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Caspase-Mediated p65 Cleavage Promotes TRAIL-Induced Apoptosis

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Abstract

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is cytotoxic to a wide variety of transformed cells, but not to most normal cells, implying potential therapeutic value against advanced cancer. However, signal transduction in TRAIL-mediated apoptosis is not clearly understood compared with other TNF family members. Specifically, it is not yet understood how TRAIL controls nuclear factor κB (NF-κB) activation and overcomes its antiapoptotic effect. We explored the regulation of NF-κB activity by TRAIL and its role in apoptosis. TRAIL combined with IκBα-"superrepressor" induced potent apoptosis of SK-Hep1 hepatoma cells at low concentrations of TRAIL that do not independently induce apoptosis. Apoptosis by high concentrations of TRAIL was not affected by IκBα-super-repressor. Although TRAIL alone did not induce NF-κB activity, TRAIL combined with z-VAD significantly increased NF-κB activation. Analysis of the NF-κB activation pathway indicated that TRAIL unexpectedly induced cleavage of p65 at Asp^{97}, which was blocked by z-VAD, accounting for all of these findings. p65 expression abrogated apoptosis and increased NF-κB activity in TRAIL-treated cells. Cleavage-resistant $p65^{D97A}$ further increased NF-κB activity in TRAIL-treated cells, whereas the COOH-terminal p65 fragment acted as a dominant-negative inhibitor. XIAP levels were increased by TRAIL in combination with z-VAD, whereas XIAP levels were decreased by TRAIL alone. Cleavage of p65 was also detected after FRO thyroid cancer cells were treated with TRAIL. These results suggest that TRAIL induces NF-κB activation, but simultaneously abrogates NF-κB activation by cleaving p65, and thereby inhibits the induction of antiapoptotic proteins such as XIAP, which contributes to the strong apoptotic activity of TRAIL compared with other TNF family members. (Cancer Res 2005; 65(14): 6111-9)

Introduction

Members of the tumor necrosis factor (TNF) and TNF receptor family play critical roles in the regulation of apoptosis, inflammation, growth, and development. As a new member of the TNF family, TNF-related apoptosis-inducing ligand (TRAIL) has been reported to kill a wide variety of transformed cells, including those resistant to other TNF family members (1, 2). TRAIL does not kill most normal cells (1, 3) except certain hematopoietic cells (4, 5), which suggest a potential therapeutic role of TRAIL in the treatment of advanced cancer. However, intracellular signal transduction in TRAIL-mediated apoptosis is less clearly defined than are other TNF family members. Activation of caspases (1, 6) and mitochondrial events are well characterized (7–9). However, there has been controversy regarding the roles of Fas-associated death domain protein and nuclear factor κB (NF-κB) activation in TRAIL-induced apoptosis. Several TNF family members, such as TNFα, induce NF-κB activation that leads to inflammatory or antiapoptotic responses. Candidates for NF-κB-inducible antiapoptotic genes include IAP family members, FLIP, A1α, A20, and Gadd45β. Because of such induction of antiapoptotic genes, TNFα itself does not induce death in most cells. In contrast to TNFα, the physiologic function of which is inflammation or protection of host organisms against microbial infection, professional death effectors, such as Fas or TRAIL, have been reported to induce minor or no activation of NF-κB which might be related to more efficient death of target cells. Whereas TRAIL has initially been reported not to activate NF-κB (10, 11), a number of papers showed that TRAIL and its authentic receptors, such as DR4 or DR5, activate NF-κB (11–13). For TRAIL to have such a strong apoptotic activity on a variety of cells, it would be expected that TRAIL should overcome the antiapoptotic effect of NF-κB. However, it is far from clear how TRAIL controls NF-κB activation and leads to the suppression of its antiapoptotic effect.

In our study investigating NF-κB activation by TRAIL, we found that caspases activated by TRAIL cleave p65, which leads to the suppression of NF-κB activation and more efficient execution of apoptosis. This could explain the strong apoptotic activity of TRAIL compared with other TNF family members.

Materials and Methods

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells were seeded in 96-well microtiter plates (3×10^4 per well) and treated with TRAIL (Calbiochem, La Jolla, CA) for the indicated time periods at 250 ng/mL concentration unless otherwise indicated. In some experiments, cells were pretreated with z-Val-Ala-Asp(Ome)-CH_2F (z-VAD), a pancaspase inhibitor (Enzyme Systems, Livermore, CA) for 30 minutes. After TRAIL treatment, the medium was removed and 0.5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added, followed by incubation in a CO_2 incubator at 37°C for 2 hours. After a brief centrifugation, the supernatant was removed and DMSO was added. After insoluble crystals were completely dissolved, absorbance at 540 nm was read using a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA). In some experiments, cells were infected with adenovirus harboring XIAP, p65, or IκBα-superrepressor (kindly provided by Dr. Y. Lipp at the University of Vienna, Vienna, Austria, Dr. C.D. Logsdon, University of Michigan, MI, and Dr. C-T. Lee at Seoul National University Hospital, Seoul, Korea, respectively) prior to treatment with TRAIL.

Measurement of caspase activity. Caspase-3-like activity was measured using a commercial caspase assay kit (PharMingen, La Jolla, CA)

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

H.S. Kim and I. Chang contributed equally to this work.

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according to the instructions of the supplier. In brief, caspase-3 fluorogenic substrates (Ac-DEVD-AMC) were incubated with cytokine-treated cell lysate at 37°C for 1 hour. Liberated AMC was measured using a fluorometric plate reader with an excitation wavelength of 380 nm and an emission wavelength of 420 nm.

Electrophoretic mobility shift assay. Nuclear extracts were prepared from SK-Hep1 cells treated with TRAIL for indicated times with or without z-VAD pretreatment. Electrophoretic mobility shift assay was then done using the nuclear extracts incubated with labeled probe containing consensus NF-κB binding sequence (Promega, Madison, WI) as previously described (14). For supershift assays, a total of 0.2 μg of antibodies against the p65 or p50 subunit of NF-κB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were included in the reaction.

Nuclear factor κB reporter assay. NF-κB reporter activity was measured using the Dual-Luciferase Reporter Assay System (Promega). In brief, cells in 24-well plates were cotransfected with 0.5 μg of NF-κB-responsive reporter gene construct carrying two copies of κB sequences linked to luciferase gene (IgGκ NF-κB-luciferase, generously provided by Dr. G.D. Rosen, Stanford University, Stanford, CA) together with 0.01 μg of *Renilla* luciferase gene under herpes simplex virus thymidine kinase promoter (pRL-TK, Promega) using LipofectAMINE reagent (Life Technologies, Gaithersburg, MD). At 24 hours after transfection, cells were treated with TRAIL with or without z-VAD pretreatment. After an additional 5 hours, activities of firefly luciferase and *Renilla* luciferase were measured using the Dual-Luciferase Reporter Assay System. Results were presented as firefly luciferase activity normalized to *Renilla* luciferase activity. In some experiments, cells were cotransfected with 0.5 μg of p65, $p65^{D97A}$, or Δp65 expression plasmid along with NF-κB-responsive reporter plasmid (0.5 μg) and pRL-TK (0.01 μg).

Transient transfection of p65. After transfection of p65 or its mutants, the number of apoptotic cells was counted as described (14). In brief, SK-Hep1 cells in 24-well plates were cotransfected with 1 μg of p65 (kindly provided by Dr. W.C. Greene, Duke University, Durham, NC), $p65^{D97A}$, or Δp65 expression plasmid together with 0.1 μg of lacZ (pCH110) using LipofectAMINE reagent. At 24 hours after transfection, cells were treated with TRAIL. After another 24 hours, cells were fixed with 0.5% glutaraldehyde for 10 minutes at room temperature and stained with 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in 4 mmol/L potassium ferricyanide / 4 mmol/L potassium ferrocyanide / 2 mmol/L magnesium chloride at 37°C for the counting of blue cells. Viable blue cells and dark blue apoptotic cells with rugged condensed morphology in 10 random high-power fields (×200) were counted. At least 200 blue cells were counted for each experiment, and transfection efficiency was 15% to 30%.

Western blot analysis. Cells were lysed in a buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EDTA, 0.5 mmol/L Na_3VO_4, 50 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration in cell lysate was determined using a commercial protein assay kit (Bio-Rad, Hercules, CA). An equal amount of protein for each sample was separated by 10% SDS-PAGE and transferred to Hybond enhanced chemiluminescence membranes (Amersham Pharmacia, Uppsala, Sweden). After incubation in a 1:1,000 dilution of primary antibodies to phospho-IKKα/β, IKKβ (Cell Signaling Technology, Beverly, MA), IκBα, p65, p50 (Santa Cruz), or XIAP (MBL, Nagoya, Japan), membranes were probed with appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia). Bound antibody was visualized using an enhanced chemiluminescence reagent (Amersham Pharmacia).

RT-PCR analysis. Total RNA was extracted using TRIzol Reagent (Life Technologies). Reverse transcription was carried out using Superscript II (Invitrogen, Carlsbad, CA) and oligo$(dT)_{12\text{-}18}$ primer. PCR amplification using primer sets specific for XIAP (forward, GCAAGATGAGTCAAGTCA-GACTTC; reverse, AGACATAAAAATTTTTTGCTTG) was carried out at 55°C annealing temperature for 25 cycles.

Site-directed mutagenesis. Point mutations ($p65^{D97A}$ and $p65^{D449A}$) were introduced to a prokaryotic p65 expression vector using the Quick Change Mutagenesis Kit (Stratagene, La Jolla, CA). The oligonucleotides used for *in vitro* mutagenesis were 5′-GAAAGGACTGCCGG**GCT**GGCTTCTAT-TGAGGC-3′ and 5′-AGTTTGATGATGAA**GCC**CTGGGGGCCTTGCTTGG-3′ for $p65^{D97A}$ and $p65^{D449A}$, respectively. The mutant sequence was confirmed by direct nucleotide sequencing.

***In vitro* cleavage of p65.** *In vitro* translation of p65 and its mutants ($p65^{D97A}$ and $p65^{D449A}$) was carried out using a commercial kit (Promega) according to the manufacturer's protocol. In short, an amino acid mixture without methionine, p65 DNA templates, S^{35}-methionine, T7 RNA polymerase, rabbit reticulocyte lysate, and RNase inhibitor was mixed in a reaction buffer for incubation at 30°C for 90 minutes. After treatment of SK-Hep1 cells with 250 ng/mL TRAIL, 0.5×10^6 cells were collected for each sample, washed once with cold PBS, and resuspended in 50 μL of chilled cell lysis buffer containing 10 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, and 2 mmol/L $MgCl_2$. Further cell lysis was done by freezing and thawing for three to four cycles. After centrifugation at 14,000 rpm at a temperature of 4°C for 15 minutes, 10 μg of cell lysate was incubated for 1 hour at 37°C with 5 μL of ^{35}S-labeled wild-type or p65 mutants in a reaction buffer containing 10 mmol/L HEPES (pH 7.4), 0.04% CHAPS, 4% glycerol, 0.4 mmol/L EDTA, and 2.5 mmol/L DTT. After boiling, samples were loaded onto 10% SDS-PAGE gel.

Statistical analysis. All values were expressed as mean ± SD (n = 3). Student's t test was employed to compare the means between the groups. P values <0.05 were regarded as statistically significant.

Results

Caspase-sensitive nuclear factor κB activation by tumor necrosis factor–related apoptosis-inducing ligand. Because the role of NF-κB activation in TRAIL-induced apoptosis is not clearly understood, we first studied whether the abrogation of NF-κB activation by an IκBα-superrepressor that is resistant to degradation (15) could affect TRAIL-induced apoptosis. As reported previously (8), TRAIL concentrations >250 ng/mL induced significant apoptosis of SK-Hep1 hepatoma cells. In contrast, TRAIL concentrations <100 ng/mL did not induce notable death of SK-Hep1 cells. However, TRAIL concentrations <100 ng/mL induced potent cell death when combined with adenoviral expression of IκBα-superrepressor, suggesting that NF-κB activation occurs and affects SK-Hep1 cell death at low concentrations of TRAIL. SK-Hep1 cell death induced by TRAIL concentrations >250 ng/mL was not significantly enhanced by IκBα-superrepressor, suggesting that NF-κB activation observed at lower concentrations of TRAIL is abrogated by higher concentrations of TRAIL (Fig. 1*A*). SK-Hep1 cell death induced by TRAIL concentrations >250 ng/mL showed classical apoptosis, which is characterized by nuclear condensation, fragmentation, sub-G_1 peak on DNA ploidy assay (data not shown), and induction of caspase-3-like activity as reported previously (Fig. 1*B*; ref. 8). z-VAD, a pancaspase inhibitor, completely blocked caspase-3 activation and SK-Hep1 cell death induced by TRAIL (Fig. 1*B* and *C*).

Because TRAIL concentrations >250 ng/mL seem to abrogate NF-κB activation and lead to caspase activation, we next explored the relationship between caspase activation and NF-κB activation. Although TRAIL alone (250 ng/mL) did not induce NF-κB activity, TRAIL in combination with z-VAD pretreatment induced significant NF-κB activation (P < 0.05; Fig. 1*D*), suggesting that activated caspases at high concentrations of TRAIL inhibit NF-κB activation.

Cleavage of p65 by tumor necrosis factor–related apoptosis-inducing ligand. To understand how NF-κB activation is abrogated in apoptosis by high concentrations of TRAIL, we

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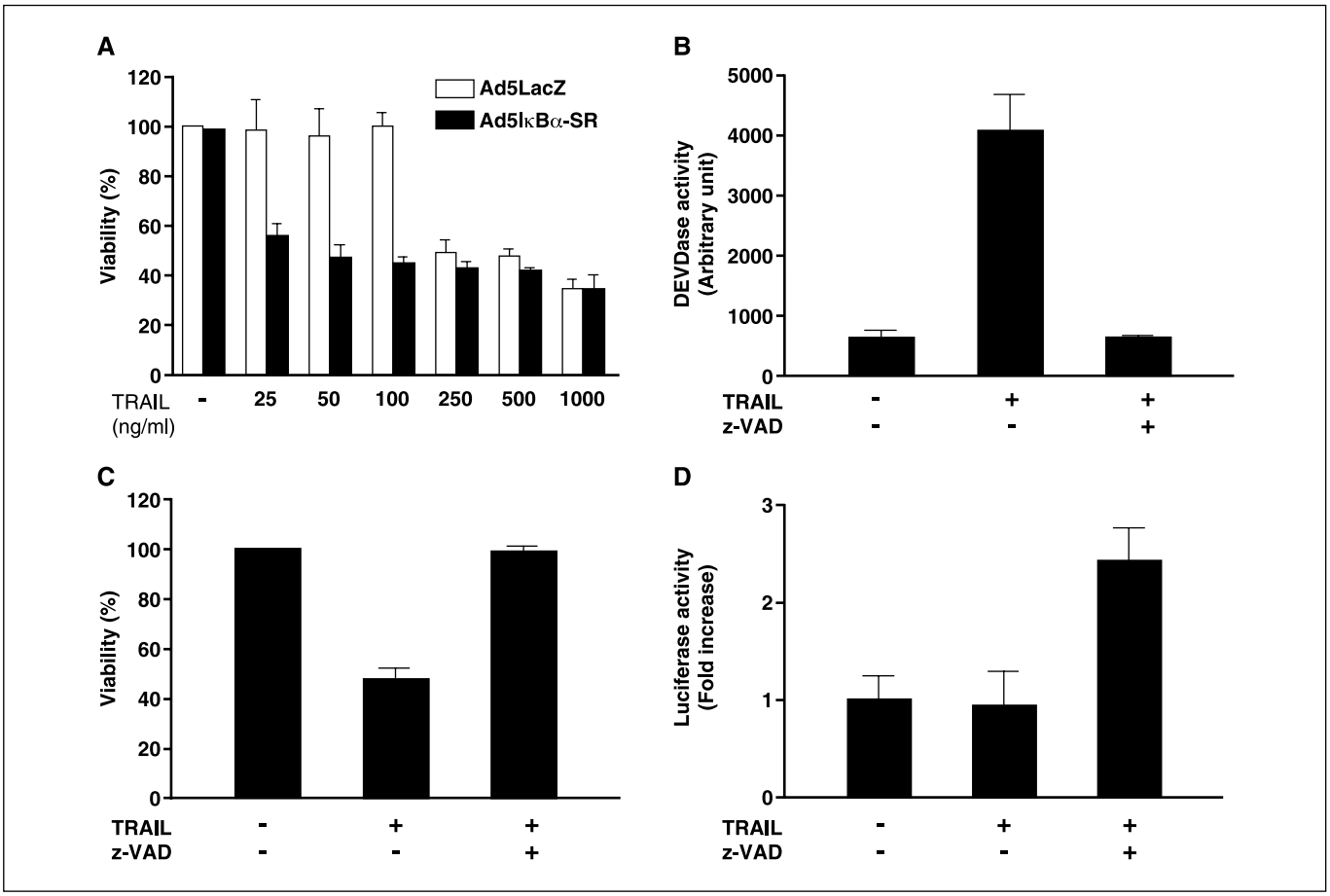

Figure 1. Increased NF-κB activity in TRAIL-treated cells by caspase inhibitors. *A,* SK-Hep1 hepatoma cells were infected with adenovirus [multiplicity of infection (MOI), 50] harboring LacZ or IκBα-superrepressor and treated with indicated concentrations of TRAIL. After 24 hours, viability was assessed using MTT assay. z-VAD inhibited caspase-3-like activity (*B*) and SK-Hep1 cell death (*C*) by TRAIL. SK-Hep1 cells were left untreated or treated with TRAIL (250 ng/mL) for 6 hours (caspase activity assay) and 24 hours (viability assay) with or without z-VAD (50 μmol/L) pretreatment for 30 minutes, respectively. Caspase-3-like activity was determined by AMC release using DEVD-AMC as a substrate. Viability was assessed as in (*A*). *D,* SK-Hep1 cells were cotransfected with 0.5 μg of NF-κB luciferase reporter construct together with 0.01 μg of pRL-TK reporter construct. After 24 hours, transfected cells were left untreated or treated with TRAIL (250 ng/mL) for 5 hours with or without z-VAD (50 μmol/L) pretreatment for 30 minutes. Relative firefly luciferase activities of NF-κB reporter normalized to *Renilla* luciferase activities are shown.

tested whether z-VAD pretreatment affects TRAIL-mediated IKK activation, IκBα degradation, and NF-κB DNA binding activity (Fig. 2). TRAIL induced a similar degree of IKKα/β phosphorylation and IκBα degradation irrespective of z-VAD pretreatment (Fig. 2*A* and *B*). The increase in IKKα/β phosphorylation was paralleled by a decrease in IκBα protein levels, which was noticeable at 1 hour and peaked at 2 hours. Compared with TNFα, the induction of IKK activity and the subsequent degradation of IκBα were relatively slow (Fig. 2*C* and *D*). We then determined NF-κB DNA-binding activity by electrophoretic mobility shift assay after TRAIL treatment with or without z-VAD pretreatment. Remarkably, whereas TRAIL alone induced only minor NF-κB DNA-binding activity, TRAIL in combination with z-VAD pretreatment significantly enhanced NF-κB DNA-binding activity (Fig. 2*E*), suggesting that TRAIL-mediated caspase activation mainly affects NF-κB DNA-binding activity after a similar degree of IκBα degradation. In contrast, TNFα-induced NF-κB activation was not affected by z-VAD pretreatment (Supplementary Fig. S1), which is consistent with our observation that TNFα induced no apoptosis of SK-Hep1 cells (data not shown). Supershift analysis indicated that NF-κB complex comprised both p50 and p65 subunits (data not shown). Thus, we studied the potential changes to the p65/p50 NF-κB components by TRAIL. Unexpectedly, immunoblot analysis disclosed a band with a smaller molecular size (~55 kDa; Δp65) which is recognized by anti-p65 antibody, indicating the cleavage of p65 after TRAIL treatment. The cleavage of p65 was blocked by z-VAD (Fig. 3*A*), suggesting that caspases activated by TRAIL induce p65 cleavage and abrogate the NF-κB-dependent antiapoptotic process as z-VAD enhances NF-κB activity after TRAIL treatment (see Figs. 1*D* and 2). The cleaved p65 was recognized by an antibody to the COOH terminus of p65, but not by that to the NH_2 terminus, which suggests that p65 is cleaved at the NH_2 terminus. No cleavage of p50 was detected during TRAIL-induced apoptosis of SK-Hep1 cells (Fig. 3*A*). Because the above results indicated *in vivo* cleavage of p65 in intact cells after TRAIL treatment, we further investigated whether p65 could be cleaved *in vitro*. *In vitro* translated p65 was, indeed, cleaved after incubation with cell lysate prepared from TRAIL-treated cells (Fig. 3*B*). The cleavage of p65 *in vitro* was abrogated by z-VAD, suggesting that p65 cleavage is induced by activated caspases in TRAIL-treated cells (Fig. 3*B*).

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To determine the cleavage site, we introduced point mutations at previously reported putative caspase cleavage sites (16, 17). When the mutant p65 protein harboring D97A substitution ($p65^{D97A}$) was translated *in vitro* and incubated with TRAIL-treated cell lysate, no cleavage product was detected. Another mutant harboring D449A substitution ($p65^{D449A}$; ref. 17) was cleaved like wild-type p65, indicating that Asp^{97}, but not Asp^{449}, is the site preferentially cleaved in TRAIL-induced apoptosis. These results are consistent with the recognition of the cleaved p65 fragment by the antibody to the COOH terminus but not by that to the NH_2 terminus (Fig. 3*A*). The results are also consistent with the abrogated NF-κB DNA-binding activity in TRAIL-treated cells because Asp^{97} is located in the DNA-binding domain of p65 (Fig. 2*E*).

Abrogation of tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis by p65. Because these results suggested that the cleavage of p65 by activated caspases plays an important role in the execution of apoptosis by TRAIL, we next studied whether p65 overexpression would reverse such a process. Indeed, adenoviral p65 expression significantly inhibited apoptosis of SK-Hep1 cells by TRAIL (Fig. 4*A*). Additionally, we examined the effect of $p65^{D97A}$, which is resistant to the cleavage. Transfection of wild-type p65 reduced apoptosis from 42% to 34%, and $p65^{D97A}$ transfection further reduced apoptosis to 25%, which was significantly protective compared with wild-type p65 (Fig. 4*B*, third versus fourth column from the left; $P < 0.05$). The caspase-3-cleaved COOH-terminal fragment of p65 (98-551; Δp65) did not inhibit cell death. The expression of p65 also significantly increased NF-κB reporter activity in TRAIL-treated cells (Fig. 4*C*). TRAIL significantly decreased NF-κB reporter activity by the expression of p65 alone. This is probably due to activated caspases cleaving both endogenous and exogenous p65 (Fig. 4*C*, third versus fourth column from the left; $P < 0.05$). The expression of $p65^{D97A}$ further increased NF-κB reporter activity in TRAIL-treated cells compared with wild-type p65 expression in TRAIL-treated cells

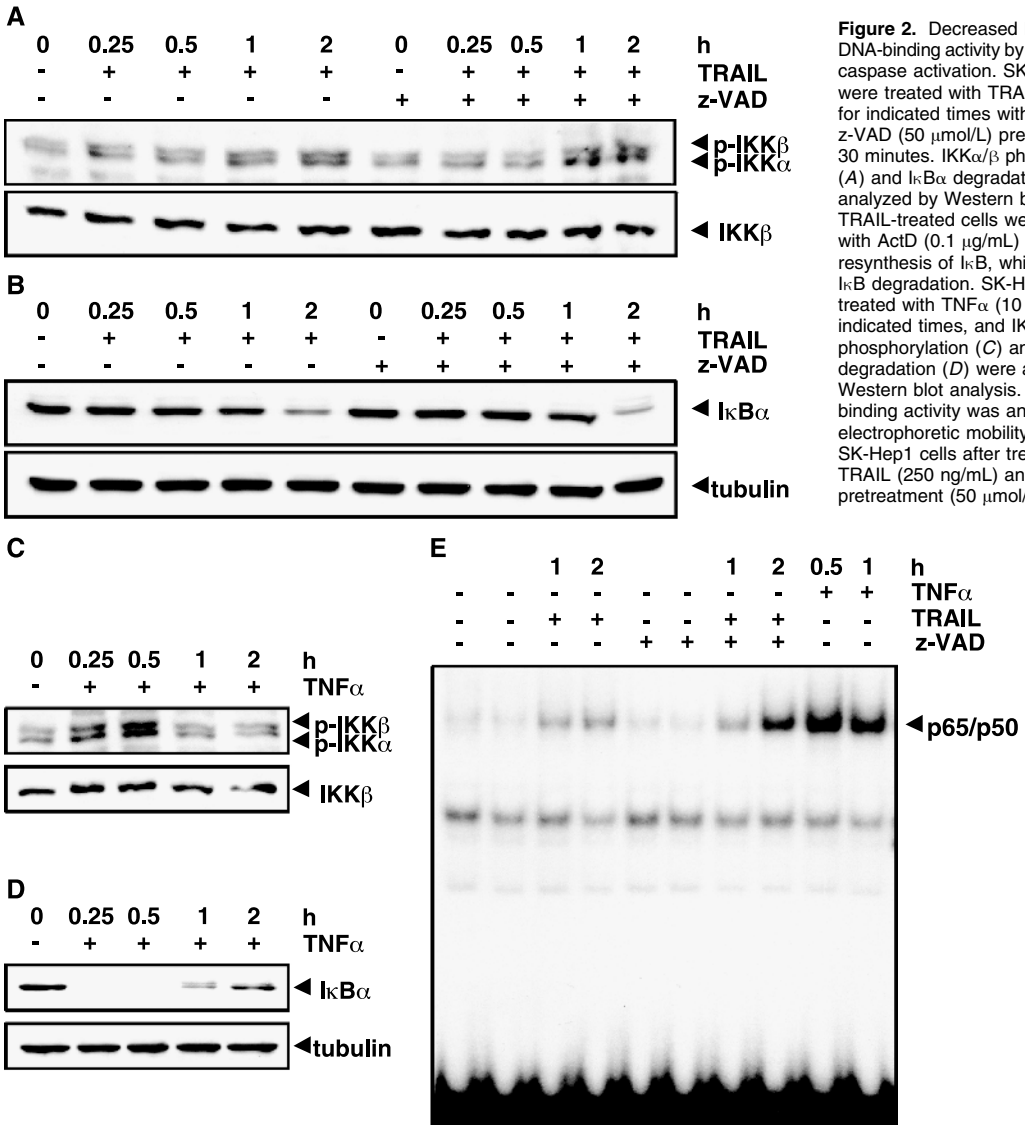

Figure 2. Decreased NF-κB DNA-binding activity by TRAIL-mediated caspase activation. SK-Hep1 cells were treated with TRAIL (250 ng/mL) for indicated times with or without z-VAD (50 μmol/L) pretreatment for 30 minutes. IKKα/β phosphorylation (*A*) and IκBα degradation (*B*) were analyzed by Western blot analysis. TRAIL-treated cells were pretreated with ActD (0.1 μg/mL) to prevent resynthesis of IκB, which might mask IκB degradation. SK-Hep1 cells were treated with TNFα (10 ng/mL) for indicated times, and IKKα/β phosphorylation (*C*) and IκBα degradation (*D*) were analyzed by Western blot analysis. *E*, NF-κB DNA binding activity was analyzed by electrophoretic mobility shift assay in SK-Hep1 cells after treatment with TRAIL (250 ng/mL) and/or z-VAD pretreatment (50 μmol/L).

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(Fig. 4*C*, fourth versus sixth column from the left; $P < 0.05$). The expression of Δp65 did not increase NF-κB reporter activity before or after TRAIL treatment (Fig. 4*C*). Furthermore, Δp65 inhibited NF-κB activation by wild-type p65 in a dose-dependent manner, suggesting a dominant-negative role of Δp65 in NF-κB activation (Fig. 4*D*). However, Δp65 transfection did not significantly increase apoptosis after TRAIL treatment, likely because of endogenously produced Δp65 (Fig. 4*B*, second versus fifth column from the left; $P > 0.1$).

Degradation and synthesis of XIAP in apoptosis by tumor necrosis factor–related apoptosis-inducing ligand. We next studied whether TRAIL induced changes in the expression of XIAP, an important NF-κB-inducible antiapoptotic molecule. XIAP expression began to decrease from 6 hours after TRAIL treatment and reached the nadir after 24 hours (Fig. 5*A*). Adenoviral expression of XIAP almost completely inhibited caspase-3-like activity and SK-Hep1 cell death induced by TRAIL, suggesting that the disappearance of XIAP plays an important role in the progression of the apoptotic process induced by TRAIL (Fig. 5*B*). The disappearance of XIAP after TRAIL treatment was abrogated by z-VAD, suggesting the degradation of XIAP by activated caspases (Fig. 5*A*). Because XIAP is a well-known target protein of NF-κB and because activated caspase inhibited NF-κB activation after TRAIL treatment, we further investigated the effect of caspase activation on the production of XIAP. Although the potential increase in the expression of XIAP protein by TRAIL/z-VAD was not apparent, probably due to high basal levels of XIAP (Fig. 5*A*), RT-PCR analysis showed that RNA levels of XIAP were markedly increased by TRAIL/z-VAD but not by TRAIL alone, suggesting that NF-κB activation by TRAIL plus caspase inhibitors not only inhibited the degradation of XIAP by activated caspases, but also increased XIAP synthesis, probably through the activation of NF-κB (Fig. 5*C*).

Because high basal expression interfered with the study of XIAP synthesis at the protein level, we studied the changes in XIAP protein in a low-serum condition (1% fetal bovine serum) where baseline expression was low (data not shown). As hypothesized, an increase in XIAP protein levels after treatment with TRAIL/z-VAD was clearly shown. TRAIL alone decreased the XIAP protein level by activating caspases as expected (Fig. 5*D*).

Cleavage of p65 in other cells. We finally studied whether the cleavage of p65 in TRAIL-induced apoptosis is restricted to SK-Hep1 cells or could be observed in other cells. All of the three tested cells, including highly malignant FRO anaplastic thyroid cancer cells, HeLa cells, and HEK293 cells, underwent TRAIL-induced apoptosis, which was inhibited by z-VAD (Fig. 6*A*). In contrast, p65 cleavage was detected after TRAIL treatment of FRO cells, but not after treatment of HeLa or HEK293 cells (Fig. 6*B*). Whereas TRAIL alone did not induce NF-κB activity in FRO cells, TRAIL in combination with z-VAD induced a strong NF-κB activation, similar to that observed in SK-Hep1 cells (Fig. 6*C*). TRAIL in combination with z-VAD induced even stronger NF-κB activation in HeLa cells, suggesting that the abrogation of NF-κB by activated caspases occurs through pathways other than p65 cleavage, such as the cleavage of RIP (18).

Discussion

Our results, which indicated that the expression of IκBα-superrepressor accelerated TRAIL-induced target cell death, are similar to the results of previous reports (18, 19). However, the effect of IκBα-superrepressor was observed only when TRAIL concentration was low and was not enough to independently kill SK-Hep1 hepatoma cells. These results suggest that NF-κB is activated by TRAIL, but the activation is abrogated by high concentrations of TRAIL. Our results also suggested a reciprocal relationship between NF-κB and caspase activation because the latter was observed only at high concentrations of TRAIL, whereas the effect of the former was manifest at both high and

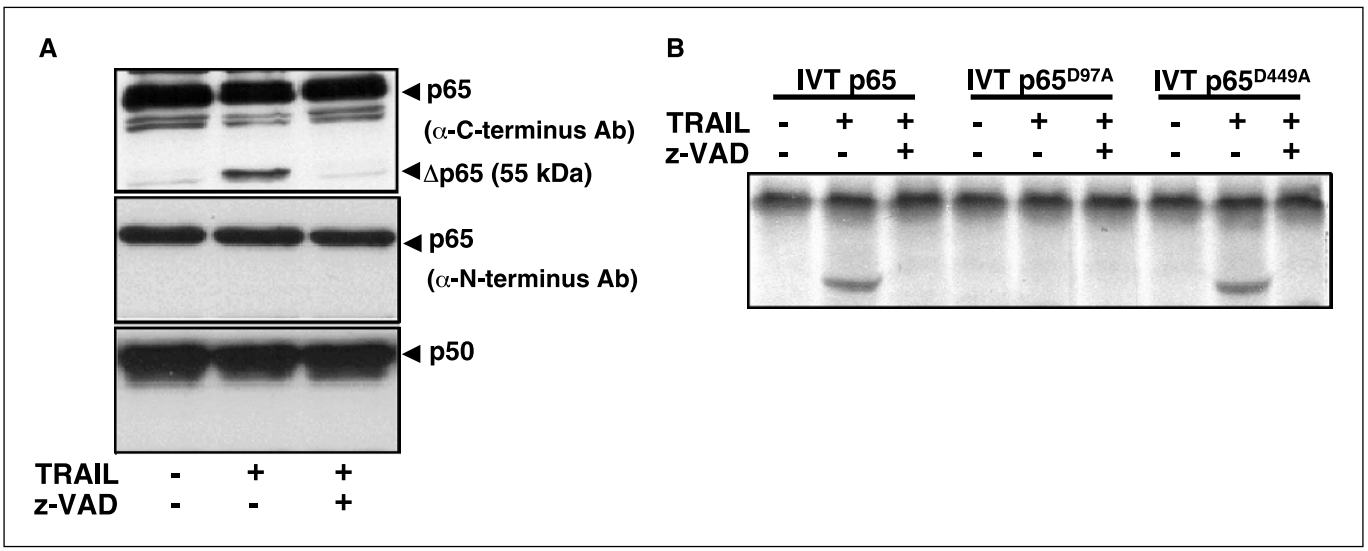

Figure 3. Cleavage of p65 at Asp[97] by TRAIL. *A,* SK-Hep1 cells were treated with TRAIL (250 ng/mL) for 2 hours with or without z-VAD (50 μmol/L) pretreatment for 30 minutes. Cell lysates were subjected to SDS-PAGE (10%), followed by Western blot analysis using specific antibodies to the COOH terminus and the NH_2 terminus of p65 or p50. *B,* SK-Hep1 cells were treated with TRAIL (250 ng/mL) for 6 hours and cell lysates were prepared. Aliquots (5 μL) of *in vitro* translated [^{35}S]Met-p65 and mutants ([^{35}S]Met-p65^{D97A} or [^{35}S]Met-p65^{D449A}) were incubated with 10 μg of cell lysate in the presence or absence of z-VAD (50 μmol/L) for 1 hour. Reaction mixtures were resolved on 10% SDS-PAGE, and autoradiography was obtained on the dried gel.

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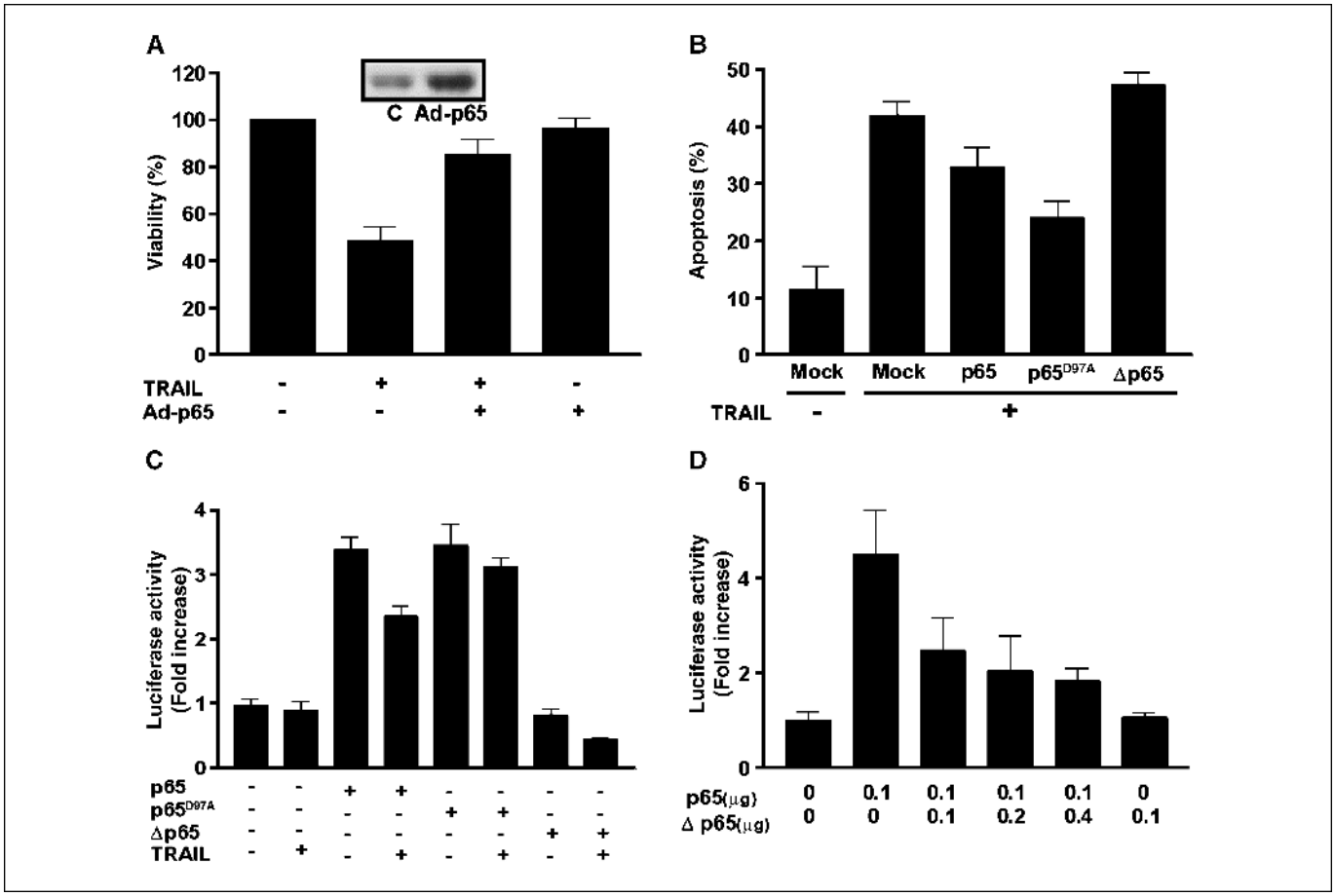

Figure 4. Inhibition of TRAIL-induced apoptosis by p65 or its cleavage-resistant mutant. *A,* SK-Hep1 cells were infected with adenovirus (50 MOI) harboring p65 and treated with TRAIL (250 ng/mL). After 24 hours, viability was assessed using MTT assay. Inset, expression level of p65 after adenoviral transduction of p65 determined by Western blot analysis. *B,* SK-Hep1 cells were cotransfected with 1 μg of p65, $p65^{D97A}$, or Δp65 construct together with 0.1 μg of lacZ construct. After 24 hours, transfected cells were left untreated or treated with TRAIL (250 ng/mL) for 24 hours and cells with apoptotic morphologic changes were counted in lacZ-expressing cells. *C,* SK-Hep1 cells were cotransfected with expression plasmid encoding p65, $p65^{D97A}$, or Δp65 (0.5 μg each), together with NF-κB luciferase construct (0.5 μg) and pRL-TK construct (0.01 μg). After 24 hours of transfection, cells were untreated or treated with TRAIL (250 ng/mL) for 5 hours and luciferase reporter assay was done. *D,* SK-Hep1 cells were cotransfected with p65 (0.1 μg), NF-κB luciferase construct (0.1 μg), and pRL-TK construct (0.01 μg) together with increasing amounts of Δp65. Cells were harvested 24 hours after transfection, and luciferase reporter assay was done.

low concentrations of TRAIL. In our efforts to study the relationship between NF-κB activation and caspase activation, we observed that caspase inhibition by z-VAD enhanced NF-κB activation, which is similar to a previous paper which showed an increase in NF-κB activity by z-VAD in HeLa cells after TRAIL treatment (18). In our effort to examine each component of the NF-κB complex, we unexpectedly found that p65 was cleaved during TRAIL-induced SK-Hep1 hepatoma cell apoptosis, which seems to be responsible for the abrogation of NF-κB activation at high TRAIL concentrations. The cleavage of p65 has been reported in apoptosis by serum withdrawal, certain chemicals, and Fas (16, 17, 20). However, p65 cleavage has not been shown in other types of apoptosis, particularly apoptosis induced by TRAIL and other TNF family members. The cleavage of p65 and abrogation of NF-κB activation in TRAIL-induced apoptosis are particularly interesting because TRAIL induces much stronger apoptosis on a wider variety of cancer cells than do other types of TNF family members, such as TNFα and Fas. p65 cleavage after TRAIL treatment was not restricted to SK-Hep1 cells because such cleavage was also noted after the treatment of FRO cells with TRAIL. Active NF-κB components might be more accessible to the cleavage by activated caspases than inactive NF-κB components associated with IκBα because the p65 cleavage fragment after TRAIL treatment constituted a higher fraction of the total p65 in nuclear extracts compared with cytosol extracts (Supplementary Fig. S2). Other cells that do not show p65 cleavage after TRAIL treatment might have other ways of abrogating NF-κB activation, such as the cleavage of RIP (18). However, we cannot rule out the involvement of other mechanism(s) for negating NF-κB activation after TRAIL treatment in these cells, and further studies are required to determine this possibility.

The cleavage site of p65 in TRAIL-induced apoptosis seemed to be in the NH_2-terminal DNA-binding domain because TRAIL primarily decreased the NF-κB DNA-binding activity and the cleaved p65 was recognized by an antibody to the COOH terminus of p65, but not by an anti-NH_2 terminus antibody. Our *in vitro* translation study also showed that caspases activated by TRAIL cleave the Asp-Cys-Arg-Asp^{97}-Gly^{98} motif of p65 at the DNA-binding domain (21), similar to a previous report employing a menadione analogue as an apoptosis inducer (16). Consistent with our hypothesis, transfection of a cleavage-resistant p65

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mutant ($p65^{D97A}$) inhibited apoptosis and increased NF-κB reporter activity after TRAIL treatment further than that of wild-type p65. The caspase-3-cleaved COOH-terminal fragment of p65 (Δp65) that might be present in TRAIL-treated SK-Hep1 cells could block the wild-type p65-induced NF-κB activation by acting as a dominant-negative inhibitor. This may explain the abrogation of NF-κB activation despite the persistent presence of wild-type p65 together with the cleaved p65 in TRAIL-treated cells.

In our effort to search for the target antiapoptotic proteins that are induced by NF-κB in TRAIL-treated cells, we observed that XIAP levels were markedly decreased after TRAIL treatment, similar to previous reports (22, 23). The decrease in XIAP after TRAIL treatment was completely reversed by caspase inhibitors, which suggests that XIAP is degraded by activated caspases. The decrease in XIAP level or XIAP degradation was not simply a result of apoptosis but contributed to apoptosis because XIAP overexpression abrogated caspase activation and SK-Hep1 cell death by TRAIL. Activated caspases not only induced the cleavage of XIAP, but also inhibited XIAP synthesis by cleaving p65 and blocking NF-κB activation, which was shown by an increase in XIAP RNA and protein levels after the treatment of SK-Hep1 cells with TRAIL/z-VAD. These were not seen after treatment with TRAIL alone.

At present, it is not clear whether caspase-mediated NF-κB inactivation is a primary determinant in TRAIL-mediated apoptosis and defects in this pathway contribute to inherent TRAIL resistance or the development of resistance to TRAIL. However, recent studies showed that sensitivity to TRAIL-mediated apoptosis was reciprocally regulated by NF-κB activation and was significantly enhanced by abrogating NF-κB activation in some cancer cell lines (19, 24, 25), suggesting that NF-κB is a key molecule underlying the TRAIL-resistant mechanism. Furthermore, certain cancer cells were reported to have either direct or indirect inactivating mutations of caspase that might affect both the caspase-mediated NF-κB inactivation and cellular apoptosis by TRAIL (26–28).

Taken together, these results indicate that p65 is cleaved by high concentrations of TRAIL in some TRAIL-sensitive cells, leading to the strong apoptotic activity of TRAIL compared with other TNF family members. Δp65 that can form dimers but cannot bind to specific DNA sequences, thus acting as a dominant-negative inhibitor, could be used for sensitization of resistant cancer cells to TRAIL or other TNF family members.

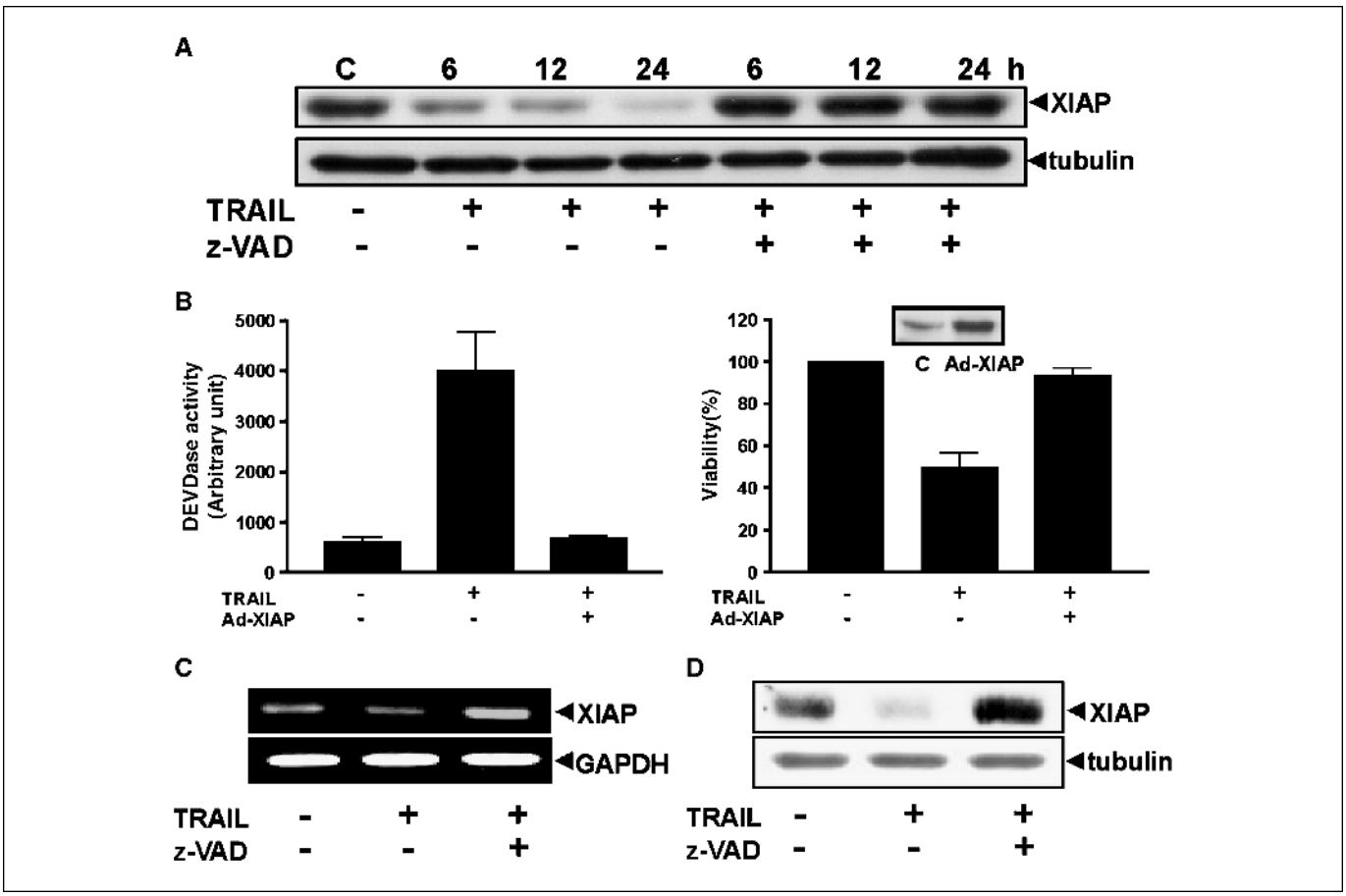

Figure 5. Induction of XIAP by TRAIL/z-VAD. *A,* SK-Hep1 cells were treated with TRAIL (250 ng/mL) for indicated times with or without z-VAD (50 μmol/L) pretreatment for 30 minutes, and the XIAP protein level was analyzed by Western blot analysis. *B,* SK-Hep1 cells were infected with adenovirus (50 MOI) harboring XIAP and treated with TRAIL (250 ng/mL) for 6 hours (caspase activity assay) and 24 hours (viability assay) with or without z-VAD (50 μmol/L) pretreatment for 30 minutes, respectively. Caspase-3-like activity was determined as in Fig. 1*B* and viability was assessed using MTT assay. Inset, expression level of XIAP after adenoviral transduction of XIAP determined by Western blot analysis. *C,* SK-Hep1 cells were treated with TRAIL (250 ng/mL) for 6 hours with or without z-VAD (50 μmol/L) pretreatment for 30 minutes. Cells were harvested for total RNA extraction and XIAP mRNA level was analyzed by RT-PCR. *D,* SK-Hep1 cells were cultured in a low-serum condition (1% fetal bovine serum) for 24 hours. Cells were then treated with TRAIL (250 ng/mL) for 24 hours with or without z-VAD (50 μmol/L) pretreatment for 30 minutes and the XIAP protein level was analyzed by Western blot analysis.

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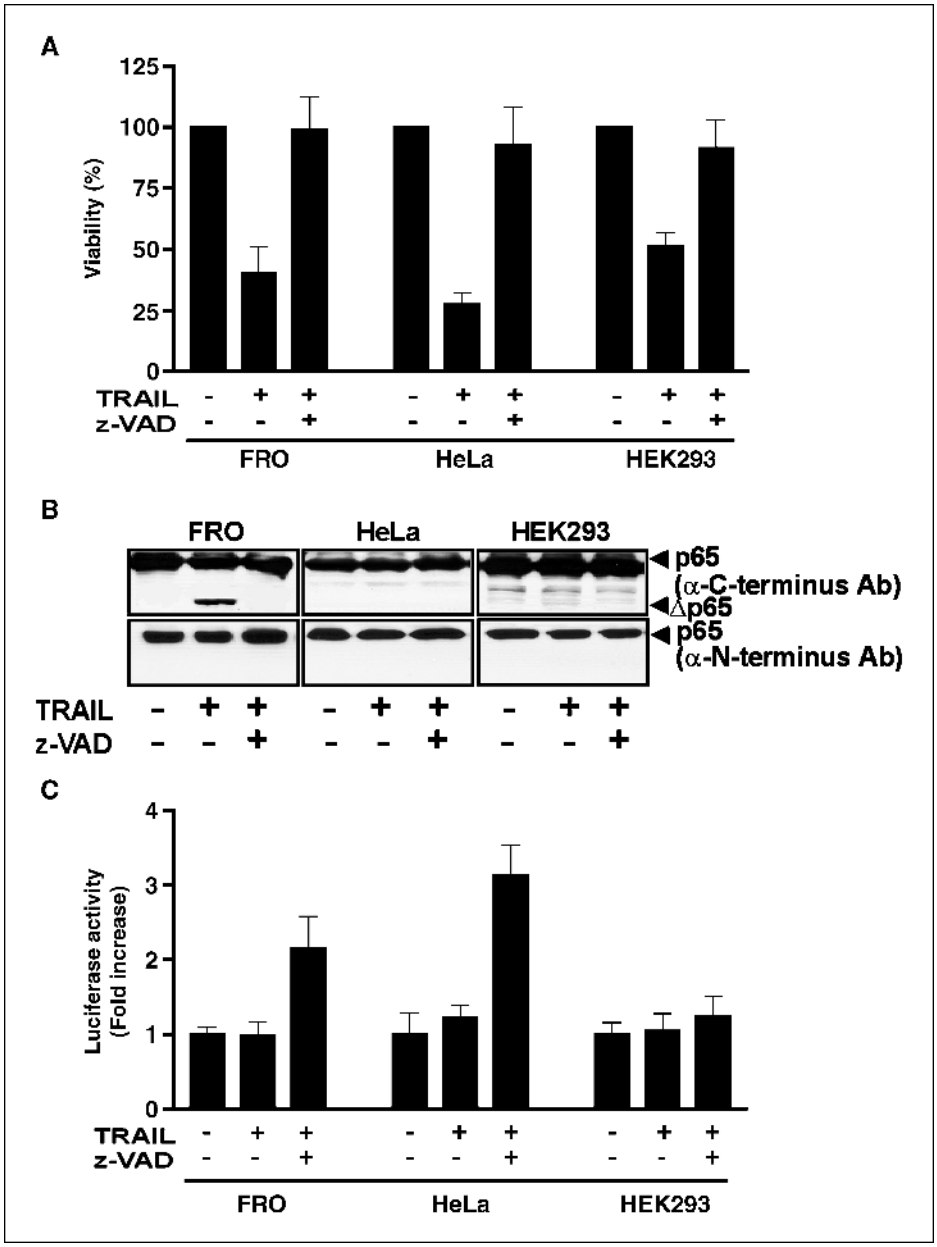

Figure 6. p65 cleavage in other cells. *A,* FRO thyroid cancer, HeLa cervical cancer, and HEK293 cells were treated with TRAIL (250 ng/mL) for 24 hours with or without z-VAD (50 μmol/L) pretreatment for 30 minutes and viability was assessed using MTT assay. *B,* FRO, HeLa, and HEK293 cells were treated with TRAIL (250 ng/mL) for 2 hours with or without z-VAD (50 μmol/L) pretreatment for 30 minutes. Cell lysates were subjected to SDS-PAGE (10%), followed by Western blot analysis using specific antibodies to the COOH terminus and the NH_2 terminus of p65. *C,* FRO, HeLa, and HEK293 cells were cotransfected with 0.5 μg of NF-κB luciferase reporter construct together with 0.01 μg of pRL-TK reporter construct. After 24 hours, transfected cells were left untreated or treated with TRAIL (250 ng/mL) for 5 hours and luciferase reporter assay was done.

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References

1. Griffith TS, Chin WA, Jackson GC, Lynch DH, Kubin MZ. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. J Immunol 1998;161: 2833–40.
2. Thomas WD, Hersey P. TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in Fas ligand-resistant melanoma cells and mediates CD4 T cell killing of target cells. J Immunol 1998;161: 2195–200.
3. Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. Nat Med 1999;5:157–63.
4. Wang J, Zheng L, Lobito A, et al. Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. Cell 1999; 98:47–58.
5. Jeremias I, Herr I, Boehler T, Debatin K. TRAIL/Apo-2-ligand-induced apoptosis in human T cells. Eur J Immunol 1998;28:143–52.
6. Yamada H, Tada-Oikawa S, Uchida A, Kawanishi S. TRAIL causes cleavage of bid by caspase-8 and loss of mitochondrial membrane potential resulting in apoptosis in BJAB cells. Biochem Biophys Res Com 1999;265: 130–3.
7. Deng Y, Lin Y, Wu X. TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. Genes Dev 2002;16:33–45.
8. Kim JY, Kim YH, Chang I, et al. Resistance of mitochondrial DNA-deficient cells to TRAIL: role of Bax in TRAIL-induced apoptosis. Oncogene 2002;21: 3139–48.
9. Kandasamy K, Srinivasula SM, Alnemri ES, et al. Involvement of proapoptotic molecules Bax and Bak in

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tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial disruption and apoptosis: differential regulation of cytochrome *c* and Smac/DIABLO release. Cancer Res 2003;63:1712–21.

10. Pan G, O'Rourke K, Chinnaiyan AM, et al. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science 1997;276:111–3.

11. Sheridan JP, Marsters SA, Pitti RM, et al. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science 1997;277:818–21.

12. Hu WH, Johnson H, Shu HB. Tumor necrosis factor-related apoptosis-inducing ligand receptors signal NF-κB and JNK activation and apoptosis through distinct pathways. J Biol Chem 1999;43:30603–10.

13. Schneider P, Thome M, Burns K, et al. TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-κB. Immunity 1997;7:831–6.

14. Suk K, Chang I, Kim YH, et al. Interferon γ (IFNγ) and tumor necrosis factor α synergism in ME-180 cervical cancer cell apoptosis and necrosis. IFNγ inhibits cytoprotective NF-κB through STAT1/IRF-1 pathways. J Biol Chem 2001;276:13153–9.

15. Chang I, Kim S, Kim JY, et al. Nuclear factor κB protects pancreatic β-cells from tumor necrosis factor-α-mediated apoptosis. Diabetes 2003;52:1169–75.

16. Kang KH, Lee KH, Kim MY, Choi KH. Caspase-3-mediated cleavage of the NF-κB subunit p65 at the NH2 terminus potentiates naphthoquinone analog-induced apoptosis. J Biol Chem 2001;276:24638–44.

17. Levkau B, Scatena M, Giachelli CM, Ross R, Raines EW. Apoptosis overrides survival signals through a caspase-mediated dominant-negative NF-κB loop. Nat Cell Biol 1999;1:227–33.

18. Harper N, Farrow SN, Kaptein A, Cohen GM, MacFarlane M. Modulation of tumor necrosis factor apoptosis-inducing ligand-induced NF-κB activation by inhibition of apical caspases. J Biol Chem 2001;276:34743–52.

19. Chen X, Kandasamy K, Srivastava RK. Differential roles of RelA (p65) and c-Rel subunits of nuclear factor κB in tumor necrosis factor-related apoptosis-inducing ligand signaling. Cancer Res 2003;63:1059–66.

20. Ravi R, Bedi A, Fuchs EJ, Bedi A. CD95 (Fas)-induced caspase-mediated proteolysis of NF-κB. Cancer Res 1998;58:882–6.

21. Chen FE, Huang DB, Chen YQ, Ghosh G. Crystal structure of p50/p65 heterodimer of transcription factor NF-κB bound to DNA. Nature 1998;391:410–3.

22. Chawla-Sarkar M, Bauer JA, Lupica JA, et al. Suppression of NF-κB survival signaling by nitrosylcobalamin sensitizes neoplasms to the anti-tumor effects of Apo2L/TRAIL. J Biol Chem 2003;278:39461–9.

23. Muhlethaler-Mottet A, Bourloud KB, Auderset K, Joseph JM, Gross N. Drug-mediated sensitization to TRAIL-induced apoptosis in caspase-8-complemented neuroblastoma cells proceeds via activation of intrinsic and extrinsic pathways and caspase-dependent cleavage of XIAP, Bcl-xL and RIP. Oncogene 2004;23:5415–25.

24. Oya M, Ohtsubo M, Takayanagi A, Tachibana M, Shimizu N, Murai M. Constitutive activation of nuclear factor-κB prevents TRAIL-induced apoptosis in renal cancer cells. Oncogene 2001;20:3886–96.

25. Ehrhardt H, Fulda S, Schmid I, Hiscott J, Debatin KM, Jeremias I. TRAIL induced survival and proliferation in cancer cells resistant towards TRAIL-induced apoptosis mediated by NF-κB. Oncogene 2003;22:3842–52.

26. Shin MS, Kim HS, Kang CS, et al. Inactivating mutations of CASP10 gene in non-Hodgkin lymphomas. Blood 2002;99:4094–9.

27. Kim HS, Lee JW, Soung YH, et al. Inactivating mutations of caspase-8 gene in colorectal carcinomas. Gastroenterology 2003;125:708–15.

28. Zhang Q, Siebert R, Yan M, et al. Inactivating mutations and overexpression of BCL10, a caspase recruitment domain-containing gene, in MALT lymphoma with t(1;14)(p22;q32). Nat Genet 1999;22:63–8.

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