays a critical role in the induction of apoptosis, we were interested in determining
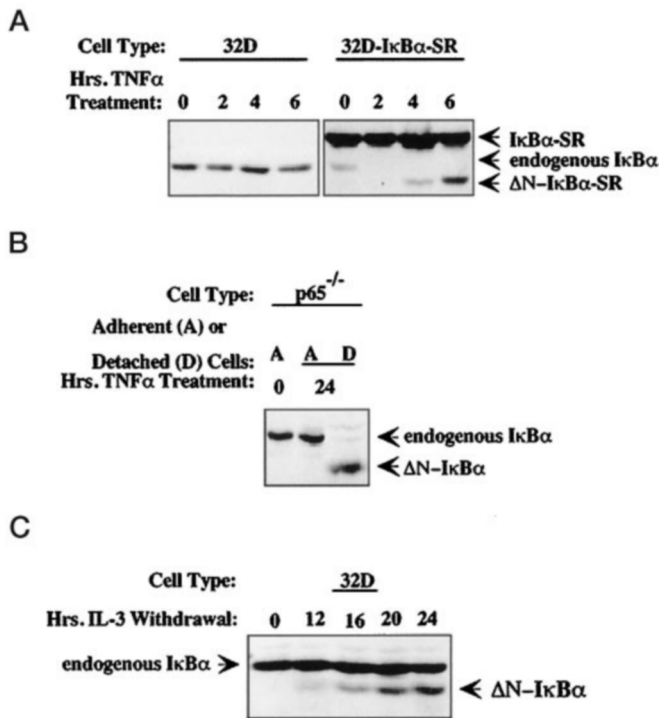
FIG. 1. **Apoptosis leads to the amino-terminal truncation of IκBα.** *A*, TNFα-induced apoptosis leads to cleavage of IκBα in 32D/IκBα-SR cells. Cell extracts were prepared from 32D and 32D/IκBα-SR cells treated with TNFα for 0, 2, 4, or 6 h by lysing an equal number of cells in sample buffer. Extracts 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. 1*A*). In contrast, TNFα treatment of 32D cells did not lead to the accumulation of ΔN-IκBα (Fig. 1*A*), 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
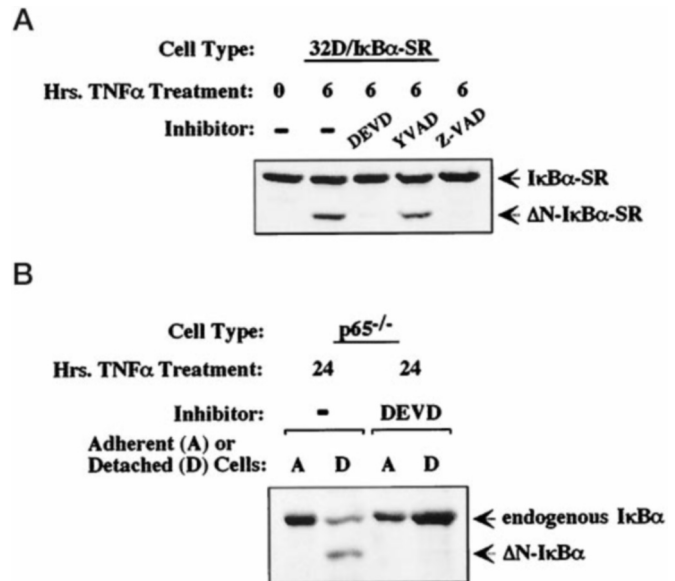

FIG. 2. **Cleavage of IκBα during TNFα-induced apoptosis requires caspase-3.** *A*, 32D/IκBα-SR cells were treated with TNFα for 0 or 6 h. Three hours after TNFα treatment, the cells were treated with Me$_2$SO or the caspase inhibitor DEVD, YVAD, or Z-VAD. Cell extracts were prepared by lysing equal numbers of cells in sample buffer. Extracts were analyzed by Western blotting with a carboxyl-terminal IκBα antibody. Mobilities of IκBα-SR and ΔN-IκBα-SR are indicated with *arrows*. *B*, p65$^{-/-}$ cells were pretreated with Me$_2$SO or DEVD for 3 h and treated with TNFα for 24 h. The detached cells (*D*) were collected separately from the adherent cells (*A*). An equivalent number of adherent cells and an equivalent number of detached cells were lysed in sample buffer and analyzed as in *A*. Mobilities of endogenous IκBα and ΔN-IκBα are indicated with *arrows*.

(Fig. 1*B*). 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. 1*B*).

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 carboxyl-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. 1*C*).

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. 2*A*). 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. 2*A*). These data suggest that the generation of ΔN-IκBα-SR in response to TNFα requires caspase-3 activity or a DEVD-directed caspase.
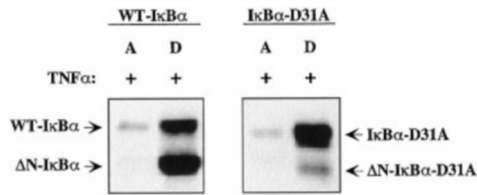
FIG. 3. **Mutation of the proposed caspase cleavage sequence in IκBα suppresses the ability of IκBα to be cleaved during apoptosis.** p65$^{-/-}$ cells were transiently transfected with expression plasmids for WT-IκBα or IκBα-D31A and treated with TNFα for 24 h. The detached cells (*D*) were collected separately from the adherent cells (*A*), and cell lysates were analyzed by Western blotting with a carboxyl-terminal IκBα antibody. Mobilities of transfected WT-IκBα and IκBα-D31A and cleaved WT-IκBα (ΔN-IκBα) and IκBα-D31A (ΔN-IκBα-D31A) are indicated with *arrows*.

We also wanted to determine whether the caspase-3 inhibitor had any effect on the generation of ΔN-IκBα in p65$^{-/-}$ cells treated with TNFα. As described previously, treatment of p65$^{-/-}$ cells with TNFα for 24 h generates two populations of cells, adherent cells that express full-length IκBα and detached cells that contain ΔN-IκBα (Figs. 1*B* and 2*B*). Exposure of TNFα-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 IκBα had not been cleaved to ΔN-IκBα (Fig. 2*B*). These data suggest that caspase-3 or a DEVD-sensitive caspase cleaves IκBα in p65$^{-/-}$ cells treated with TNFα.

Barkett *et al.* (44) have previously shown that caspase-3 cleaves IκBα NH$_2$-terminal to Ser-32 in human IκBα *in vitro*. In order to provide direct evidence that caspases cleave IκBα *in vivo*, it was important to mutate the caspase target sequence within IκBα. Since caspases cleave carboxyl-terminal to Asp within consensus target sequences (50), we reasoned that mutation of Asp-31 to Ala in IκBα (IκBα-D31A) would result in an IκBα that is resistant to degradation in response to apoptosis. Therefore, p65$^{-/-}$ cells were transiently transfected with WT IκBα or IκBα-D31A and induced to undergo apoptosis with TNFα for 24 h. Western blot analysis of IκBα in the detached cells (Fig. 3) revealed that although WT IκBα was cleaved to ΔN-IκBα, cleavage of IκBα-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-IκBα-D31A. These data indicate that cleavage of IκBα indeed occurs within the proposed caspase target sequence and most likely by caspase-3. Lastly, we were interested in determining whether IκBα 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 IκBα revealed that both are cleaved 16 h after the induction of apoptosis (data not shown). These data indicate that IκBα is cleaved with kinetics similar to the activation of caspase-3 and that IκBα is not cleaved as a consequence of but as a component of the apoptotic process.

ΔN-IκBα Retains the Ability to Bind NF-κB—Since we were interested in determining whether ΔN-IκBα can act as a constitutive inhibitor of NF-κB, it was important to show that ΔN-IκBα can bind NF-κB *in vivo*. In these experiments, we induced the generation of ΔN-IκBα-SR by treating 32D/IκBα-SR cells with TNFα for 6 h. The whole cell extracts of untreated or TNFα-treated 32D/IκBα-SR cells were incubated with an antibody specific for the RelA/p65 subunit of NF-κB (p65) or with the corresponding preimmune serum. Immunoprecipitated proteins were analyzed by Western blotting with
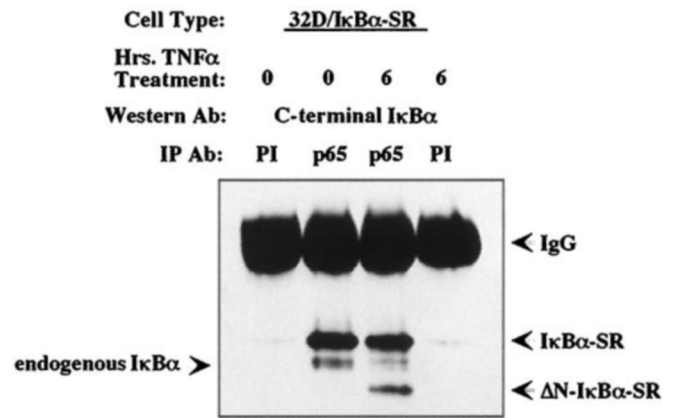
FIG. 4. **ΔN-IκBα-SR associates with the p65 subunit of NF-κB.** The lysate of 32D/IκBα-SR cells treated with TNFα for 0 or 6 h was incubated with preimmune serum (*PI*) or a carboxyl-terminal p65 antibody (p65). Immunoprecipitated proteins were eluted in sample buffer and analyzed by Western blotting using a carboxyl-terminal IκBα polyclonal antibody. Mobilities of the immunoglobulin heavy chain (IgG), endogenous IκBα, IκB-SR, and ΔN-IκBα-SR are indicated with *arrows*.

an antibody specific for the carboxyl terminus of IκBα. The p65 subunit of NF-κB interacted with IκBα-SR as well as with endogenous IκBα in untreated and in TNFα-treated 32D/IκBα-SR cells (Fig. 4, *lanes 2* and *3*). Interestingly, ΔN-IκBα-SR generated in response to TNFα treatment also associated with the p65 subunit of NF-κB (Fig. 4, *lane 3*). Therefore, ΔN-IκBα binds NF-κB *in vivo*.

ΔN-IκBα Is Resistant to Degradation by an Inducer of NF-κB Activation—In order to determine whether ΔN-IκBα is a constitutive inhibitor of NF-κB, we tested whether ΔN-IκBα is resistant to degradation by inducers of NF-κB. TNFα treatment of parental cells leads to the activation of NF-κB through the phosphorylation and degradation of IκBα. Activation of NF-κB leads to the transcription of IκBα, resulting in an autoregulatory loop that culminates in the reinhibition of NF-κB in the cytoplasm by newly synthesized IκBα (52, 53). The degradation and resynthesis of IκBα can be easily monitored through Western blotting as shown in Fig. 5. This analysis reveals that treatment of parental 32D cells with TNFα over time leads to the degradation and reaccumulation of IκBα (Fig. 5, *lanes 1–4*, *upper panel*). However, treatment of 32D cells with TNFα in the presence of the protein synthesis inhibitor cycloheximide (*CHX*) results in degradation of IκBα but does not allow the resynthesis of IκBα (Fig. 5, *lanes 10–13*, *upper panel*). When 32D cells are grown in the absence of IL-3, they undergo apoptosis and accumulate ΔN-IκBα (Fig. 1*C* and Fig. 5, *lanes 5* and *14*, *upper panel*). Treatment of IL-3-deprived cells with TNFα leads to the degradation and resynthesis of endogenous IκBα, but ΔN-IκBα protein levels are unaffected (Fig. 5, *lanes 5–9*, *upper panel*). In addition, TNFα treatment of CHX pretreated cells results in the degradation of endogenous IκBα without resynthesis of protein. However, ΔN-IκBα protein levels remain constant, indicating that the presence of ΔN-IκBα is not a result of degradation of newly synthesized IκBα (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 TNFα was suppressed in cells that had accumulated ΔN-IκBα following IL-3 withdrawal when compared with control cells (described below; Fig. 6). These results indicate that ΔN-IκBα cannot be degraded by inducers of NF-κB.

Release of NF-κB from IκBα in the cytoplasm relies on the phosphorylation of IκBα 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-IκBα may be unable to be ubiquitinated on lysines 21 and 22 because these sequences are

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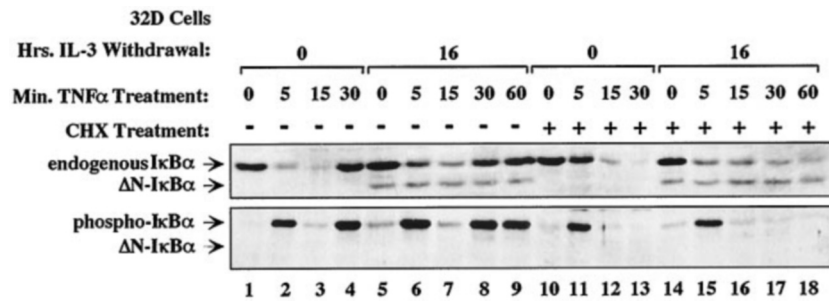

FIG. 5. **ΔN-IκBα is resistant to degradation induced by TNFα.** 32D cells were grown in the presence or absence of IL-3 for 15 h and subsequently treated with TNFα for 0, 5, 15, 30, or 60 min (*lanes 1–9*). Cell extracts were prepared by lysis in sample buffer and analyzed by Western blotting with a carboxyl-terminal IκBα antibody (*upper panel*) or a phosphoserine 32-specific IκBα antibody (*lower panel*). Cycloheximide was added 1 h prior to TNFα treatment (*lanes 10–18*). Mobilities of endogenous IκBα, ΔN-IκBα, and phospho-IκBα are indicated with *arrows*.

probably lost during cleavage of IκBα. Presumably, ΔN-IκBα is not degraded because recognition by the 26 S proteasome cannot occur. We were, however, interested in determining whether ΔN-IκBα can be inducibly phosphorylated on Ser-32 and Ser-36 *in vivo*. Western analysis was performed on 32D cell extracts with a phosphoserine 32-specific polyclonal antibody. Endogenous IκBα was strongly phosphorylated within 5 min following exposure to TNFα in the presence or absence of IL-3 (Fig. 5, *lanes 2*, *6*, *11*, and *15*, *lower panel*). Phosphorylated IκBα 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 IκBα was phosphorylated on Ser-32, indicating that IκB-kinase complex is still active at this time point. However, phosphorylation of ΔN-IκBα following TNFα 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 ΔN-IκBα to be phosphorylated by IκB-kinase complex, suggesting that the absence of these sequences may lead to the loss of a potential recognition site for IκB-kinase complex. It is also possible that the phosphoserine 32-specific antibody cannot recognize phosphoserine 32 in ΔN-IκBα in the absence of the 5′-flanking sequences. Nevertheless, ΔN-IκBα is not degraded following stimulation by an inducer of NF-κB, 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, ΔN-IκBα is resistant to phosphorylation-induced degradation by an inducer of NF-κB.

ΔN-IκBα Prevents NF-κB Activation—The generation of ΔN-IκBα during apoptotic conditions, its association with the p65 subunit of NF-κB, and its resistance to degradation by inducers of NF-κB imply that ΔN-IκBα is an inhibitor of NF-κB. To verify that the amino-terminal truncation of IκBα produces a dominant inhibitory molecule, we tested whether endogenous ΔN-IκBα could suppress the activation of NF-κB. NF-κB 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 ΔN-IκBα and were subsequently treated with TNFα to induce the nuclear translocation of NF-κB. EMSAs performed with these nuclear extracts revealed that the amount of TNFα-induced NF-κB DNA binding is decreased in cells grown in the absence of IL-3 (Fig. 6*A*, *lanes 1–4*) when compared with control cells grown in the presence of IL-3 (Fig. 6*A*, *lanes 5–9*). Since IL-3 withdrawal leads to the accumulation of ΔN-IκBα (see Figs. 1*C* and 5), these data suggest that the presence of ΔN-IκBα suppresses the activation of NF-κB. Although TNFα-induced NF-κB DNA binding was decreased in the presence of ΔN-IκBα (Fig. 6*A*, *lanes 5–9*), it was not completely abolished. This is probably due to the fact that not all of these cells contain ΔN-IκBα (see Fig. 5) and that NF-κB acti-

vation can only be suppressed in these cells.

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

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

The presence of ΔN-IκBα in cells may also cause increased sensitivity to cellular death through the inhibition of NF-κB. Therefore, 293T cells were transiently transfected with pCDNA3 or pCDNA3-ΔN-IκBα and subsequently treated with TNFα. Consistent with the known antiapoptotic function of NF-κB, TNFα treatment led to apoptosis in cells expressing ΔN-IκBα, 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 IκBα to generate ΔN-IκBα. Specifically, we have shown that caspase-3 or a DEVD-directed caspase leads to the cleavage of IκBα under TNFα and cytokine deprivation-induced apoptosis. The cleavage of IκBα does not appear to be absolutely essential for apoptosis, because p65^{−/−}

A, 32D cells
B, 293T Cells
C
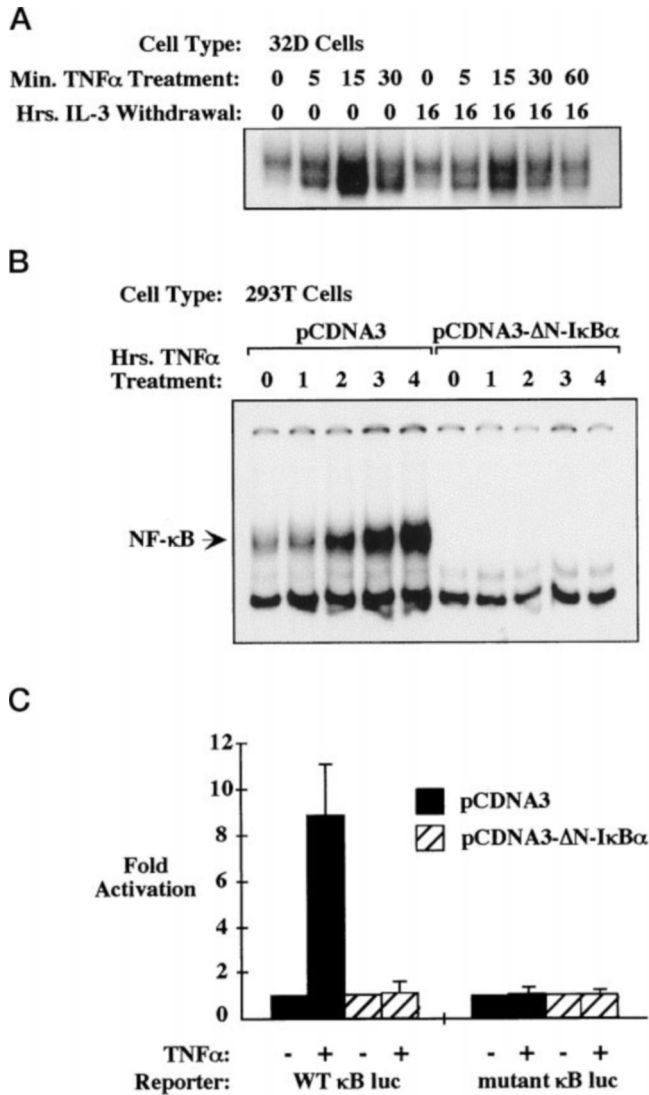
FIG. 6. **ΔN-IκBα suppresses NF-κB activity.** *A*, 32D cells were grown in the presence or absence of IL-3 for 15 h and subsequently treated with TNFα for 0, 5, 15, 30, or 60 min. EMSA was performed utilizing the nuclear extracts from these cells and an oligonucleotide probe containing a NF-κB binding site. *B*, 293T cells were transiently co-transfected with pCDNA3 empty vector or with a pCDNA3-ΔN-IκBα expression vector. Nuclear extracts were prepared from these cells following treatment with TNFα for 0, 1, 2, 3, or 4 h. EMSA was performed using these nuclear extracts as described for *A*. The NF-κB binding complex is indicated with an *arrow*. *C*, ΔN-IκBα suppresses NF-κB-dependent gene expression. Luciferase reporter assays were performed with cell extracts from 293T cells co-transfected with a NF-κB luciferase reporter (WT κB luc) or a mutant NF-κB reporter (mutant κB luc) and pCDNA3 empty vector or pCDNA3-ΔN-IκBα expression vector. Cells were treated with TNFα for 8 h. The data shown are from a representative experiment of three experiments, each performed in triplicate. Fold activation is indicated ± S.D.

cells expressing IκBα-D31A undergo apoptosis upon TNFα treatment although IκBα-D31A is not appreciably cleaved. Similarly, apoptosis induced in v-Rel-transformed cells leads to the appearance of a truncated form of chicken IκBα whose appearance can be blocked with CrmA (an inhibitor of caspases) without affecting apoptosis in these cells (54–56).

The cleavage of IκBα may facilitate the process of apoptosis, since we have shown that ΔN-IκBα acts as a constitutive inhibitor of NF-κB activation. Research from our laboratory and others have shown that inhibition of NF-κB sensitizes cells to killing by various stimuli including TNFα and cancer therapy drugs (17–20) and also induces apoptosis in B cells (40). NF-κB

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-radiation-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.* p65, 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-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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