Differential Roles of RelA (p65) and c-Rel Subunits of Nuclear Factor κB in Tumor Necrosis Factor-related Apoptosis-inducing Ligand Signaling

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ABSTRACT

Apo-2L/Trail (tumor-necrosis factor-related apoptosis-inducing ligand) is a member of the tumor necrosis factor superfamily and has recently been shown to induce apoptosis through engagement of the death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5). The transcription factor nuclear factor (NF)-κB regulates the expression of genes involved in cancer cell invasion, metastasis, and resistance to chemotherapeutics. In normal unstimulated cells, NF-κB is maintained in the cytoplasm with its inhibitor protein IκBα, whereas in cancer cells, NF-κB is in the nucleus and constitutively activates target genes. To understand the function of NF-κB in TRAIL-induced apoptosis, we have analyzed the specific roles of NF-κB subunits. Overexpression of a transdominant-negative mutant of the inhibitory protein IκBα results in down-regulation of constitutively active NF-κB, induction of DR5, and tumor necrosis factor receptor (TNFR) 1-associated death domain expression and enhancement of TRAIL sensitivity. Overexpression of RelA or a transcriptional-deficient mutant of c-Rel inhibits TRAIL-induced apoptosis. Depletion of RelA in mouse embryonic fibroblasts increases cytokine-induced apoptosis, whereas depletion of c-Rel blocks this process. Overexpression of RelA subunit inhibits caspase-8 and DR4 and DR5 expression and enhances expression of cIAP1 and c-IAP2 after TRAIL treatment. By comparison, overexpression of c-Rel enhances DR4, DR5, and Rel-X, and inhibits cIAP1, cIAP2, and survival after TRAIL treatment. These results suggest that the RelA subunit acts as a survival factor by inhibiting expression of DR4/DR5 and caspase-8 and up-regulating cIAP1 and cIAP2. The dual function of NF-κB, as an inhibitor or activator of apoptosis, depends on the relative levels of RelA and c-Rel subunits. Thus, NF-κB activity may play an important role in tumor progression, and down-regulation of RelA or up-regulation of c-Rel represents a possible therapeutic target for the treatment of cancer.

INTRODUCTION

Apoptosis is genetically controlled process that plays an essential role in embryogenesis, homeostasis, and the cellular response to stressful stimuli (1–3). Genetic aberrations that render cells incapable of executing their suicide program promote tumorigenesis and underlie the observed resistance of human cancers to genotoxic anticancer agents (4). Thus, revealing the mechanisms of apoptotic program in tumor cells might aid the monitoring of molecular targets and design of effective therapeutic interventions against resistant human cancers.

Recently, Apo-2L/TRAIL3 has been shown to be a potential candidate for cancer therapy (5, 6). We and others (7–14) have shown that TRAIL induces apoptosis in various cancer cell lines, including those that resist to chemotherapeutic agents or ionizing radiation because of inactivating mutations of the p53 tumor suppressor gene, but is less effective in nontransformed cells (15–17). TRAIL induces apoptosis by binding to TRAIL-R1 (DR4/Apo-2A) and TRAIL-R2 (DR5/TRICK/Killer; Refs. 7, 9, 18–22). Both TRAIL-R1 and TRAIL-R2 contain a conserved cytoplasmic sequence, termed death domains, that can recruit adapter proteins and activate caspase-8 (12, 23, 24). The cleavage and activation of caspase-8, in turn, activate downstream effector caspses such as caspase-3 and caspase-7 (25, 26). Activation of caspase-8 by TRAIL may also cleave Bid (a Bcl-2 inhibitory protein), the cleavage product of which triggers mitochondrial depolarization (decrease in ΔΨm) and subsequent release of cytochrome c from mitochondria (10, 25, 27). Once released into the cytosol, cytochrome c binds to apoptotic protease-activating factor 1 and, in the presence of dATP, recruits and activates procaspase-9 to form the apoptosome (27). Activated caspases cleave several downstream death substrates and activate endonucleases, resulting in the apoptosis (28–30). Other three TRAIL receptors, TRAIL-R3 (TRID/Dec/1/LIT; Refs. 19, 31–33), TRAIL-R4 (TRUNDD/Dec2; Refs. 13, 34), and osteoprotegerin (35), also bind to TRAIL. TRAIL-R3 and TRAIL-R4 have extracellular domains similar to TRAIL-R1 and TRAIL-R2 but lack a functional cytoplasmic death domain. TRAIL-R3 and TRAIL-R4 may serve as decoy receptors, whereas the fifth receptor, osteoprotegerin, is a secreted protein with no known membrane anchor.

NF-κB is a transcription factor that plays an important role in controlling immune and inflammatory responses, cellular proliferation and adhesion molecules (36–39). NF-κB is a heterodimeric or homodimeric complex formed from five distinct subunits, RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52; Refs. 36, 38, 40). RelA (p65), RelB, and c-Rel are transcriptionally active members of the NF-κB family, whereas p50 and p52 primarily serve as DNA binding subunits (36–38). The p50 and p52 NF-κB subunits are derived from larger precursor products, p105 and p100, respectively, or from differential translation of their mRNAs. The classical form of NF-κB, the heterodimer of p50 and p65 subunits, is normally retained in the cytoplasm in association with inhibitor proteins IκBα and IκBβ. When phosphorylated on serine 32 and serine 36, IκBα is degraded by the ubiquitin/26S proteasome pathway, allowing NF-κB to translocate to the nucleus and regulate gene expression (36, 38, 39, 41). Recently, NF-κB has been implicated in protecting cells from apoptosis (42–45), whereas much evidence highlights an apparently paradoxical proapoptotic role for NF-κB (44, 46–48). On the basis of these studies, it appears that opposite functions of NF-κB lies on the expression of its subunits where c-Rel and RelA functions as proapoptotic and antiapoptotic proteins, respectively.

It has recently been shown that TRAIL can activate NF-κB (44, 49–51). However, the intracellular signaling pathways responsible for TRAIL receptor-mediated NF-κB activation and the role of distinct subunits of NF-κB in TRAIL-induced signaling are unclear. Here, we demonstrate the opposite roles of RelA (p65) and c-Rel subunits of NF-κB in apoptosis. RelA deficiency enhances, whereas c-Rel deficiency blocks TRAIL-induced apoptosis. Thus, NF-κB may play an important role in the sensitivity of cancer cells to the apoptotic response to TRAIL.

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2 The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IκB, inhibitor of nuclear factor-κB; FBS, β-galactosidase; fetal bovine serum; EMSA, electrophoretic mobility shift assay; DAPI, 4',6-diamidino-2-phenylindole; MEF, mouse embryonic fibroblast; TNF, tumor necrosis factor; Fast, β-galactosidase; TRADD, tumor necrosis factor receptor (TNFR1)-associated death domain; RIP, receptor interacting protein; IAP, inhibitor of apoptosis protein.

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MATERIALS AND METHODS

Reagents. Antibodies against Rel A (p65), p50, IxBα, Bcl-XL, and c-Rel were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); antibodies against DR4 and DR5 were from Imgenex, Inc. (San Diego, CA); and antibody against actin was from Oncogene Research (Boston, MA). ELISA kits for DR4 and DR5 were purchased from BioSource International, Inc. (Camarillo, CA).

Enhanced chemiluminescence Western blot detection reagents were from Amersham Life Sciences, Inc. (Arlington Heights, IL). LipofectAMINE reagent was from Invitrogen Life Technologies (Carlsbad, CA). p50/p65, IxBα, and empty vector (pCMV-4-p50) were used to transfect MDA-MB-231 cells because MDA-MB-231 cells express low level of p50/p65. p50/p65, IxBα, and empty vector (pCMV-4-p50) or empty vector (pCMV-4-neo) were used to transfect MCF-7 cells. MCF-7 cells are from Amersham Life Sciences, Inc. (Arlington Heights, IL). LipofectAMINE reagent was from Invitrogen Life Technologies, Inc. (Carlsbad, CA).

Cells and Culture Conditions. MDA-MB-231 and MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA). They were used in the current study because MDA-MB-231 cells express low level of constitutive active NF-kB compared with MCF-7 cells. Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin mixture. All of the cells were maintained at 37°C with 5% CO2.

Transient Transfection. Cells were plated in 60-mm dishes in RPMI 1640 containing 10% FBS and 1% penicillin-streptomycin mixture at a density of 1 x 10⁵ cells/dish. The next day, transfection mixtures were prepared. Cells were transfected with expression constructs encoding mutant IxBα (pCMV4-IxBαM and S32/36), IxBα (pCMV4-p50), p50 (pCMV4-p50), or empty vector (pCMV4-neo) as control in the presence of a vector pCMV-LacZ (Invitrogen Life Technologies, Inc.) expressing β-gal. For each transfection, 2 μg of DNA were diluted in 50 μl of medium without serum. After the addition of 3 μl of LipofectAMINE into 50 μl of Opti-MEM, the transfection mixture was incubated for 10 min at room temperature. Cells were washed with serum-free medium, the transfection mixture was added, and cultures were incubated for 24 h in the incubator. The next day, culture medium was replaced with fresh RPMI 1640 containing 10% FBS and 1% penicillin-streptomycin mixture, and TRAIL was added. At the end of incubation, cells were harvested and washed with ice-cold PBS.

Flow Cytometry. Cells were treated with TRAIL (20 ng/ml) for various time points, harvested, and fixed in 85% ethanol. Cells were then stained with propidium iodide in PBS with 0.5% NP40 and RNase A. Propidium iodide-stained cells were analyzed using a Beckton Dickinson FACStar flow cytometer. Data were analyzed by ModFit LT.

EMSA. Nuclear extracts were prepared from cells. Double-stranded oligonucleotides containing consensus binding sites for NF-kB (GATCAGGCGCACCTTCCCTAGC; Promega) were used. The 5’-end was labeled using polynucleotide kinase and [γ-32P]ATP. Nuclear extracts (5.0 μg) were incubated with 1 μl of labeled oligonucleotide (20,000 cpm) in 20 μl of incubation buffer [10 mM Tris-HCl, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 2% glycerol, and 2 μg of poly(deoxyinosinic-deoxycytidic acid)] for 20 min at room temperature. The specificity of NF-kB DNA binding activity was confirmed by competition with excess cold wild-type or nonconsensus oligonucleotide. Supershift was assayed by additional incubations with rabbit polyclonal antibodies against p65, p50, or c-Rel for 45 min at room temperature before incubation of the labeled oligonucleotide. Antibody-β-actin was used as control. DNA protein complexes were resolved by electrophoresis in 6% nondenaturing polyacrylamide gels and analyzed by autoradiography.

Reporter Transfection and Luciferase Assay. The transcription activity of NF-kB was assayed by the transfection of luciferase reporter containing specific consensus sequence of NF-kB and β-gal as a control into cells, and luciferase activity was measured and normalized by β-gal activity as per manufacturer’s directions (Promega).

RNase Protection Assay. Total RNAs were extracted using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). The RNase Protection Assay was performed as per manufacturer’s directions (Pharmingen). Briefly, 2 μg of RNA was incubated with α-32P-UTP labeled single-stranded RNA probes overnight at 56°C and treated with RNase for 45 min at 30°C. The RNA-RNA complexes were resolved by electrophoresis in 6% denaturing polyacrylamide gels and analyzed by autoradiography.

Immunohost Assays. Cells were lysed in a buffer containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.5 mM EDTA, 1 mM EGTA, 1% SDS, 1 mM sodium orthovanadate, and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, and 2 μg/ml aprotinin). Lysates were centrifuged for 20 min at 12,000 × g and stored at −70°C. Proteins were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membrane (Bio-Rad), and probed with appropriate dilutions of primary antibodies. Immunoreactive protein complexes were visualized by enhanced chemiluminescence kit (Amersham).

DR4 and DR5 ELISA. MDA-MB-231 and MCF-7 cells were treated with TRAIL for 48 h. At the end of incubation period, cells were harvested and washed twice with ice-cold PBS. Cells were lysed in extraction buffer for 30 min on ice with vortexing at 10-min intervals. The extracts were centrifuged at 13,000 rpm for 10 min at 4°C. Lysates were aliquoted and assayed for DR4 and DR5 proteins by ELISA as per manufacturer’s instructions (Biosource International, Inc., Camarillo, CA).

RESULTS

TRAIL Activates NF-kB in Breast Cancer MDA-MB-231 and MCF-7 Cells. Recent studies have shown the involvement of NF-kB in the regulation of apoptosis (52–54). In some breast cancer cells, NF-kB is constitutively activated (54, 55) and, therefore, may change the apoptotic response of several anticancer drugs and irradiation. To determine whether NF-kB plays a role in TRAIL-induced apoptotic signaling in breast cancer cells, we first examined the activation of NF-kB (Fig. 1A). MDA-MB-231 and MCF-7 cells were treated with soluble TRAIL for various time periods. Translocation of NF-kB to nucleus was examined by EMsAs. The NF-kB binding activity was increased in a time-dependent manner in MDA-MB-231 cells after treatment with TRAIL, reaching a maximum at 2 h (Fig. 1A). In MCF-7 cells, a robust activation of NF-kB was observed in untreated cells, which was additionally increased by TRAIL treatment as early as 20 min (Fig. 1A).

We next examined the transcriptional activation of NF-kB by luciferase assay. MDA-MB-231 and MCF-7 cells were transiently transfected with NF-kB-luciferase reporter plasmid construct and the NF-kB transactivation was measured by the luciferase assay (Fig. 1B). The data showed that NF-kB transactivation was induced 25-fold in MDA-MB-231 cells at 2 h and 3.5-fold in MCF-7 cells at 40 min by TRAIL (Fig. 1B). Thus, these data suggest that TRAIL can induce NF-kB-DNA binding and NF-kB transcriptional activity of NF-kB in MDA-MB-231 and MCF-7 cells.

Effects of RelA (p65), p50, and c-Rel Subunits on TRAIL-induced NF-kB-DNA Binding Activity. Recent studies have shown conflicting data on the physiological role of NF-kB on cell survival and apoptosis (37, 43–48). It is possible that the dual functions of NF-kB depend on its subunits. To additionally identify which NF-kB subunit(s) contributes to enhanced DNA binding activity, we performed NF-kB assay with specific antibodies against different NF-kB subunits, i.e., RelA (p65), p50, and c-Rel (Fig. 2). An antibody against β-actin was also used in the experiment as a negative control. Treatment of cells with TRAIL enhanced the NF-kB-DNA binding activity in MDA-MB-231 cells. Incubation of nuclear extracts with unlabelled consensus NF-kB oligonucleotide completely displaced complexes I and II, suggesting the specificity of NF-kB bands. An antibody specific for the p65 subunit of NF-kB, which can recognize p65 homodimers and p50/p65 heterodimers of NF-kB, supershifted complex I and was unable to shift complex II in MDA-MB-231 cells (Fig. 2). An antibody specific for p50 also caused a supershift in MDA-MB-231 cells. By comparison in MCF-7 cells, anti-p50 antibody caused a major supershift. No significant supershift was observed with anti-c-Rel antibody in both cell lines, probably because of the nature of antibody (Fig. 2). Anti-β-actin antibody did not cause supershift in both MDA-MB-231 and MCF-7 cells.
Differential Effects of RelA (p65) and c-Rel on TRAIL-induced Apoptosis. The physiological role of NF-κB in cell survival and apoptosis is not clear (37, 43–48). NF-κB has been found to act as an antiapoptotic or a proapoptotic factor. The opposing effects of NF-κB on cell survival and apoptosis may be attributable to differential expression of its subunits. We therefore sought to examine the specific role of NF-κB subunits on TRAIL-induced signaling. MDA-MB-231 and MCF-7 cells were transiently transfected with plasmid cDNAs containing different subunits of NF-κB (RelA/p65, p50, and c-Rel) and a mutant NF-κB inhibitory protein IκBα (serine 32/serine 36) termed as mIκBα. We first confirmed the transfection of various subunits of NF-κB (RelA, p50, and c-Rel) and mIκBα by the Western blot analysis (Fig. 3A). The data revealed that transfected genes were overexpressed significantly in MDA-MB-231 cells.

To determine the specific role of NF-κB subunits in TRAIL-induced apoptosis, transfected cells were treated with or without TRAIL (20 ng/ml) for various time periods, fixed, and stained with DAPI. The mIκBα (serine 32/serine 36) expression slightly enhanced the apoptotic response to TRAIL (20 ng/ml) in both MDA-MB-231 and MCF-7 cells (Fig. 3B). Overexpression of RelA (p65) subunit significantly attenuated TRAIL-induced apoptosis in MDA-MB-231 and MCF-7 cells at 12 and 24 h (Fig. 3B). By comparison, transfection of cells with c-Rel enhanced TRAIL-induced apoptosis in both MDA-MB-231 and MCF-7 cells at 24 h. Overexpression of p50 subunit had no significant effect on TRAIL-induced apoptosis in both MDA-MB-231 and MCF-7 cells. Considering the different activation status of NF-κB induced by TRAIL, the results suggested that RelA (p65) subunit of NF-κB blocks, whereas c-Rel enhances TRAIL-induced apoptosis at 24 h.

Because RelA (p65) subunit of NF-κB inhibited TRAIL-induced apoptosis, we next examined its effect on DNA binding and NF-κB activity (Fig. 4). MDA-MB-231 cells were transiently transfected with plasmid cDNAs containing RelA subunit of NF-κB or a mIκBα. MDA-MB-231 cells showed increased NF-κB-DNA binding activity.

**Fig. 1.** Time course of TRAIL-induced NF-κB activation in breast cancer cells. A, MDA-MB-231 and MCF-7 cells were treated with TRAIL (25 ng/ml) for 20, 40, 60, 120, and 300 min. After the incubated period, cells were harvested and nuclear extracts were prepared. Nuclear extracts were analyzed for NF-κB binding with its consensus sequence by EMSA. B, MDA-MB-231 and MCF-7 cells were transfected with NF-κB/luciferase reporter plasmid and a cytomegalovirus 4 promoter-driven β-gal expression plasmid to normalize the transfection efficiency. The luciferase activity was assayed for NF-κB activation and normalized to β-gal.

**Fig. 2.** Involvement of various subunits of NF-κB in TRAIL-induced NF-κB activation. MDA-MB-231 and MCF-7 cells were treated with or without TRAIL (20 ng/ml) for 2 h and 40 min, respectively. Nuclear extracts were incubated in the presence or absence of antibodies against anti-p65, anti-p50, and anti-c-Rel to determine the presence of NF-κB subunits in the nuclear extract by super shift assay. An anti-β-actin antibody was used as a control.
of RelA (p65) subunit, whereas mIκBα decreased the NF-κB binding activity in MDA-MB-231 cells (Fig. 4A). Treatment of cells with MG132, a proteosome inhibitor, inhibited TRAIL-induced NF-κB-DNA binding activity.

Because RelA (p65) subunit of NF-κB enhanced NF-κB-DNA binding activity, we sought to examine whether this subunit increases the transcriptional activity of NF-κB. MDA-MB-231 cells were transiently transfected with plasmid cDNAs containing various subunits of NF-κB or mIκBα and treated with or without TRAIL for 2 h (Fig. 4B). Treatment of cells with TRAIL alone caused an enhancement in luciferase activity. Overexpression of mIκBα inhibited,
 whereas RelA (p65) enhanced TRAIL-induced luciferase activity. Under these conditions, overexpression of p50 had no effect on luciferase activity, whereas c-Rel subunit slightly enhanced luciferase activity in MDA-MB-231 cells treated with various subunits of NF-κB or mLxBo with a plasmid (pCMV-LacZ) encoding β-gal enzyme. More than 85% of cells were transfected, and there were no significant differences in transfection efficiency among groups. Transfectants were treated with or without TRAIL (25 ng/ml) for 12 h. Total RNA was used in RNase protection assay (hAPO-3c; PharMingen) to measure the expression of genes. L32 and glyceraldehyde-3-phosphate dehydrogenase were shown as housekeeping genes. C, effects of various subunits of NF-κB on the expression of death receptors DR4 and DR5 in breast cancer cells. MDA-MB-231 cells overexpressing various subunits of NF-κB were treated with or without TRAIL (25 ng/ml) for 24 h. The Western blots were performed with anti-DR4 antibody or anti-DR5 antibody. An anti-β-tubulin antibody was used as a loading control. D–G, effects of various subunits of NF-κB on the expression of death-related genes (caspase-8, FasL, Fas, DcR1, DR3, DR4, DR5, TRADD, and RIP) in breast cancer cells. A and B, MDA-MB-231 cells were transfected with various subunits of NF-κB or mLxBo with a plasmid (pCMV-LacZ) encoding β-gal enzyme. More than 85% of cells were transfected, and there were no significant differences in transfection efficiency among groups. Transfectants were treated with or without TRAIL (25 ng/ml) for 12 h. Total RNA was used in RNase protection assay (hAPO-3c; PharMingen) to measure the expression of genes. L32 and glyceraldehyde-3-phosphate dehydrogenase were shown as housekeeping genes. C, effects of various subunits of NF-κB on the expression of death receptors DR4 and DR5 in breast cancer cells. MDA-MB-231 cells overexpressing various subunits of NF-κB were treated with or without TRAIL (25 ng/ml) for 24 h. The Western blots were performed with anti-DR4 antibody or anti-DR5 antibody. An anti-β-tubulin antibody was used as a loading control. D–G, effects of various subunits of NF-κB on the expression of death-related genes (caspase-8, FasL, Fas, DcR1, DR3, DR4, DR5, TRADD, and RIP) in breast cancer cells. A and B, MDA-MB-231 cells were transfected with various subunits of NF-κB or mLxBo with a plasmid (pCMV-LacZ) encoding β-gal enzyme. More than 85% of cells were transfected, and there were no significant differences in transfection efficiency among groups. Transfectants were treated with or without TRAIL (25 ng/ml) for 12 h. Total RNA was used in RNase protection assay (hAPO-3c; PharMingen) to measure the expression of genes. L32 and glyceraldehyde-3-phosphate dehydrogenase were shown as housekeeping genes. C, effects of various subunits of NF-κB on the expression of death receptors DR4 and DR5 in breast cancer cells. MDA-MB-231 cells overexpressing various subunits of NF-κB were treated with or without TRAIL (25 ng/ml) for 24 h. The Western blots were performed with anti-DR4 antibody or anti-DR5 antibody. An anti-β-tubulin antibody was used as a loading control. D–G, effects of various subunits of NF-κB on the expression of death-related genes (caspase-8, FasL, Fas, DcR1, DR3, DR4, DR5, TRADD, and RIP) in breast cancer cells. A and B, MDA-MB-231 cells were transfected with various subunits of NF-κB or mLxBo with a plasmid (pCMV-LacZ) encoding β-gal enzyme. More than 85% of cells were transfected, and there were no significant differences in transfection efficiency among groups. Transfectants were treated with or without TRAIL (25 ng/ml) for 12 h. Total RNA was used in RNase protection assay (hAPO-3c; PharMingen) to measure the expression of genes. L32 and glyceraldehyde-3-phosphate dehydrogenase were shown as housekeeping genes.
caspase-8 was analyzed by RNase protection assay and immunoblotting. MDA-MB-231 and MCF-7 cells were transfected with different subunits of NF-κB or mIκBα and treated with or without of TRAIL. The RNase protection assays were performed with probe-set of hAPO-2c (caspase-8, Fas, Fasl, DcR1, DR3, DR5, DR4, TRAIL, TNFRp55, TRADD, and RIP) as per manufacturer’s directions (PharMingen). Overexpression of p65 subunit of NF-κB inhibited the mRNA expression of DR4, DR5, and caspase-8 in MDA-MB-231 cells treated with TRAIL (Fig. 6A). In contrast, overexpression of c-Rel subunit of NF-κB enhanced the mRNA expression of DR4, DR5, and caspase-8 in MDA-MB-231 cells treated with TRAIL (Fig. 6A). Overexpression of p50 subunit of NF-κB had no effect on the expression of caspase-8, FAS, Fasl, DcR1, DR3, DR5, DR4, TRADD, and RIP in the absence or presence of TRAIL. Overexpression of mIκBα significantly enhanced expression of DR5 and TRADD in MDA-MB-231 cells (Fig. 6B). Similarly, overexpression of p65 subunit of NF-κB inhibited the mRNA expression of DR4 and caspase-8 in MCF-7 cells treated with TRAIL (data not shown).

We additionally confirmed the expression of proteins in MDA-MB-231 cells by the Western blot analysis (Fig. 6C). Overexpression of RelA (p65) subunit of NF-κB in MDA-MB-231 cells inhibited death receptors DR4 and DR5 after TRAIL treatment. In contrast, overexpression of c-Rel subunit of NF-κB in MDA-MB-231 cells enhanced DR4 and DR5 proteins after TRAIL treatment. There was no difference in loading as evident from antitubulin antibody.

We next measured the effects of overexpression of p65 and c-Rel subunits of NF-κB on DR4 and DR5 proteins by ELISA (Biosource International). TRAIL induced DR4 and DR5 proteins in MDA/Neo cells (Fig. 6, D and E). Overexpression of RelA (p65) subunit of NF-κB in MDA-MB-231 cells inhibited death receptors DR4 and DR5 after TRAIL treatment. In contrast, overexpression of c-Rel subunit of NF-κB in MDA-MB-231 cells enhanced DR4 and DR5 proteins after TRAIL treatment. These data confirmed our previous findings that overexpression of Rel A (p65) inhibits DR4 and DR5, whereas overexpression of c-Rel enhances DR4 and DR5 proteins in MDA-MB-231 cells.

**Regulation of Bcl-2 Family Members by NF-κB.** Bcl-2 family proteins play important roles in apoptotic response of cancer cells treated with chemotherapeutic drugs or irradiation (30, 56). The regulation of Bcl-2 family members by NF-κB is still unclear. Recently, it has been shown that NF-κB can induce expression of Bcl-X<sub>L</sub> and a Bcl-2 homologue A1/Bfl-1 (57, 58). In our experiment, no significant changes were found in the transcriptional level of Bcl-2 family members (except Bcl-X) in TRAIL-induced apoptotic signaling in both of MDA-MB-231 and MCF-7 cells overexpressing different subunits of NF-κB and mIκBα (Fig. 7, A and B). Interestingly, overexpression of c-Rel significantly enhanced the expression of survivin, NAIP, c-IAP1, cIAP2, and TRAM2) in breast cancer cells. MDA-MB-231 cells were transfected with various subunits of NF-κB or mIκBα with a plasmid (pCMV-LacZ) encoding β-gal enzyme. More than 85% of cells were transfected, and there were no significant differences in transfection efficiency among groups. Transfectants were treated with or without of TRAIL (25 ng/ml) for 24 h. Bcl-X<sub>L</sub> protein was examined by the Western blot analysis. Tubulin was used as a loading control.

**Fig. 7.** Effects of various subunits of NF-κB on the expression of Bcl-2 family members (bcl-w, bcl-x, bfl1, bad, bik, bak, bax, bcl-2, and mcl-1) in breast cancer cells. (A and B), MDA-MB-231 and MCF7 cells were transfected with various subunits of NF-κB or mIκBα with a plasmid (pCMV-LacZ) encoding β-gal enzyme. More than 85% of cells were transfected, and there were no significant differences in transfection efficiency among groups. Transfectants were treated with or without TRAIL (25 ng/ml) for 12 h. Total RNA was used in RNase protection assay (hAPO-2c; PharMingen) to measure the expression of genes. L32 and glyceraldehyde-3-phosphate dehydrogenase were shown as housekeeping genes. C effects of various subunits of NF-κB on the expression of Bcl-X<sub>L</sub> protein. MDA-MB-231 cells were transfected with various subunits of NF-κB or mIκBα with a plasmid (pCMV-LacZ) encoding β-gal enzyme. There were no significant differences in transfection efficiency among groups. Transfectants were treated with or without TRAIL. (25 ng/ml) for 24 h. Bcl-X<sub>L</sub> protein was examined by the Western blot analysis. Tubulin was used as a loading control.

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Bcl-X in MDA-MB-231 cells after TRAIL treatment, this band was later identified as Bcl-XL protein by immunoblot analysis (Fig. 7C). Thus, the results suggested that c-Rel could promote the expression of proapoptotic gene Bcl-XL.

**Regulation of IAPs by NF-κB.** NF-κB can protect cells from apoptosis by promoting expression of survival genes such as Bfl-1/A1, TRAF1, TRAF2, c-IAP1, and c-IAP-2 (59, 60). However, which subunit of NF-κB is important in the regulation of survival factors in cells are unknown. We therefore examined the expression of IAP, c-IAP1, c-IAP2, and survivin in MDA-MB-231 cells overexpressing p65, p50, and c-Rel subunits of NF-κB or mIκBα (Fig. 8). The Western blot analysis revealed that overexpression of RelA/p65 subunit significantly enhanced the expression of cIAP1 and cIAP2, whereas overexpression of c-Rel subunit inhibited the expression of these proteins after TRAIL treatment in MDA-MB-231 cells (Fig. 8). Overexpression of p50 had no effect on these genes after TRAIL treatment. Furthermore, overexpression mIκBα inhibited cIAP1 and cIAP2 after TRAIL treatment. Overexpression of RelA (p65), p50, or mIκBα had no effect on the expression of survivin, whereas overexpression of c-Rel significantly inhibited survivin after TRAIL treatment.

**DISCUSSION**

In this study, we examined the biological roles of various subunit of NF-κB in TRAIL-induced apoptosis in breast cancer cells. The oncogenic role of NF-κB was observed earlier in leukemia and lymphoma (61), breast cancer (52), and pancreatic cancer (62). Apparently, the physiological roles of NF-κB in cell survival and apoptosis (37, 43, 45–48, 57, 59, 63–66) have provided conflicting views. In certain situations, NF-κB acts as an antiapoptotic, whereas in others it functions as proapoptotic transcription factor. Therefore, additional studies are needed to examine the physiological roles of NF-κB subunits.

In our study, overexpression of RelA inhibits TRAIL-induced apoptosis, whereas overexpression of c-Rel enhances TRAIL-induced apoptosis. Similarly, inhibition of RelA subunit of NF-κB results in hepatic cell death and embryonic lethality of mice (43). Furthermore, depletion of Rel A or inhibition of NF-κB phosphorylation, via the expression of the super-repressor form of IκBα, sensitizes cells to cytokine-induced apoptosis (43, 45, 53, 65). This suggests that NF-κB plays a survival role in oncogenesis because inhibition of NF-κB in transformed cells can induce apoptosis. In contrast to these studies, NF-κB can function as proapoptotic in other situations. Interestingly, constitutively active NF-κB through targeted disruption of IκBα causes a massive thymic and splenic cell death in mouse embryos (63). Furthermore, high levels of the c-Rel subunit of NF-κB have been noticed during apoptosis in the developing avian embryo (46).

On the basis of our studies, it appears that dual functions of NF-κB, as proapoptotic or antiapoptotic, depend on its subunits c-Rel or RelA (p65), respectively. Activation of c-Rel subunit of NF-κB results in induction of apoptosis-related genes such as DR4, DR5, and Bcl-XL, and inhibition of survival genes such as cIAP1, cIAP2, and survivin. By comparison, activation of RelA (p65) subunit of NF-κB inhibits expression of apoptotic genes such as caspase-8, DR4, and DR5 and enhances expression of cIAP1 and cIAP2. Thus, the ratio between c-Rel and RelA (p65) subunits will determine whether the activation of NF-κB will trigger apoptotic or survival signal.

In our study, MCF-7 cells express higher level constitutively active NF-κB and are less sensitive to TRAIL-induced apoptosis compared with MDA-MB-231 cells, which express lower level of constitutively active NF-κB. It appears that constitutive activation of NF-κB can prevent TRAIL-induced apoptosis. The mechanism by which the constitutive activation of NF-κB antagonizes TRAIL-induced apoptosis remains to be elucidated. There are several possible candidates that may regulate apoptosis. NF-κB suppression of apoptosis appears to be at transcriptional level because it regulates expression of TRAF1, TRAF2, cIAP-1, and cIAP-2. Other antiapoptotic genes that are transcriptionally activated by NF-κB are the Bcl-2 homologues A1/Bfl1 and Bcl-XL, LEX-1, and XIAP. NF-κB can antagonize p53 function, possibly through the cross-competition for transcriptional coactivators. Conversely, p53 may in some cases act through NF-κB to induce apoptosis (48).

It has been reported that constitutively active NF-κB in breast cancer cells generally does not consist of p50-p65 heterodimer but rather complexes that contain p50, p52, and Bcl-3 (55). The nuclear accumulation of Bcl-3 is independent of inhibition by other IκB proteins and of IκB kinase stimulation. Thus, IκB expression does not generate a true NF-κB null phenotype and may, in fact, lead to the up-regulation of functionally different NF-κB complexes. Therefore, different NF-κB complexes (some lacking NF-κB) may control growth and differentiation in different cell types or in response to different stimuli. It is possible to speculate that there are opposing roles for NF-κB subunits in transformation.

In summary, we have provided direct evidence that the dual function of NF-κB, as an inhibitor or activator of apoptosis, depends on the relative levels of RelA (p65) or c-Rel subunits, respectively. The ratio between RelA (p65) and c-Rel subunits will determine whether activation of NF-κB will trigger apoptotic or survival signal. In addition, up-regulation of RelA (p65) subunit of NF-κB can enhance TRAIL resistance, whereas up-regulation of c-Rel can enhance TRAIL sensitivity. Thus, regulation of NF-κB subunits may be a novel strategy for cancer therapy.

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Differential Roles of RelA (p65) and c-Rel Subunits of Nuclear Factor κB in Tumor Necrosis Factor-related Apoptosis-inducing Ligand Signaling

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