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# Caspase Cleavage Product Lacking Amino-Terminus of IκBα Sensitizes Resistant Cells to TNF-α and TRAIL-Induced Apoptosis

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**Abstract** In response to a diverse array of signals, IκBα is targeted for phosphorylation-dependent degradation by the proteasome, thereby activating NF-κB. Here we demonstrate a role of the cleavage product of IκBα in various death signals. During apoptosis of NIH3T3, Jurkat, Rat-1, and L929 cells exposed to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), Fas, serum deprivation, or TNF-α, respectively, IκBα was cleaved in a caspase-dependent manner. In vitro and in vivo cleavage assays and site-directed mutagenesis showed that caspase-3 cleaved IκBα between Asp31 and Ser32. Expression of the cleavage product lacking amino-terminus (1–31), ΔIκBα, sensitized otherwise resistant NIH3T3 fibroblast cells to apoptosis induced by TNF-α or TRAIL, and HeLa tumor cells to TNF-α. ΔIκBα was more pro-apoptotic compared to wild type or cleavage-resistant (D31E)IκBα mutant and the sensitization elicited by ΔIκBα was as effective as that by the dominant negative mutant, (S32,36A)IκBα, in NIH3T3 cells. ΔIκBα suppressed the transactivation of NF-κB induced by TNF-α or TRAIL, as reflected by luciferase-reporter activity. Conversely, expression of the p65 subunit of NF-κB suppressed TNF-α-, TRAIL-, and serum deprivation-induced cell death. On the contrary, ΔIκBα was less effective at increasing the death rate of HeLa cells that were already sensitive to death signals including TRAIL, etoposide, or taxol. These results suggest that ΔIκBα generated by various death signals sensitizes cells to apoptosis by suppressing NF-κB activity. J. Cell. Biochem. 85: 334–345, 2002. 

**Key words:** IκBα; apoptosis; caspase; TNF-α; TRAIL

Programmed cell death or apoptosis is crucially involved in processes of development, tissue homeostasis, and disease [Kerr et al., 1994; Schul et al., 1999; Yuan and Yankner, 2000]. Caspases have been shown to play a critical role in apoptosis of both cell culture and animal models. For instance, caspase inhibitors including both peptide inhibitors and CrmA, a serpin encoded by the cowpox virus [Ray et al., 1992; Komiyama et al., 1994], suppress programmed cell death induced by anti-Fas Ab [Enari et al., 1995; Tewari and Dixit, 1995], TNF-α [Miura et al., 1995], and growth factor deprivation [Milligan et al., 1995; Garland and Halestrap, 1997]. Moreover, caspase (–/–) mutant mice exhibit defective tissue development resulting from suppression of apoptosis [Kuida et al., 1996, 1998; Varfolomeev et al., 1998; Nakagawa et al., 2000].

The members of caspase family include at least 14 isoforms [Alnemri et al., 1996; Ahmad et al., 1998; Humke et al., 1998; Thornberry and lazebnik, 1998; Nicholson, 1999]. They are known to be synthesized as inactive zymogens and are activated through proteolysis when cells receive apoptotic signals. Dozens of proteins have been identified as substrates for caspases [Cryns and Yuan, 1998; Nunez et al., 1998; Thornberry and Lazebnik, 1998; Nicholson, 1999; Utz and Anderson, 2000]. All caspases cleave protein substrates at the C-terminus side

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of aspartic acids, and individual caspases also show distinct substrate specificity [Nicholson and Thornberry, 1997]. Overall, however, the caspase cascade and critical caspase substrates remain largely uncharacterized, although it is likely that in many cell types, signal-specific caspases are activated sequentially or in parallel to execute cell death.

The NF-κB family of transcription factors plays essential roles in apoptosis and immune responses. Heterodimeric NF-κB [p50/RelA (p65)] family proteins are sequestered in the cytoplasm through their association with IκB proteins (e.g., IκBα, a conserved family of proteins that act as inhibitors of NF-κB [Gilmore and Morin, 1993]). In response to a variety of signals including tumor necrosis factor (TNF)-α and TNF-related apoptosis-inducing ligand (TRAIL), IκBα is phosphorylated at $Ser^{32}$ and $Ser^{36}$, which marks it for degradation by the proteasome [Brown et al., 1995; Chen et al., 1995; Finco and Baldwin, 1995; Traenckner et al., 1995; Baldi et al., 1996; Hu et al., 1999]. The resultant free NF-κB can then enter into the nucleus and affect gene transcription. Fibroblasts from p65 (−/−) mutant mouse embryos are sensitive to death induced by several forms of stimuli including TNF-α, ionizing radiation, and cancer chemotherapeutic agents [Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996]. More recent evidence shows that NF-κB controls expression of a number of genes involved in inhibition of cell death [Chu et al., 1997; Wang et al., 1998; Stehlik et al., 1999].

The fact that TNF-α generates at least two conflicting signals in certain types of tumor cells, a proapoptotic signal through caspases and a pro-survival signal through activation of NF-κB, prompted us to examine the interaction between NF-κB and caspases. IκBα was previously reported to be cleaved by caspase or calpain [Barkett et al., 1997; Han et al., 1999; Reuther and Baldwin, 1999]. We report here that caspase-mediated cleavage product of IκBα lacking the amino-terminal region is generated during apoptosis induced by various signals. Moreover, expression of the caspase cleavage product of IκBα sensitized otherwise resistant cells to receptor-mediated apoptosis. Based on these observations, we suggest that an amino-terminal cleavage product of IκBα acts as a potent dominant inhibitor of NF-κB to facilitate the death process.

## MATERIALS AND METHODS

### Reagents

The caspase peptide inhibitors, z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), z-Asp-Glu-Val-Asp-fmk (DEVD-fmk), and z-Ile-Glu-Thr-Asp-fmk (IETD-fmk) were purchased from Enzyme Systems Products (Livermore, CA); calpeptin and MG132 were obtained from CALBIOCHEM (Darmstadt, Germany); anti-IκBα Abs (C-21, C-15) and anti-α-tubulin Ab were from Santa Cruz (Santa Cruz, CA) and Sigma Chemical (St. Louis, MO), respectively. Fas ligand (7C11) was from MBL (Nagoya, Japan); TRAIL was a gift from Genentech, Inc. (San Francisco, CA); etoposide (VP-16), okadaic acid, taxol, and luciferase assay system were from Sigma (St. Louis, MO) and Promega (Madison, WI), respectively. All other molecular biology grade reagents were from Sigma or New England Biolabs (Hertfordshire, England).

### Plasmid Constructs

Full-length cDNA encoding human IκBα was cloned into the *EcoR*I site of the eukaryotic expression vector pcDNA3 (Invitrogen, Carlsbad, CA). pcDNA3-Flag-(S32,36A)IκBα was constructed by subcloning 1.5 kb of the *Kpn*I and *Xba*I fragments of pCMV4-Flag-(S32,36A)IκBα into pcDNA3. The N-terminal deletion mutant of IκBα encoding amino acids from 32 to 317 (ΔIκBα) was amplified by PCR using synthetic oligonucleotides (5′-GCGGA TCCAT GAGCG GCCTG GACTCC-3′ and 5′-CATTT AGGTG ACACTA-3′) as primers and inserted into the *BamH*I and *Xba*I sites of pcDNA3. pcDNA3-Flag-(D31E)IκBα [p(D31E)IκBα] and pcDNA3-Flag-(S32A)IκBα [p(S32A)IκBα] were established by site-directed mutagenesis using a protocol modified from the overlapping extension PCR method [Higuchi et al., 1998]. PCR was performed using the following synthetic oligonucleotides as primers. For p(D31E)IκBα: 5′-AATAC GACTC ACTAT AGGGA-3′; 5′-GGAGT CCAGG CCGCT CTCGT GGCGG TCGTCC-3′; 5′-GGACG ACCGC CACGA GAGCG GCCTG GACTCC-3′; and 5′-CATTT AGGTG ACACTA-3′. For p(S32A)IκBα: 5′-AATAC GACTCA CTATA GGGA-3′; 5′-ATGGA GTCCA GGCCG GCGTC GTGGC GGTCGTC-3′; and 5′-GACGA CCGCC ACGAC GCCGG CCTGG ACTCCAT-3′. The p65 subunit of NF-κB was inserted into pGL plasmid (Promega). Bacterial expression plasmid of caspase-3

was previously described by Chung et al. [2001].

### In Vitro Caspase Cleavage Assays

Bacterial plasmids expressing caspases were transformed into BL21(DE3) and incubated with 0.2 mM IPTG for 3 h, harvested, and lyzed by sonication in a buffer containing 0.05% Nonidet P-40, 20 mM HEPES (pH 7.4), and 100 mM NaCl. The lysates were cleared by centrifugation, and the protein concentration was determined with a Bio-Rad protein assay. The pIκBα, p(D31E)IκBα, p(S32A)IκBα, and p(S32,36A)IκBα plasmids were transcribed and translated in vitro using TNT® Systems (Promega) in the presence of T7 RNA polymerase and $^{35}$S-methionine. In vitro caspase cleavage reactions were carried out in a buffer containing 0.5% Nonidet P-40, 20 mM HEPES (pH 7.4), 100 mM NaCl, and 20 mM dithiothreitol for 30 min at 30°C. Cleavage products were separated by SDS–PAGE and exposed to X-ray film.

### Cell Culture, DNA Transfection

Rat-1, Rat-1/CrmA (Rat-1 cells expressing *CrmA*), NIH3T3, HeLa, and COS-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented by 100 U/ml penicillin-G, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) (Biofluids: Rockville, MD). Jurkat and L929 cells were maintained in RPMI1640 with 10% FBS. DNA transfection was performed using LipofectAMINE PLUS™ Reagent following the protocol from Gibco-BRL (Grand Island, NY).

### Western Blot Analysis

Western blot analysis was performed as described by Jung et al. [1996]. Briefly, cells were lyzed in buffer containing 60 mM Tris-Cl (pH 6.8), 1% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.5% β-mercaptoethanol. The lysates were separated by electrophoresis on polyacrylamide gels and then transferred to PVDF membranes (Bio-Rad) using Semi-Dry Transfer system (Bio-Rad). The membranes were treated overnight at 4°C with TBST blocking buffer (20 mM Tris-Cl at pH 7.5, 150 mM NaCl, 0.2% Tween-20) containing 5% nonfat dried milk, incubated with primary Ab (1:500–5,000 dilution), washed, and incubated with horse-radish peroxidase-conjugated secondary Ab (1:5,000 dilution). Proteins were visualized on X-ray film using Enhanced Chemiluminescence (ECL™, Amersham).

### Apoptosis Assay

Cells were incubated with soluble Fas ligand (7C11), TNF-α, TRAIL, okadaic acid, cadmium, etoposide, and taxol for the indicated times. Rat-1 and Rat-1/CrmA cells were incubated in serum-free medium after washing three times with DMEM and cell viability was determined with trypan exclusion assay. To determine viability of cells transfected with plasmids, cells were initially cotransfected with pβactgal and effector plasmids at a ratio of 1:3. After 24 h, the cells were fixed for 10 min in 1% glutaraldehyde, rinsed three times with PBS, and then stained in β-galactosidase (X-gal) reaction buffer (0.5 mg/ml 5-bromo-4-chloro-3-indolyl β-galactoside, 3 mM $K_3Fe(CN)_6$-$3H_2O$, 3 mM $K_4Fe(CN)_6$-$3H_2O$, 1 mM $MgCl_2$, 10 mM KCl, and 0.1% Triton X-100 in 0.1 M sodium phosphate buffer, pH 7.5) for 6 h at 37°C. Cell viability was determined based on cell morphology of X-gal positive cells. Round cells were eventually detached from the culture plate and were counted as dead cells.

### NF-κB Activity Assays: Luciferase and β-Galactosidase Assays

Cells were collected and lyzed in 100 μl of lysis buffer containing 25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% glycerol, and 1% Triton X-100. After spinning briefly, 50 μl aliquots of supernatant were mixed with 50 μl of luciferase assay reagent, and luciferase activity was measured using a luminometer (Lumat LB9501, Berthold, Germany). For the β-galactosidase assay, cell lysates were incubated for 30 min at 37°C with β-galactosidase assay buffer containing 100 mM sodium phosphate (pH 7.3), 1 mM $MgCl_2$, 50 mM β-mercaptoethanol, and 0.66 mg/ml ONPG. Absorbance at 420 nm was then measured using a Microplate reader (Bio-Rad).

### Statistical Analysis

All results are presented as means ± SD of n independent experiments. Student's *t*-test was used and *P* values smaller than 0.05 were considered significant. Asterisks indicate the statistical significance with respect to the other (Φ).

## RESULTS

### IκBα is Cleaved by Caspase in a Variety of Cell Types During Apoptosis

Figure 1A shows that consistent with our earlier findings [Jung et al., 1996], when Rat-1 fibroblast cells were incubated with serum-free medium, they underwent apoptosis that was blocked in the presence of 5 μg/ml insulin or 0.1 μM dexamethasone (DEX), a synthetic glucocorticoid hormone. Western blot analysis showed that IκBα was cleaved in serum-deprived (SD) cells exhibiting a 30% rate of viability, which yielded a cleavage product designated as ΔIκBα. Treatment of the cells with insulin or DEX suppressed SD-induced cleavage of IκBα. The cleavage of IκBα to ΔIκBα was also observed in Jurkat cells incubated with soluble Fas ligand (7C11) (Fig. 1B) and in L929 cells exposed to TNF-α (Fig. 1C, left panel). NIH3T3 fibroblast cells, otherwise resistant to TNF-α or TRAIL alone, became sensitive to TNF-α or TRAIL-induced apoptosis after being exposed to low concentration of okadaic acid (Fig. 1C, right panel) or cadmium (Fig. 1D), generating ΔIκBα during apoptosis. These results show that a distinct cleavage of IκBα is induced during apoptosis by various signals.

The protease responsible for cleaving IκBα during apoptosis was characterized in Rat-1 and Jurkat cells. Expression of *CrmA* in Rat-1 cells (Fig. 1A) or preincubation of Jurkat cells with the caspase inhibitors, zVAD-fmk (a pan caspase inhibitor), DEVD-fmk (caspase-3 inhibitor), and IETD-fmk (caspase-8 inhibitor), suppressed cleavage of IκBα (Fig. 1B). On the other hand, calpeptin (calpain inhibitor) and MG132 (a proteasome inhibitor) did not affect the cleavage of IκBα (Fig. 1B). These results indicate that IκBα is cleaved by a caspase or a caspase-downstream protease.

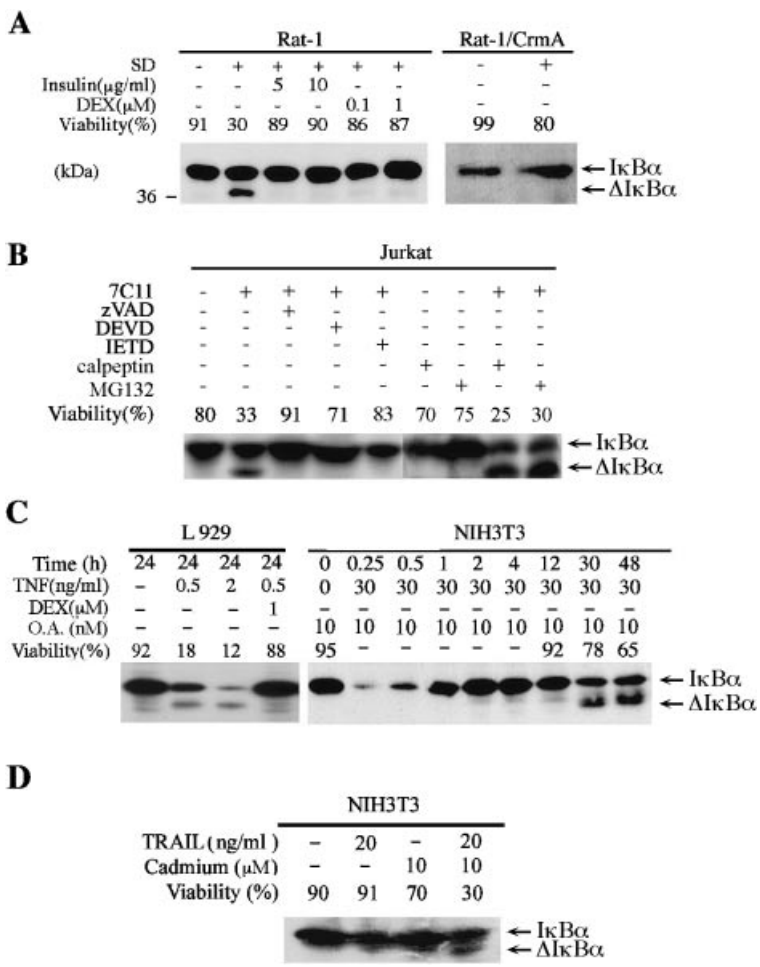


**Fig. 1.** IκBα is cleaved by caspase during apoptosis induced by various death signals. **A**: Serum-deprivation (SD)-induced apoptosis. Rat-1 and Rat-1/CrmA cells were incubated for 24 h in serum-free medium in the presence or absence of either insulin (5 and 10 μg/ml) or dexamethasone (DEX, 0.1 and 1 μM). Cell viability was determined with Trypan Blue exclusion assay and rates of viability (%) are shown above the respective lanes. Cell lysates were then analyzed by Western blot using anti-IκBα Ab (C-21). ΔIκBα denotes the cleavage products of IκBα. **B**: Fas-mediated apoptosis. Jurkat cells were preincubated with zVAD-fmk (100 μM), DEVD-fmk (100 μM), IETD-fmk (100 μM), calpeptin (20 μg/ml), or MG132 (1 μM) for 2 h and then exposed to soluble Fas ligand (7C11) for 8 h. **C**: TNF-α-induced apoptosis. L929 (**left panel**) and NIH3T3 (**right panel**) cells were incubated for the indicated times with TNF-α in the presence or absence of okadaic acid (OA, 10 nM). **D**: TRAIL-induced apoptosis. NIH3T3 cells were exposed to TRAIL for 24 h in the presence of cadmium (10 μM).

### Asp31 of IκBα is Cleaved in Apoptotic Cells

The anti-IκBα Ab (C-21) used in Figure 1 recognized the carboxyl-terminus of IκBα. On the contrary, Western blot analysis using anti-IκBα Ab (C-15) recognizing the amino-terminus of the human IκBα failed to detect the ΔIκBα in dying cells (Fig. 2C), suggesting that the cleavage site must be in the amino-terminus that contains a putative cleavage site: 28Asp-Arg-His-**Asp**-Ser-Gly-Leu-Asp-Ser36 [Nicholson and Thornberry, 1997]. In vitro cleavage assays showed that the (D31E)IκBα mutant, in which Asp31 was substituted by Glu, was resistant to cleavage by caspase-3 (Fig. 2A). In contrast, the (S32A)IκBα and (S32,36A)IκBα mutants, which contain mutations (Ser to Ala) at the signal-induced phosphorylation sites, remained subject to cleavage by caspase-3, consistent with previous reports [Barkett et al., 1997; Han et al., 1999; Reuther and Baldwin, 1999]. ΔIκBα generated in Jurkat cells undergoing apoptosis co-migrated with the cleavage product of IκBα generated by caspase-3 in vitro (Fig. 2B). To confirm the cleavage in vivo, COS-7 cells were transiently transfected with pIκBα or p(D31E)IκBα mutant and then exposed to okadaic acid (Fig. 2D). Western blot analysis showed that ΔIκBα was detected in apoptotic cells expressing IκBα (Fig. 2D, left panel) but not in cells expressing (D31E)IκBα mutant (Fig. 2D, right panel). These results indicate that during apoptosis, caspase-3 cleaves IκBα between Asp31 and Ser32.

### Overexpression of ΔIκBα Sensitizes NIH3T3 Fibroblast Cells to TNF-α- and TRAIL-Induced Apoptosis

To characterize a role of ΔIκBα, NIH3T3 embryo fibroblast cells, which are resistant to TNF-α and TRAIL, were transiently transfected with pIκBα, pΔIκBα, p65NF-κB, or

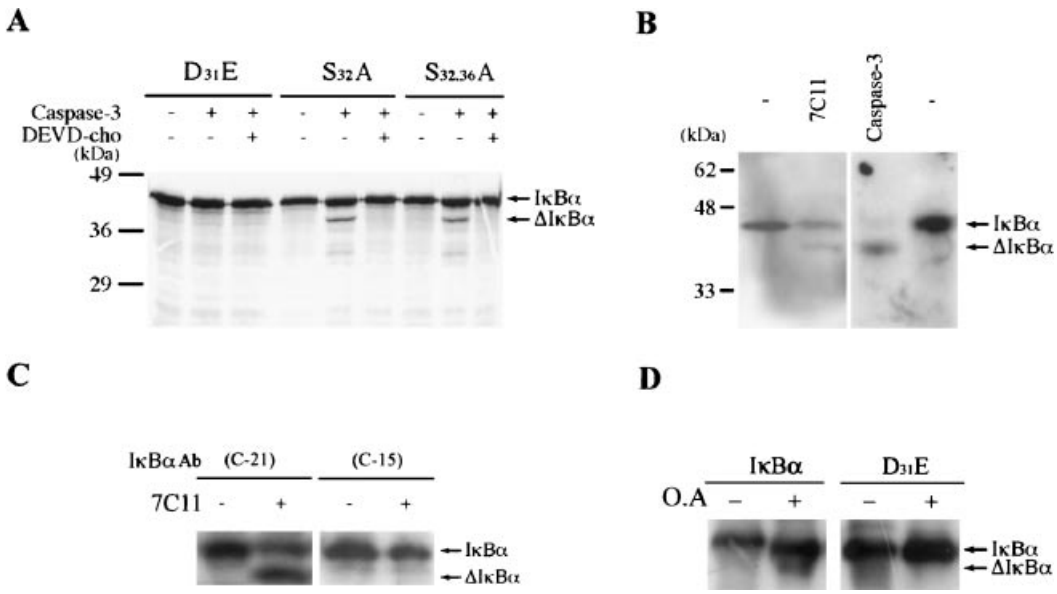


**Fig. 2.** Caspase-3 cleaves IκBα at Asp31 in vitro and in vivo. **A**: Asp31 of IκBα is the cleavage site of caspase-3. Site-directed mutagenesis was used to substitute Asp31, Ser32, or Ser32/Ser36 of IκBα with Glu, Ala, or Ala/Ala, respectively, generating (D31E)IκBα, (S32A)IκBα, and (S32,36A)IκBα. These IκBα mutants translated in vitro in the presence of $^{35}$S-methionine were incubated with 4 μl (20–25 μg) of caspase-3 extract. In some experiments, IκBα mutants were incubated with caspase-3 in the presence of 100 nM DEVD-cho. The reaction mixtures were then analyzed by SDS–PAGE followed by autoradiography. **B**: In vitro cleavage product of IκBα by caspase-3 co-migrates with the ΔIκBα generated in apoptotic cells. In vitro caspase-3 cleavage assay of IκBα was performed as in Figure 2A. Reaction mixtures were then analyzed by SDS–PAGE followed by autoradiography (**right panel**). Jurkat cells were exposed to 7C11 for 8 h, and cell lysates were then analyzed by Western blot using anti-IκBα Ab (C-21) (**left panel**). **C**: Generation of ΔIκBα by cleavage in the amino-terminus of IκBα. Jurkat cells exposed to 7C11 for 8 h were analyzed for the cleavage of the endogenous IκBα with Western blot using anti-IκBα Abs (C-21) and (C-15) recognizing the carboxyl-terminus (**left panel**) and the amino-terminus (**right panel**) of IκBα, respectively. **D**: In vivo cleavage assay of IκBα and (D31E)IκBα. COS-7 cells were transfected with IκBα (**left panel**) or (D31E)IκBα mutant (**right panel**). After 24 h, cells were treated for 24 h with okadaic acid (OA, 50 nM) and then analyzed for the cleavage of the transfected IκBα with Western blot.

p(S32,36A)IκBα, and then exposed to TNF-α. Western blot analysis showed that expression levels of the exogenous IκBα in the transfected cells were similar (Fig. 3A). After being exposed to TNF-α, dying cells shrank in size and eventually detached from the culture plate (Fig. 3B). Determination of cell viability showed that expression of ΔIκBα increased death rates of NIH3T3 cells exposed to TNF-α compared to death rate observed in wild-type IκBα-expressing cells. The effects of ΔIκBα on cell death were further examined in TRAIL-signaling. While expression of IκBα, (D31E)IκBα, or (S32A)IκBα showed little effects on TRAIL-induced apoptosis (Fig. 3C), expression of ΔIκBα increased death rates by twofold (11–22%), which was equivalent to that of the increase observed in cells expressing (S32,36A)IκBα. These results indicate that ΔIκBα sensitizes NIH3T3 cells to TNF-α and TRAIL-mediated apoptosis.

### ΔIκBα Inhibits NF-κB Activation Induced by TNF-α and TRAIL in NIH3T3 Cells

TNF-α, TRAIL, and ionizing radiation have all been found to activate NF-κB via ubiquitination-mediated degradation of IκBα. To address whether ΔIκBα affected NF-κB activity, NIH3T3 cells were cotransfected with a luciferase reporter gene under the control of the NF-κB regulatory element, designated as pNF-κB-luc, and various NF-κB expression plasmids including pΔIκBα (Fig. 4). NF-κB activity assays showed that NF-κB activity, as reflected by luciferase reporter activity, was suppressed by the expression of IκBα, (D31E)IκBα, or (S32A)IκBα, and further decreased by ΔIκBα and (S32,36A)IκBα (Fig. 4, left panel), showing that ΔIκBα inhibited TNF-α-induced activation of NF-κB as effectively as the dominant negative mutant. The suppressive effects of ΔIκBα on the TRAIL-induced activation of NF-κB was also observed in NIH3T3 cells transfected with ΔIκBα and subsequently exposed to TRAIL (Fig. 4, right panel).

### Expression of p65NF-κB Overcomes Cell Death

If ΔIκBα generated by caspase in apoptotic NIH3T3 cells increases apoptosis by inhibiting NF-κB activity, overexpression of the p65 subunit of NF-κB may antagonize TNF-α- and TRAIL-induced apoptosis by overcoming the inhibitory effects of ΔIκBα on NF-κB activity. NIH3T3 cells were therefore transfected with

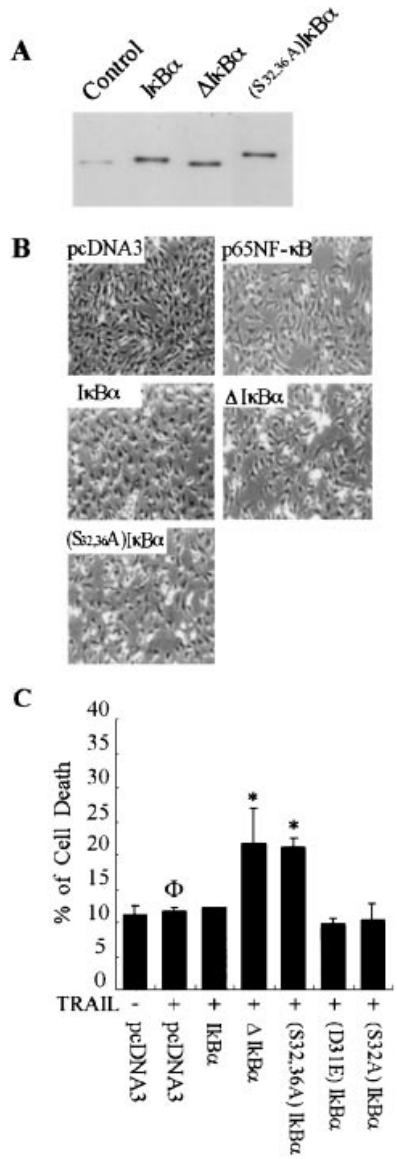


**Fig. 3.** Expression of ΔIκBα sensitizes NIH3T3 cells to TNF-α and TRAIL. **A**: NIH3T3 cells were transfected with pcDNA3 or expression plasmids encoding either p65NF-κB, IκBα, N-terminal deletion Δ(1-31)IκBα (ΔIκBα), or dominant negative Flag-(S32,36A)IκBα. After 24 h, expression level of the exogenous IκBα was examined with Western blot analysis using anti-IκBα Ab (C-21), or (**B**) the transfectants were exposed to 30 ng/ml of murine TNF-α for an additional 12 h, and morphological profiles of cells undergoing apoptosis were observed under phase contrast microscopy and indicated by arrows. **C**: NIH3T3 cells transfected with the indicated expression plasmids were exposed to 20 ng/ml TRAIL for 24 h and then incubated with X-gal reaction buffer for 6 h at 37°C. Cell viability was assessed based on the morphology of X-gal-positive cells. Death rates are expressed as the percent (%) apoptotic cells among the total number of cells. The data shown are the means ± SD from at least three independent experiments.

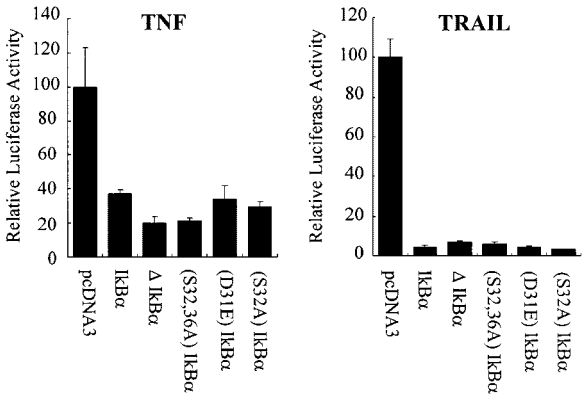


**Fig. 4.** ΔIκBα suppresses transactivation of NF-κB. NIH3T3 cells were cotransfected with 0.1 μg of luciferase reporter plasmid containing NF-κB binding element (pNF-κB-luc) and 0.5 μg of either pcDNA3, pIκBα, pΔIκBα, p(S32,36A)IκBα, p(D31E)IκBα, or p(S32A)IκBα. pβactgal (0.3 μg) was included in every transfection as an internal control. After 24 h, cells were treated with murine TNF-α (30 ng/ml) (**left panel**) or TRAIL (20 ng/ml) (**right panel**) for 8 h, and luciferase activity was then measured. The luciferase activity of control (pcDNA3) was arbitrarily set to a value of 100. Other values are expressed as percentages of the control cells and represented as means ± SD.

p65NF-κB and then exposed to TNF-α or TRAIL in the presence of cadmium and okadaic acid, respectively (Fig. 5A). Cell viability assays showed that NIH3T3 cells overexpressing p65NF-κB became resistant to apoptosis. Similarly, Rat-1 cells expressing exogenous p65NF-κB became resistant to serum deprivation-induced cell death (Fig. 5B). These results suggest that the pro-apoptotic effects of ΔIκBα result from its inhibition of NF-κB activity.

## Overexpression of ΔIκBα Sensitizes HeLa Cervical Carcinoma Cells to TNF-α in the Absence of Protein Synthesis Inhibitor

We then examined the sensitization effects of ΔIκBα on tumor cells. HeLa cells are derived from human cervical carcinoma cells and are resistant to TNF-α in the absence of cycloheximide or actinomycin D. HeLa cells were transfected with various IκBα expression plasmids and then incubated with 30 ng/ml TNF-α (Fig. 6A). Determination of cell viability showed that, while TNF-α alone exerted only marginal effects on death rates of control cells (pcDNA3: 19–26% cell death) or cells expressing IκBα (25% cell death), ΔIκBα increased death rates to 52%, indicating that ΔIκBα sensitized these tumor cells to TNF-α-induced cell death. Similarly, (S32,36A)IκBα mutant, but not the (D31E)IκBα and (S32A)IκBα mutants, effectively increased death rates of HeLa cells (51% cell death). These results showed that ΔIκBα functioned to sensitize HeLa cells to apoptosis as effectively as the dominant-negative IκBα mutant.

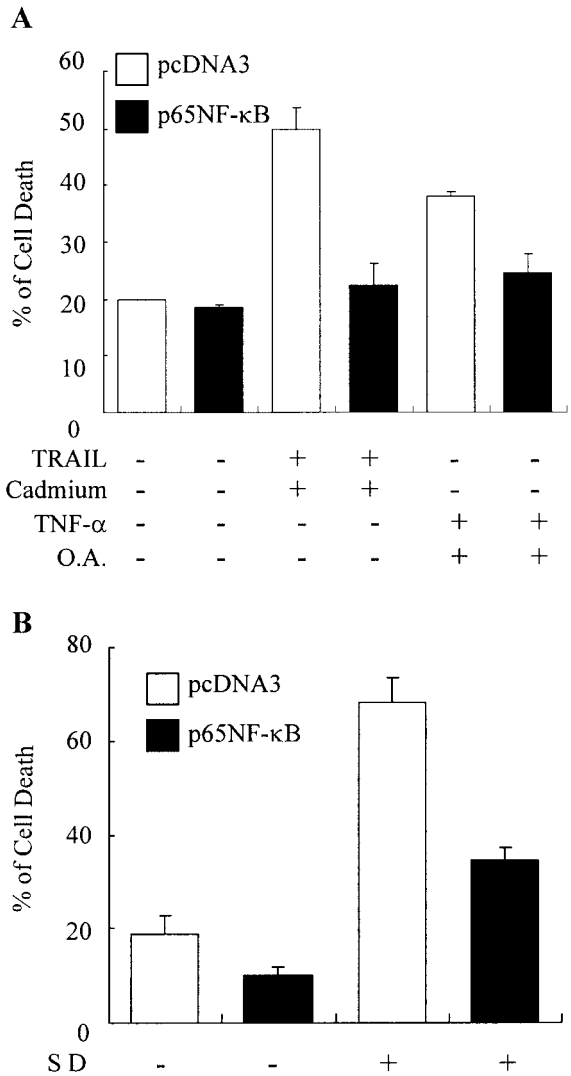


**Fig. 5.** Overexpression of p65 NF-κB overcomes cell death induced by TRAIL, TNF-α, or serum deprivation in NIH3T3 (**A**) and Rat-1 cells (**B**), respectively. NIH 3T3 and Rat-1 cells were transfected at a 1:3 ratio with pβactgal and either pcDNA3 or p65NF-κB. After 24 h, the cells were incubated with TNF-α (30 ng/ml)/okadaic acid (OA, 40 nM) for 36 h, TRAIL (20 ng/ml)/cadmium (10 μM) for 20 h, or in serum-free medium for 6 h. Cell viability (% of cell death) was assessed after X-gal staining as described in Materials and Methods. Data are expressed as means ± SD.

## ΔIκBα Partially Promotes Apoptosis Induced by TRAIL, Etoposide, and Taxol in HeLa Cells

TRAIL is a member of the TNF family and induces apoptosis mainly in tumor-derived cell

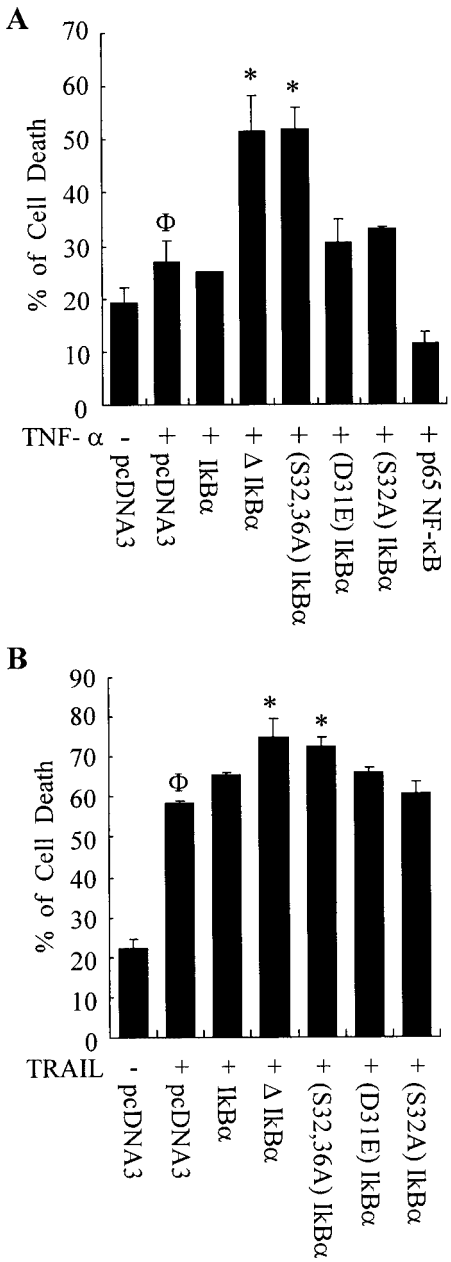


**Fig. 6.** Overexpression of ΔIκBα sensitizes HeLa cells to TNF-α and partially promotes the TRAIL-induced cell death. HeLa cells were cotransfected with pβactgal along with pcDNA3 or expression plasmids encoding either IκBα, ΔIκBα, (S32,36A) IκBα, (D31E)IκBα, or (S32A)IκBα. After 24 h, cells were exposed to 30 ng/ml human TNF-α for an additional 24 h (**A**) and 20 ng/ml TRAIL for 3 h (**B**). Cell viability was assessed based on the morphology of X-gal-positive cells. Death rates are expressed as the percentage of apoptotic cells among the total blue cells.

lines including HeLa cells. TRAIL itself induced apoptosis in 59% of HeLa cells (Fig. 6B) and unlike TNF-α, expression of ΔIκBα and (S32,36A)IκBα mutants marginally enhanced the death rate of HeLa cells exposed to TRAIL (75% cell death) (Fig. 6B). Thus, these results indicate that the sensitization effects of ΔIκBα on cell death may be signal-specific.

To examine a correlation between the selective sensitization effects of ΔIκBα on receptor-mediated death signals and the activation of NF-κB in HeLa cells, the effects of ΔIκBα on NF-κB activity were addressed. Co-expression with pNF-κB-luc and various pIκBα expression plasmids and subsequent exposure to TNF-α (Fig. 7, left panel) or TRAIL (Fig. 7, right panel) showed that NF-κB activation induced by TNF-α or TRAIL was suppressed by the expression of ΔIκBα as much as (S32,36A)IκBα. However, efficient suppression of NF-κB activation by (D31E)IκBα and (S32A)IκBα in HeLa cells indicates that the pro-apoptotic activity of ΔIκBα may not always be tightly associated with its inhibitory effects on NF-κB activity.

It was known that the induction of apoptosis by etoposide was accompanied by a transient stimulation of AP-1 and NF-κB binding activity [Bessho et al., 1994; Kim and Beck, 1994]. Thus, we further examined the effects of ΔIκBα on apoptosis of HeLa cells triggered by other non-receptor signals such as etoposide (Fig. 8A) and taxol (Fig. 8B). Expression of ΔIκBα partially but significantly enhanced apoptosis induced by etoposide or taxol compared to IκBα, indicating that ΔIκBα promotes apoptosis of HeLa cells exposed to the non-receptor death signals as well as receptor signals.

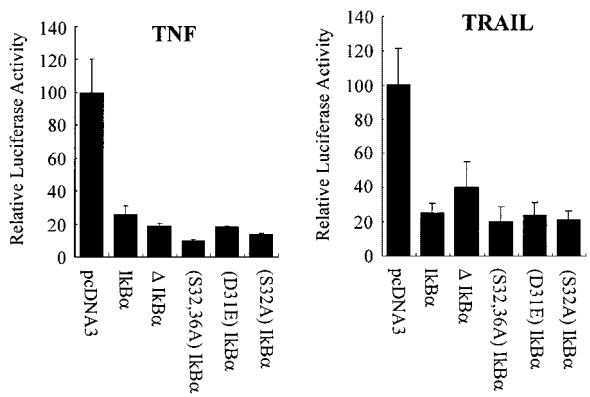


**Fig. 7.** ΔIκBα suppresses TNF-α and TRAIL-mediated transactivation of NF-κB in HeLa cells. HeLa cells were cotransfected with pNF-κB-luc and the indicated effector plasmids. After 24 h, cells were treated for 8 h with TNF-α (30 ng/ml) (**left panel**) or TRAIL (20 ng/ml) for 2 h (**right panel**). The luciferase activity of control (pcDNA3) was arbitrarily set to a value of 100, and other values (means ± SD) are expressed as percentages of the control cells.

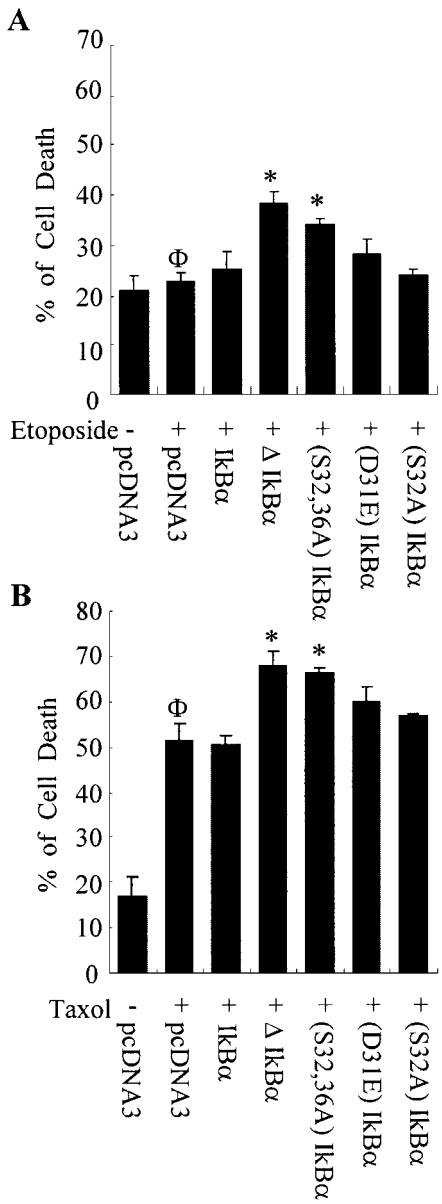


**Fig. 8.** ΔIκBα promotes apoptosis induced by etoposide and taxol. HeLa cells were transfected with the indicated expression plasmids. After 24 h, the transfectants were exposed to etoposide (100 μM) for an additional 30 h (**A**) or taxol (4 ng/ml) for 20 h (**B**). Cell viability was assessed from at least three independent experiments and represented as means ± SD. Death rates are expressed as the percent (%) apoptotic cells among the total number of cells.

## DISCUSSION

Receptors such as TNF-R and TRAIL-R have been shown to generate at least two conflicting signals: a pro-apoptotic signal leading to activation of caspase through TRADD/FADD signaling and an anti-apoptotic signal mediated by activation of NF-κB which controls the expression of inducible genes such as *IEX-1L* [Wu et al., 1998] and *IAP* [Wang et al., 1998]. Frequently, signaling molecules evoke NF-κB activation, which subsequently mediates pro-survival signaling in cells. The present report established a biochemical linkage between caspases and NF-κB signaling in a subset of cells. We provided evidence that IκBα was cleaved in dying cells by various signals likely functioning to facilitate cell death. This proposal is reinforced by other's observations showing that the truncated IκBα functions as a stable inhibitor of NF-κB [Whiteside et al., 1995; Reuther and Baldwin, 1999]. The caspase-mediated generation of pro-apoptotic molecule in the NF-κB signal has also been shown for p65 [Levkau et al., 1999], RIP [Lin et al., 1999], and TRAF1 [Irmler et al., 2000].

The sensitization effects of ΔIκBα on cell death may depend on the contribution of NF-κB activity to anti-apoptotic signaling. We have investigated the extent of apoptosis after overexpression of ΔIκBα in TNF-α- or TRAIL-resistant NIH3T3 cells and TNF-α-resistant but TRAIL-sensitive HeLa cells. Interestingly, overexpression of ΔIκBα dramatically sensitized otherwise resistant cells to death: NIH3T3 became sensitive to TNF-α or TRAIL, and HeLa cells sensitive to TNF-α. On the other hand, ΔIκBα did not significantly enhance death of HeLa cells exposed to TRAIL and non-receptor stimuli including etoposide and taxol. Although ΔIκBα suppressed TRAIL-induced activation of NF-κB in NIH3T3 and HeLa cells, death rates of cells that were already sensitive to the death signals were not significantly affected by NF-κB activity (Figs. 7 and 8). TRAIL activated NF-κB depending on tumor cell types [Degli-Esposti et al., 1997; Trauzold et al., 2001]. Thus, a downstream signaling of TRAIL-receptor may be different among various cell types.

IκBα was previously shown to be cleaved during apoptosis of 32D myeloid cells [Reuther and Baldwin, 1999]. Our result showed that expression of either (D31E)IκBα or IκBα in HeLa cells and NIH3T3 cells did not significantly sensitize cells to TNF-α-mediated death. We speculate that (D31E)IκBα may behave like IκBα and be subjected to other processing such as proteasome-mediated degradation. Thus, the effects of IκBα cleavage on cell death may depend on signals, as degradation pattern of IκBα varies depending on the extracellular signals. White et al. [1995, 1996] showed that the

appearance of a truncated form of chicken IκBα could be blocked by CrmA without affecting apoptosis of v-Rel-transformed cells.

The cleavage pattern of IκBα by caspase-3 is distinct from the typical proteasome-mediated degradation of IκBα [Chen et al., 1995; Traenckner et al., 1995]. It is for this reason that expression of (S32,36A)IκBα, which is a well-known dominant negative mutant that cannot be phosphorylated at $Ser^{32}$ and $Ser^{36}$ and is resistant to ubiquitination-based degradation [Wang et al., 1996], prevents activation of NF-κB and increases the rates of TNF-α-induced apoptosis. Caspase-mediated cleavage of IκBα, by contrast, does not likely require phosphorylation [Barkett et al., 1997], implying that signal-induced phosphorylation may determine the degradation pathway of IκBα.

An additional IκBα cleavage pattern was recently reported to be mediated by calpain or a calcium-sensitive protease [Han et al., 1999]. While cleavage of IκBα by proteasome or calpain led to activation of NF-κB, caspase-mediated cleavage of IκBα generated a dominant negative inhibitor of NF-κB activation. IκBα interacts with Rel/NF-κB complexes via ankyrin repeats at the central core resulting in retention of the Rel complex within the cytosol. Caspase-mediated deletion of the IκBα amino-terminus removes the Lys residues at positions 21 and 22 responsible for ubiquitination, but the ankyrin repeats are retained [Baldi et al., 1996]. Consequently, the IκBα cleavage product retains its ability to bind NF-κB [Reuther and Baldwin, 1999] and would be expected to escape both signal-induced and ubiquitination-mediated degradation and remain associated with the Rel/NF-κB complex in the cytosol [Whiteside et al., 1995]. A functional role of different truncation form of IκBα, which was isolated by expression cloning, was demonstrated to affect radiation sensitivity in Ataxia Telangiectasia cells [Jung et al., 1995]. At the present time, there is much that still needs to be clarified with respect to cell types and apoptotic signals converting IκBα to a truncated form to determine the fate of affected cells.

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