

Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptors Signal NF- κ B and JNK Activation and Apoptosis through Distinct Pathways*

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Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family that interacts with several receptors, including TRAIL-R1, TRAIL-R2, and TRAIL-R4. TRAIL-R1 and TRAIL-R2 can induce apoptosis of cancer cells and activate the transcription factor NF- κ B. TRAIL-R4 can activate NF- κ B and protect cells from TRAIL-induced apoptosis. Here we show that TRAIL-R1-, TRAIL-R2-, and TRAIL-R4-induced NF- κ B activation are mediated by a TRAF2-NIK-I κ B kinase α/β signaling cascade but is MEKK1 independent. TRAIL receptors also activate the protein kinase JNK. JNK activation by TRAIL-R1 is mediated by a TRAF2-MEKK1-MKK4 but not the TRAF2-NIK/I κ B kinase α/β signaling pathway. We also show that activation of NF- κ B or overexpression of TRAIL-R4 does not protect TRAIL-R1-induced apoptosis. Moreover, inhibition of NF- κ B by I κ B α sensitizes cells to tumor necrosis factor- but not TRAIL-induced apoptosis. These findings suggest that TRAIL receptors induce apoptosis, NF- κ B and JNK activation through distinct signaling pathways, and activation of NF- κ B is not sufficient for protecting cells from TRAIL-induced apoptosis.

“death domain” that is required for TRAIL-R1- and TRAIL-R2-induced apoptosis. Three additional receptors, TRAIL-R3 (TRID/DcR1/LIT) (7, 10, 13, 14), TRAIL-R4 (15, 16), and osteoprotegerin (17), also bind to TRAIL. TRAIL-R3 does not have a cytoplasmic domain and can protect cells from TRAIL-induced apoptosis, probably by functioning as a “decoy” receptor (7, 10). TRAIL-R4 retains a cytoplasmic fragment containing one-third of the consensus death domain motif. Overexpression of TRAIL-R4 activates the transcription factor NF- κ B and protects cells against TRAIL-induced apoptosis, suggesting that TRAIL-R4, unlike TRAIL-R3, has a functional intracellular signaling domain (15, 16). Thus, TRAIL-R4 may protect cells from TRAIL-induced apoptosis by either acting as a decoy receptor or transducing an anti-apoptotic signal. In this context, several studies have established that NF- κ B activation can protect cells from TNF-induced apoptosis, probably through its ability to induce the expression of anti-apoptosis genes (18–20). In addition to TRAIL-R4, it has been shown recently that TRAIL-R1 and TRAIL-R2 can also activate NF- κ B (8, 10, 21). These observations suggest that activation of NF- κ B alone is not sufficient to block apoptosis induced by TRAIL receptors.

Tumor necrosis factor (TNF)¹-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family, which also includes TNF and FasL (1–3). Unlike TNF and FasL, which are mainly expressed by activated immune cells, TRAIL is constitutively expressed in most normal tissues (4, 5). Previous studies suggest that TRAIL is capable of inducing apoptosis of various cancer cell lines but not of normal cells (3–5), pointing to the possibility of developing TRAIL as a reagent for cancer treatment.

TRAIL induces apoptosis through two receptors, TRAIL-R1 (DR4) (3, 6) and TRAIL-R2 (DR5) (7–12). Both TRAIL-R1 and TRAIL-R2 contain a conserved cytoplasmic region called

Currently, the intracellular signaling pathways responsible for TRAIL receptor-mediated NF- κ B activation are unclear, and the mechanisms responsible for TRAIL receptor-induced apoptosis are controversial. It has been reported that TRADD and FADD, two death domain-containing cytoplasmic proteins involved in TNF-R1 signaling, interact with TRAIL-R1 and TRAIL-R2 and are involved in apoptosis mediated by these receptors (8, 21). However, other studies have reached opposite conclusions (6, 7, 10). Moreover, studies using FADD knockout embryonic fibroblasts suggest that FADD is not required for apoptosis induced by overexpression of TRAIL-R1 (22).

The signaling pathways mediated by TNF receptor family members have been best illustrated by studies with TNF-R1. TNF-R1 is a death domain-containing receptor that can induce apoptosis and activate NF- κ B and JNK kinase (23–26). The death domain of TNF-R1 interacts with TRADD in a TNF-dependent process (24, 25, 27). Once TRADD is recruited to TNF-R1, it functions as an adapter protein to recruit several structurally and functionally divergent proteins, including FADD, RIP, TRAF2, and cIAP1 (25, 27, 28). The interaction of TRADD with FADD leads to apoptosis through the activation of a caspase cascade (24). The interaction of TRADD with TRAF2 and RIP activates NIK, a member of the mitogen-activated protein kinase kinase kinase family (29). Once NIK is activated, it further activates two downstream kinases, IKK α and IKK β (29–34). It has been shown that IKK α and IKK β form a heterodimer complex that directly phosphorylates I κ Bs (29–35). Once I κ Bs are phosphorylated, they are degraded, and consequently the active NF- κ B is released (36, 37).

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¹ The abbreviations used are: TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; NF- κ B, nuclear factor κ B; JNK, c-Jun NH₂-terminal kinase; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; GST, glutathione S-transferase; X-gal, 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside; CMV, cytomegalovirus; TRADD, TNF receptor-associated death domain protein; FADD, Fas-associated death domain protein; NIK, NF- κ B-inducing kinase; IKK, I κ B kinase; I κ B, inhibitory κ B; TRAF, TNF receptor-associated factor; RIP, receptor interacting protein; MKK, mitogen-activated protein kinase kinase.

In addition to NIK, TRAF2 and RIP can also activate MEKK1, another member of the mitogen-activated protein kinase kinase family (26). Although it has been suggested that overexpression of MEKK1 activates NF- κ B (38), it is believed that under physiological conditions, MEKK1 mediates TNF-R1-induced JNK but not NF- κ B activation (35).

In this study, we investigated the mechanism of downstream signaling by TRAIL receptors. The results indicate that TRAIL-R1-, TRAIL-R2-, and TRAIL-R4-induced NF- κ B activation are mediated by a TRAF2-NIK-IKK α/β -dependent signaling cascade, whereas TRAIL-R1-induced JNK activation is mediated by a TRAF2-MEKK1-MKK4 dependent signaling cascade. We also show that inhibition of TRAIL-R1-induced NF- κ B and JNK activation pathways does not block TRAIL-R1-induced apoptosis. In addition, our data indicate that NF- κ B activation is not sufficient for protecting cells from TRAIL-induced apoptosis.

EXPERIMENTAL PROCEDURES

Reagents and Cell Line—Recombinant human TRAIL was provided by Dr. Bryant Darnay (University of Texas MD Anderson Cancer Center, Houston, TX). The human embryonic kidney 293 cell line was provided by Dr. Zaodan Cao (Tularik Inc., South San Francisco, CA).

Reporter Constructs and Mammalian Expression Vectors—The NF- κ B luciferase reporter construct (Dr. Gary Johnson, National Jewish Center, Denver, CO), mammalian expression vectors encoding TRAIL-R1 and TRAIL-R2 (Dr. Claudius Vincenz, University of Michigan, Ann Arbor, MI), TRADD(296S) (Dr. Vijal Baichwal, Tularik Inc., South San Francisco, CA), FADD-(80–205), TRAF2-(87–501), NIK(K429A/K430A), IKK α (K44A), IKK β (K44A), crmA (Dr. Dave Goeddel, Tularik Inc., South San Francisco, CA), I κ B(S32A/S36A) (Dr. T. Kurama, Yamanuchi Pharmaceuticals Inc., Japan), MEKK1 (K1255M), JNK1 (Dr. Gary Johnson, National Jewish Center, Denver, CO), and MKK4-DN (Dr. David Riches, National Jewish Center, Denver, CO) were obtained from the indicated sources.

TRAIL-R4 expression vector was constructed by replacing the TRAIL-R1 cDNA in the pCMV1-Flag-DR4 (TRAIL-R1) vector (6) with a polymerase chain reaction product of TRAIL-R4 cDNA. The parent vector contains a DNA fragment encoding a signal peptide at 5' of the Flag tag, and therefore the DNA fragment encoding the native N-terminal signal peptide of TRAIL-R4 was omitted by polymerase chain reaction.

Cell Transfection and Reporter Gene Assays—The human embryonic kidney 293 cell line was maintained in high glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 μ g/ml penicillin G, and 100 μ g/ml streptomycin (Life Technologies, Inc.). For reporter gene assays, $\sim 2 \times 10^5$ cells/well were seeded on 6-well (35 mm) dishes. Cells were transfected the following day by the standard calcium phosphate precipitation method (39). Luciferase reporter assays were performed using a luciferase assay kit (Pharmingen) following the manufacturer's protocols.

Western Blotting—Western blots for detection of Flag-tagged TRAIL-R1, TRAIL-R2, and TRAIL-R4 were performed with a monoclonal anti-Flag following previously described procedure (27, 40).

Yeast Two-hybrid Screenings—The cDNA encoding the intracellular domain of TRAIL-R1 was inserted in frame into the Gal4 DNA-binding domain vector pGBT9 (CLONTECH). The human leukocyte, spleen, and 293 cell two-hybrid cDNA libraries were also from CLONTECH. The isolation of positive clones and subsequent two-hybrid interaction analyses were carried out as described (23, 24, 28, 40).

Apoptosis Assays—293 cells were transfected with 0.1 μ g of pCMV- β -galactosidase plasmid and various amounts of indicated plasmids. β -Galactosidase co-transfection assays for determination of cell death were performed as described (23, 24, 28, 40). Transfected cells were stained with X-gal as described previously (41). The number of blue cells from four viewing fields in one well of a 35-mm dish was determined by counting under a microscope. The average number from one representative experiment in which each transfection was done in duplicate is shown.

Solid-Phase Kinase Assays—Cytokine-treated or -transfected cells were lysed with 600 μ l of ice-cold lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 2 mM Na₃VO₄, and 1 mM dithiothreitol). The lysate was mixed with 15 μ l of 1:1 slurry of GST-c-Jun-Sepharose beads, and the mixture was incu-

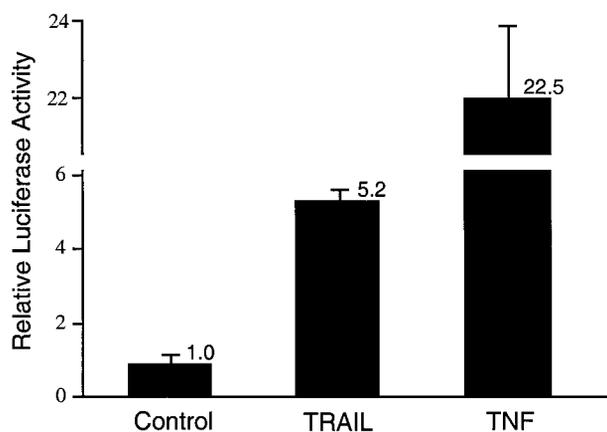


FIG. 1. Activation of NF- κ B by TRAIL. 293 cells (2×10^5) were transfected with 0.5 μ g of NF- κ B-luciferase reporter plasmid and 0.5 μ g of RSV- β -galactosidase plasmid as an internal control for transfection efficiency. 14 h after transfection, cells were treated with 200 ng/ml recombinant TRAIL, 20 ng/ml recombinant TNF, or left untreated for 8 h. Luciferase activity was measured using the luciferase assay kit (Pharmingen) and normalized on the basis of β -galactosidase expression levels. Values are averages and standard deviations for a representative experiment in which each transfection was performed in duplicate. Data shown are relative luciferase activity compared with the control treatment.

bated at 4 $^{\circ}$ C for 1 h. The beads were then washed twice with lysis buffer and once with kinase assay buffer (20 mM HEPES, pH 7.5, 10 mM β -glycerophosphate, 10 mM *p*-nitrophenylphosphate, 10 mM MgCl₂, 1 mM dithiothreitol, 50 μ M Na₃VO₄). The washed beads were resuspended in 30 μ l of kinase assay buffer containing 1 μ l of [γ -³²P]ATP (10 μ Ci/ μ L, 1 Ci = 37 GBq) and incubated at 30 $^{\circ}$ C for 30 min. The reaction was terminated by the addition of 30 μ l of 2 \times Laemmli sample buffer and boiled for 5 min. The samples were fractionated by SDS-polyacrylamide gel electrophoresis. The gels were washed in fixing buffer (10% acetic acid, 30% methanol) three times, each for 10 min, and then dried. Autoradiography was performed for 5–30 min. Fold induction of JNK kinase activity was determined by phosphoimaging analysis.

Screening of TRAIL-resistant Cells—HeLa or MCF7 cells (5×10^5) were treated with 200 ng/ml recombinant TRAIL for 24 h. Treated cells were switched to new medium containing 200 ng/ml TRAIL for additional 24 h. Surviving cells were then amplified and designated as HeLa-TL-R and MCF7-TL-R, respectively.

RESULTS

Activation of NF- κ B by TRAIL Receptors Is Mediated through a TRAF2-NIK-IKK α/β -dependent Signaling Cascade—Although several studies indicated that TRAIL could induce NF- κ B activation (8, 10, 21), one group reported results contrary to this (6). To determine whether TRAIL could activate NF- κ B, we transfected 293 cells with a NF- κ B-luciferase reporter construct and performed luciferase reporter gene assays. As shown in Fig. 1, treatment with recombinant soluble TRAIL induced NF- κ B activity. In this experiment, TRAIL was less potent than TNF in activating NF- κ B (Fig. 1). To determine the relative contributions of various TRAIL receptors to TRAIL-induced NF- κ B activation, we compared the effects of overexpression of individual TRAIL receptors on NF- κ B activity in reporter gene assays. As shown in Fig. 2, TRAIL-R1, TRAIL-R2, and TRAIL-R4 all activated NF- κ B. TRAIL-R1 was found to be more potent than TRAIL-R2 and TRAIL-R4 in activating NF- κ B in this experiment, at least partially because of its higher expression level than TRAIL-R2 and TRAIL-R4 (Fig. 2).

Because TRAIL receptors have similar biological effects as TNF receptors, we tested whether the cytoplasmic proteins involved in TNF receptor signaling, including TRADD, TRAF2, NIK, MEKK1, IKK α , and IKK β , also participate in TRAIL receptor signaling. To do this, we determined whether their dominant negative mutants could block TRAIL receptor-mediated NF- κ B activation in reporter gene assays. TRADD(296S),

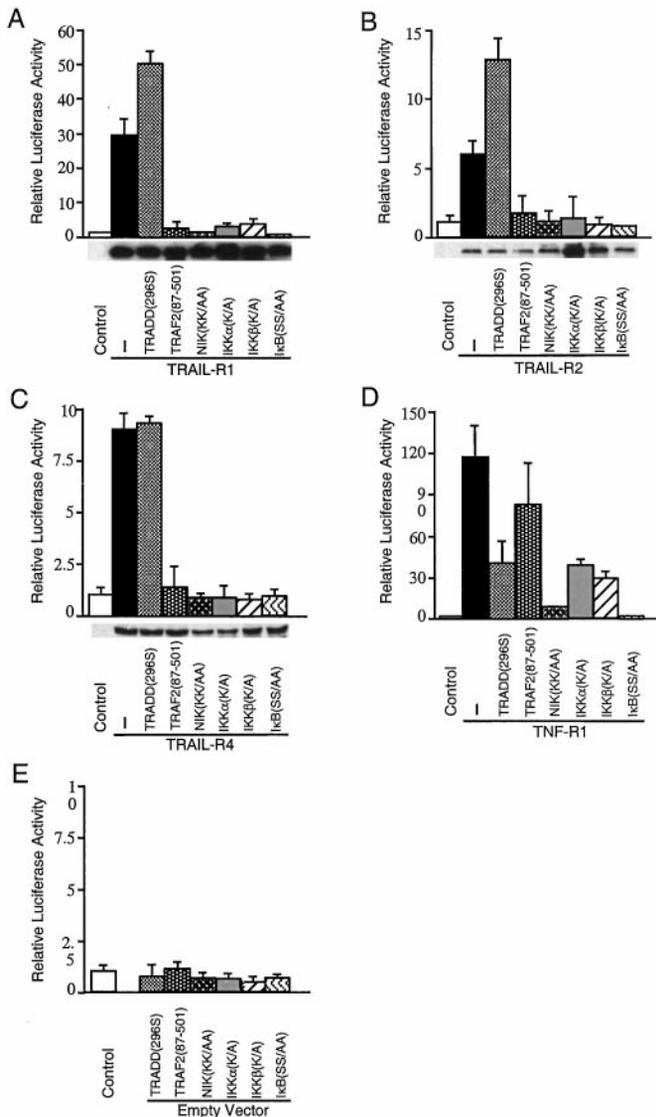


FIG. 2. Activation of NF- κ B by TRAIL receptors and effects of various dominant negative mutants on TRAIL receptor-induced NF- κ B activation. For each transfection, 293 cells (2×10^5) were transfected with 0.5 μ g of NF- κ B-luciferase reporter plasmid, 0.5 μ g of RSV- β -galactosidase, and 1 μ g of pCMV1-TRAIL-R1 (A), 1 μ g of pCMV1-TRAIL-R2 (B), 1 μ g of pCMV1-TRAIL-R4 (C), 1 μ g of pRc-TNF-R1 (D), or 1 μ g of empty control plasmid (E), together with 2 μ g of the indicated dominant negative mutant plasmids or empty vector (-). Control transfection (Control) contains equal amounts of NF- κ B-luciferase and RSV- β -galactosidase plasmids without TRAIL receptor expression plasmids and any mutant plasmids. For each transfection, 1 μ g of *crmA* expression plasmid was added to protect cells from TRAIL-R1-, TRAIL-R2-, and TNF-R1-induced apoptosis, and where necessary, enough of an amount of empty control plasmid was added to keep each transfection receiving the same amount of total DNA (5 μ g). Luciferase activity was measured 16 h after transfection and normalized on the basis of β -galactosidase expression levels. Values are averages and deviations for a representative experiment in which each transfection was performed in duplicate. Data shown are relative luciferase activity compared with the control transfection. The protein expression levels of Flag-tagged TRAIL-R1, TRAIL-R2, and TRAIL-R4 in each transfection are shown. -, empty control vector; NIK(KK/AA), NIK(K429A/K430A); IKK α (K/A), IKK α (K44A); IKK β (K/A), IKK β (K44A); I κ B(SS/AA), I κ B α (S32A/S36A).

a TRADD dominant negative mutant that inhibits TNF-R1-mediated NF- κ B activation (42) (Fig. 2D), did not block TRAIL-R1-, TRAIL-R2-, and TRAIL-R4-induced NF- κ B activation (Fig. 2), suggesting that TRADD is not involved in TRAIL receptor-mediated NF- κ B activation pathways. Consistent with this observation, we failed to detect an interaction between TRADD

and TRAIL-R1, TRAIL-R2, or TRAIL-R4 in co-transfection and co-immunoprecipitation experiments (data not shown). Interestingly, overexpression of TRADD(296S) potentiated TRAIL receptor-induced NF- κ B activation (Fig. 2).

TRAF2(87-501), a TRAF2 deletion mutant that has been shown to block TNF-R2-induced NF- κ B activation (43), potentially inhibited TRAIL-R1-, TRAIL-R2-, and TRAIL-R4-induced NF- κ B activation (Fig. 2). Consistent with previous reports (24, 43), TRAF2(87-501) only weakly inhibited NF- κ B activation by overexpression of TNF-R1 (Fig. 2D) but potentially inhibited NF- κ B activation by TNF ligation of endogenous TNF-R1 in 293 cells.² These observations are consistent with earlier reports (24, 43). The kinase-inactive mutants, NIK(K429A/K430A), IKK α (K44A), and IKK β (K44A), which have been shown to block TNF and interleukin 1-induced NF- κ B activation (28-34, 44), blocked TRAIL-R1-, TRAIL-R2-, and TRAIL-R4-induced NF- κ B activation (Fig. 2). As a positive control, the kinase inactive NIK, IKK α , and IKK β mutants inhibited TNF-R1-induced NF- κ B activation. As expected, I κ B α (S32A/S36A), an I κ B α mutant that cannot be phosphorylated and degraded and therefore has a constitutive inhibitory effect on NF- κ B (36, 37), completely inhibited TRAIL-R1-, TRAIL-R2- and TRAIL-R4-induced NF- κ B activation (Fig. 2).

MEKK1(K1255M), a kinase inactive mutant of MEKK1 (45), did not inhibit TRAIL-R4-induced NF- κ B activation but partially inhibited TRAIL-R1- and TRAIL-R2-induced NF- κ B activation (Fig. 3, A, C, and E). However, as MEKK1(K1255M) inhibited basal NF- κ B activity (Fig. 3, A, C, and E), the induction folds of NF- κ B activation by TRAIL-R1, TRAIL-R2, and TRAIL-R4 were actually higher in the presence of MEKK1(K1255M), compared with the control transfection with empty vector (Fig. 3, B, D, and F). MEKK1(K1255M) also did not inhibit TNF-R1-induced NF- κ B activation (Fig. 3, G and H), which is consistent with the belief that MEKK1 is not involved in TNF-R1-induced NF- κ B activation (35). This mutant, however, could potentially inhibit TRAIL-R1-induced JNK activation (see below). These data suggest that MEKK1 is not involved in TRAIL-R1-, TRAIL-R2-, and TRAIL-R4-induced NF- κ B activation.

TRAIL Receptors Activate JNK through a TRAF2-MEKK1-MKK4-dependent Pathway—Several TNF receptor family members are capable of activating the JNK kinase pathway. To determine whether TRAIL and its receptors have a similar effect, we performed solid-phase kinase assays with GST-c-Jun as a substrate. As shown in Fig. 4A, TRAIL treatment induced JNK activation in 293 cells. Similarly, overexpression of TRAIL-R1, TRAIL-R2, and TRAIL-R4 activated JNK (Fig. 4B) and was further enhanced by TRAIL treatment. To explore the possible signaling pathways leading to TRAIL receptor-induced JNK activation, we tested whether the dominant negative mutants of TRAF2, NIK, MEKK1, MKK4, and IKK β could block TRAIL-R1-induced JNK activation. As shown in Fig. 4C, TRAF2(87-501), MEKK1(K1255M), and the MKK4 dominant negative mutant MKK4-DN inhibited TRAIL-R1-induced JNK activation, whereas NIK(K429A/K430A) and IKK β (K44A) had no significant inhibitory effect on TRAIL-R1-induced JNK activation (Fig. 4C). These data suggest that TRAIL-R1-induced JNK activation is mediated by a TRAF2-MEKK1-MKK4 dependent pathway and is independent of the NIK-IKK α/β cascade.

Because the above data indicated that TRAIL-R1-induced NF- κ B and JNK activation pathways might bifurcate at TRAF2, we examined whether TRAF2 could directly interact with TRAIL-R1 by co-transfection and co-immunoprecipitation

² W.-H. Hu and H.-B. Shu, unpublished data.

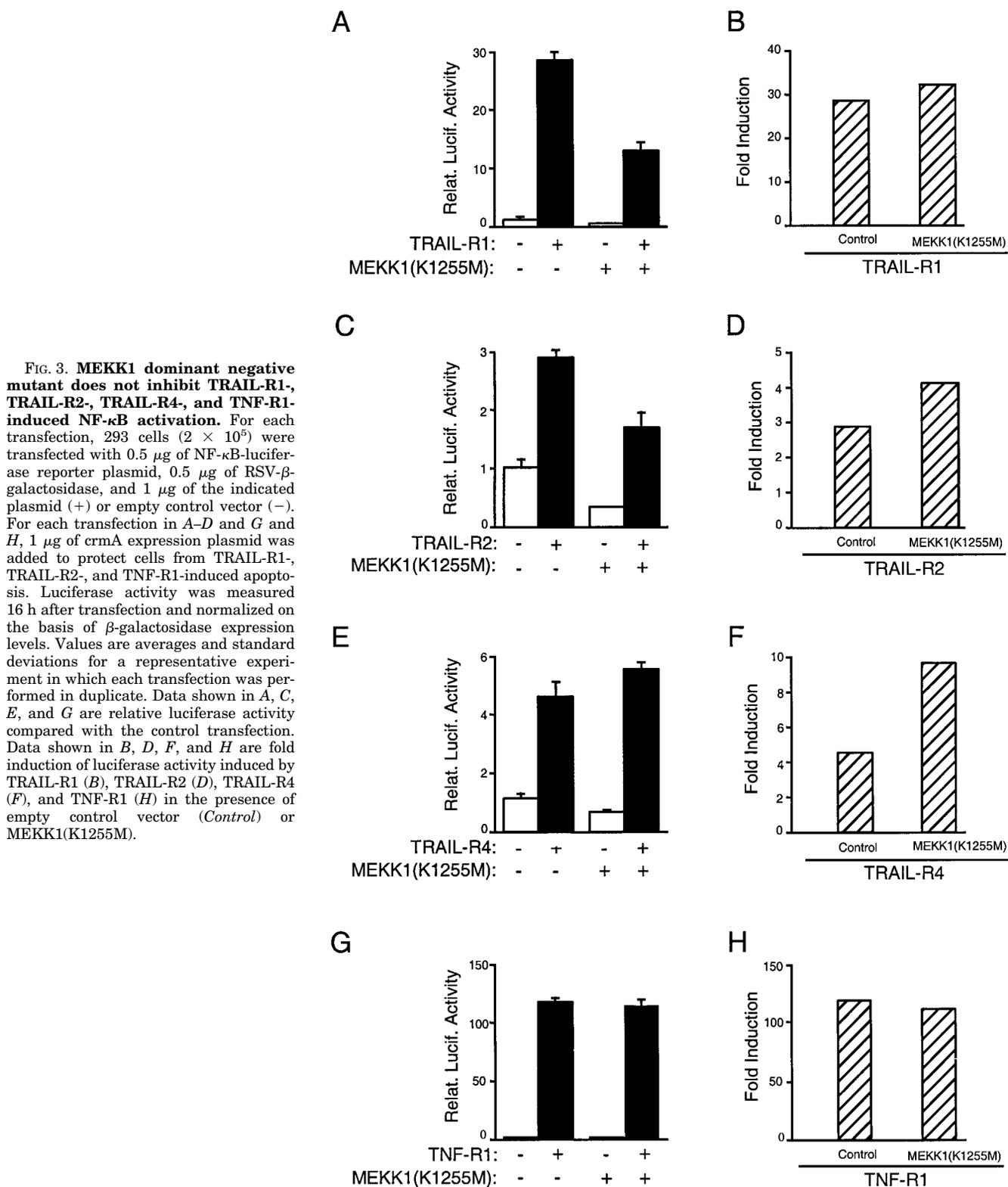


FIG. 3. MEKK1 dominant negative mutant does not inhibit TRAIL-R1-, TRAIL-R2-, TRAIL-R4-, and TNF-R1-induced NF- κ B activation. For each transfection, 293 cells (2×10^6) were transfected with 0.5 μ g of NF- κ B-luciferase reporter plasmid, 0.5 μ g of RSV- β -galactosidase, and 1 μ g of the indicated plasmid (+) or empty control vector (-). For each transfection in A-D and G and H, 1 μ g of crmA expression plasmid was added to protect cells from TRAIL-R1-, TRAIL-R2-, and TNF-R1-induced apoptosis. Luciferase activity was measured 16 h after transfection and normalized on the basis of β -galactosidase expression levels. Values are averages and standard deviations for a representative experiment in which each transfection was performed in duplicate. Data shown in A, C, E, and G are relative luciferase activity compared with the control transfection. Data shown in B, D, F, and H are fold induction of luciferase activity induced by TRAIL-R1 (B), TRAIL-R2 (D), TRAIL-R4 (F), and TNF-R1 (H) in the presence of empty control vector (Control) or MEKK1(K1255M).

experiments. We found that TRAF2 did not interact with TRAIL-R1, TRAIL-R2, and TRAIL-R4 (data not shown), suggesting that unidentified adapter molecule(s) may link TRAF2 to TRAIL receptors.

FADD Is Involved in TRAIL-R1- and TRAIL-R2-induced Apoptosis Pathway—Previously, it has been reported that TRAIL-R1 and TRAIL-R2 induce apoptosis through a FADD-dependent pathway (8, 21). In contrast, other reports have suggested that FADD is not involved in TRAIL-R1- and TRAIL-R2-induced apoptosis (6, 7, 10, 23). We screened several human

cDNA libraries using the yeast two-hybrid system with TRAIL-R1 intracellular domain as bait. These screenings identified FADD as a protein that specifically interacted with TRAIL-R1 (data not shown). Consistent with the physical interaction, FADD-(80-205), a dominant negative mutant of FADD that inhibits TNF-R1-induced apoptosis (24), significantly inhibited TRAIL-R1-induced apoptosis in a well established apoptosis assay (23, 24, 28, 40) (Fig. 5A). These data suggest that FADD is involved in a TRAIL-R1-induced apoptosis pathway.

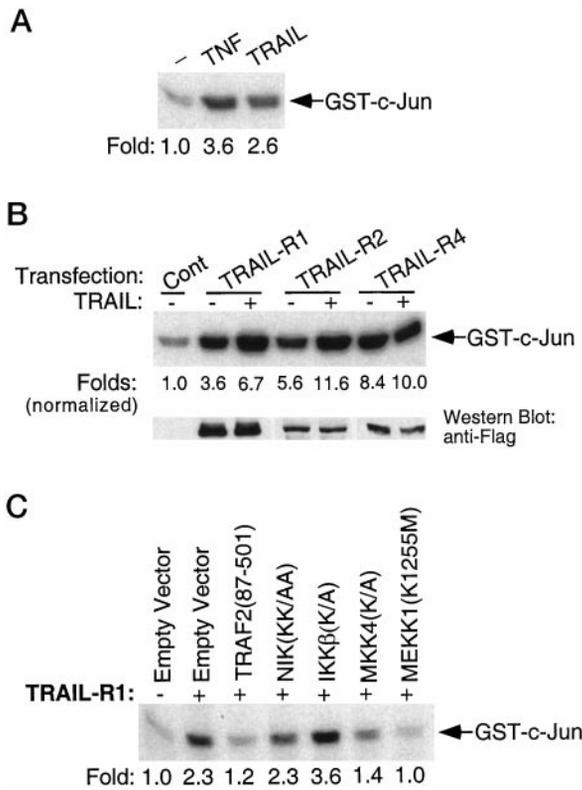


FIG. 4. TRAIL receptors activate JNK activity through a TRAF2-MEKK1-MKK4-dependent signaling pathway. *A*, TRAIL induces JNK kinase activity. 293 cells (5×10^5) were treated with 20 ng/ml TNF, 100 ng/ml TRAIL, or left untreated for 30 min. Cells were then lysed, and JNK activity was measured by a solid-phase kinase assay using recombinant GST-c-Jun as a substrate. The relative fold induction in JNK activity was determined by phosphoimaging and is indicated at the bottom of each lane. Data shown are from one representative experiment. *B*, TRAIL-R1, TRAIL-R2, and TRAIL-R4 induce JNK kinase activity. 293 cells (8×10^5) were transfected with 1 μ g of JNK1 expression plasmid, together with 5 μ g of empty control vector, TRAIL-R1, TRAIL-R2, or TRAIL-R4. 5 μ g of crmA expression plasmid was added to each transfection to inhibit cell death. 16 h after transfection, cells were lysed, and JNK activity in the lysate was determined by a solid-phase kinase assay using recombinant GST-c-Jun as a substrate. Indicated at the bottom are relative induction folds in JNK activities that were normalized based on their relative expression levels to TRAIL-R1. The expression levels of the transfected receptors are shown in the lower panel. Data shown are from one representative experiment. *C*, inhibition of TRAIL-R1-induced JNK activity by dominant negative mutants of TRAF2, MEKK1, and MKK4 but not by those of NIK and IKK β . 293 cells (2×10^5) were transfected with 3 μ g of empty control vector (lane 1) or 1 μ g of TRAIL-R1 expression plasmid together with 2 μ g of plasmid indicated at the top of the figure. 1 μ g of crmA expression plasmid was added to inhibit cell death. 16 h after transfection, cells were lysed, and JNK activity in the lysate was determined by a solid-phase kinase assay using recombinant GST-c-Jun as a substrate. The relative fold induction in JNK activity was determined by phosphoimaging and is indicated at the bottom of each lane.

TRAIL-R1-induced Apoptosis Is Independent of TRAIL-R1-induced NF- κ B and JNK Activation Pathways—Because TRAIL-R1 is capable of inducing apoptosis and NF- κ B and JNK activation, we tested whether the signaling pathways leading to the three distinct effects of TRAIL-R1 could crosstalk. We transfected 293 cells with an expression vector for TRAIL-R1 together with expression vectors for crmA, TRAF2-(87–501), NIK(K429A/K430A), MEKK1(K1255M), IKK α (K44A), IKK β (K44A), or I κ B α (S32A/S36A). We found that overexpression of TRAIL-R1 potently induced apoptosis. The caspase inhibitor crmA inhibited TRAIL-R1-induced apoptosis (Fig. 5A). The mutants TRAF2-(87–501), NIK(K429A/K430A), MEKK1 (K1255M), IKK α (K44A), IKK β (K44A), and I κ B α (S32A/S36A), which inhibited

TRAIL-R1-induced NF- κ B and/or JNK activation, had no effect on TRAIL-R1-induced apoptosis (Fig. 5A). These data suggest that TRAIL-R1-induced apoptosis and NF- κ B and JNK activation pathways are mediated through distinct pathways.

Activation of NF- κ B Is Not Capable of Protecting Cells from TRAIL-R1-induced Apoptosis—Although TRAIL induces apoptosis of various cancer cells, some cancer cells and normal cells are resistant to TRAIL-induced apoptosis, even though these cells express TRAIL-R1 and TRAIL-R2 (3). Because TRAIL-R1, TRAIL-R2, and TRAIL-R4 can activate NF- κ B, one of the possible mechanisms responsible for the resistance of a cell to TRAIL may be because of a dominant effect of NF- κ B activation, which has been shown to protect cells from TNF-induced apoptosis (18–20). In this context, it has been suggested that TRAIL-R4, which can induce NF- κ B activation but not apoptosis, can protect cells from TRAIL-induced apoptosis (16). To investigate whether NF- κ B activation is responsible for TRAIL-R4-mediated protection of cells from TRAIL-induced apoptosis, we tested the effects of overexpression of TRAIL-R4 and up-regulation of NF- κ B activity on TRAIL-R1-mediated apoptosis. As shown in Fig. 5B, overexpression of TRAIL-R4 did not protect TRAIL-R1-induced apoptosis. Co-transfection of 293 cells with expression vectors for TRAIL-R1 and NIK or IKK β greatly increased NF- κ B activity in comparison to TRAIL-R1 transfection alone (Fig. 5C). However, up-regulation of NF- κ B activity by overexpressing NIK and IKK β had no protective effect on TRAIL-R1-induced apoptosis (Fig. 5B). These data suggest that overexpression of TRAIL-R4 and activation of NF- κ B do not protect cells from TRAIL-R1-induced apoptosis.

Inhibition of NF- κ B Activation Potentiates TNF-, but Not TRAIL-induced Apoptosis—I κ B α (S32A/S36A) is an I κ B α mutant that has more potent inhibitory effect on NF- κ B activation than its wild type counterpart (36, 37). To test whether inhibition of NF- κ B activation can sensitize cells to TRAIL, we determined the effect of I κ B α (S32A/S36A) on TRAIL-induced apoptosis. To do this, we first isolated TRAIL-resistant HeLa and MCF7 cells. In these experiments, 90% of HeLa cells and 30% of MCF7 cells were killed. TRAIL-resistant cells, designated as HeLa-TL-R and MCF7-TL-R, respectively, were then amplified and transfected with expression vectors for I κ B α (S32A/S36A) and a β -galactosidase reporter gene. Fourteen hours after transfection, the cells were treated with TNF, TRAIL, or left untreated for 10 h and then stained by X-gal. As shown in Fig. 6, transfection of I κ B α (S32A/S36A) sensitized both HeLa-TL-R and MCF7-TL-R cells to TNF- but not TRAIL-induced apoptosis. These data are consistent with the hypothesis that activation of NF- κ B protects TNF- but not TRAIL-induced apoptosis.

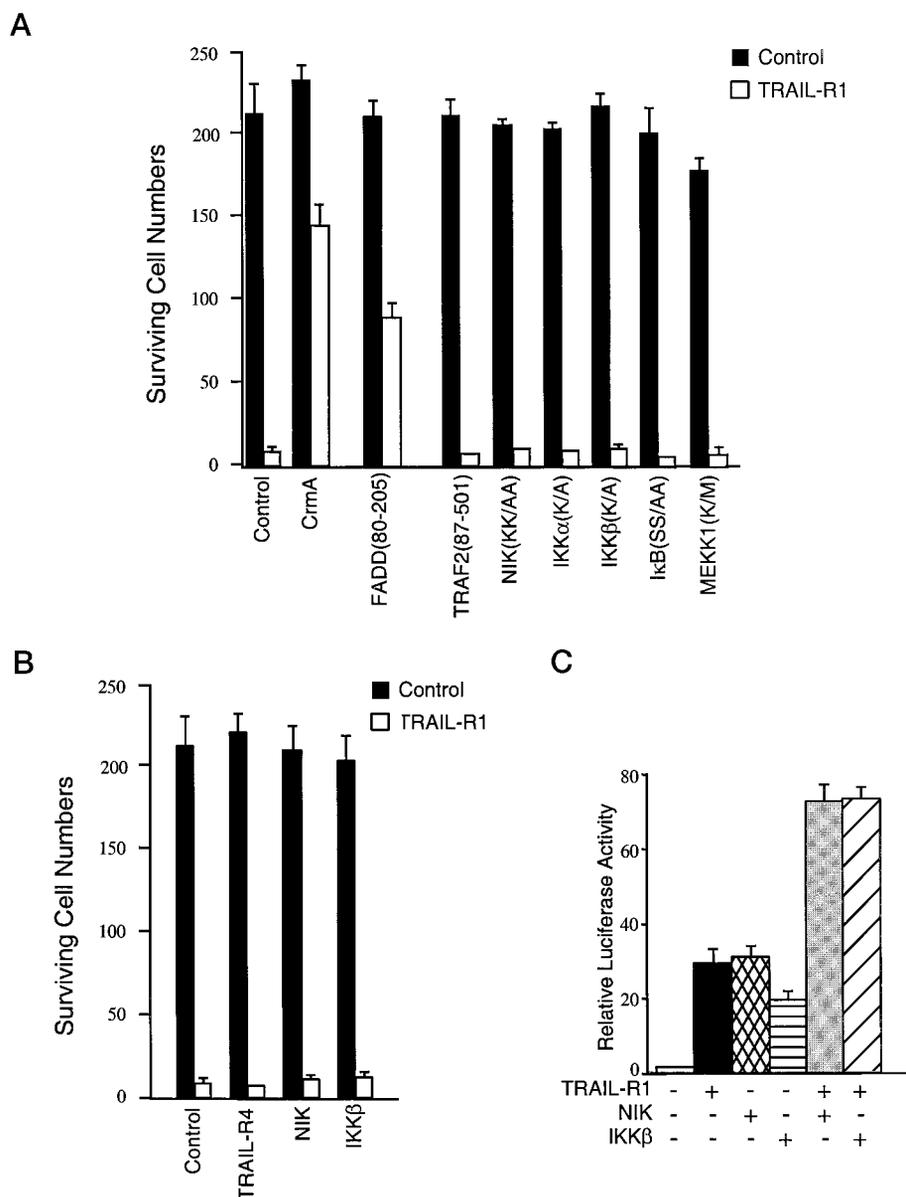
DISCUSSION

TRAIL stimulation induces three distinct biological effects: apoptosis, NF- κ B, and JNK activation. These effects of TRAIL are mediated through three signaling receptors, including TRAIL-R1, TRAIL-R2, and TRAIL-R4. In this report, we investigated the mechanisms of downstream signaling by the three mentioned TRAIL receptors.

TNF-R1 is a prototypic member of the TNF receptor family, which activates NF- κ B through a TRADD-TRAF2/RIP-NIK-IKK α / β -dependent signaling cascade (23–37). In this study, we found that TRADD did not interact with the TRAIL receptors. A dominant negative mutant of TRADD, which blocks TNF-R1-induced NF- κ B activation (42) (Fig. 2), did not inhibit TRAIL-R1-, TRAIL-R2-, and TRAIL-R4-induced NF- κ B activation. Our results are consistent with the reports that TRAIL-R1 and TRAIL-R2 do not interact with TRADD (6, 7) but contradict certain reports that TRAIL-R1 and TRAIL-R2 interact with TRADD in co-transfection/co-immunoprecipitation experi-

FIG. 5. TRAIL-R1-induced apoptosis is blocked by a FADD dominant negative mutant but not affected by inhibition or up-regulation of NF- κ B activation.

A, effects of crmA and various mutant proteins on TRAIL-R1-induced apoptosis. **B**, overexpression of TRAIL-R4 and up-regulation of NF- κ B activity do not protect cells from TRAIL-R1-induced apoptosis. **C**, up-regulation of NF- κ B activity by overexpression of NIK and IKK β . 293 cells (2×10^6) were transfected with 1 μ g of pCMV1-TRAIL-R1 (white bars) or empty control plasmid (black bars), 0.1 μ g of pCMV- β -galactosidase plasmid, 0.5 μ g of NF- κ B luciferase reporter plasmid, and 2 μ g of one of the indicated expression plasmids. 16 h after transfection, cells in two wells were stained by X-gal and surviving blue cells were counted under a microscope as described (23, 24, 28, 40) (A and B). Cells in other two wells were collected for measurement of luciferase activities (C). Data shown are averages and standard deviations for a representative experiment.



ments (8, 21), and a TRADD deletion mutant inhibits TRAIL-R1 and TRAIL-R2-induced NF- κ B activation (21). It should be pointed out that the interaction observed by the later two reports was weak, and their co-immunoprecipitation experiments did not have a nonspecific antibody control (8, 21). In addition, one group used only the intracellular domains of TRAIL-R1 and TRAIL-R2, but not full-length receptor proteins, in their co-immunoprecipitation experiments (8). The TRADD deletion mutant used by Chaudhary *et al.* (21) itself activates NF- κ B and, therefore, is not strictly a dominant negative mutant. In fact, we found that TRADD(296S), a well characterized dominant negative mutant of TRADD (42), potentiated TRAIL receptor-induced NF- κ B activation. Currently, the mechanism responsible for this observation is not clear.

In this study, we found that TRAIL-R1-, TRAIL-R2-, and TRAIL-R4-induced NF- κ B activation was potently inhibited by TRAF2(87-501), NIK(K429A/K430A), IKK α (K44A), and IKK β (K44A), but not by MEKK1(K1255M), suggesting that TRAIL-R1-, TRAIL-R2-, and TRAIL-R4-induced NF- κ B activation is mediated by a TRAF2-NIK-IKK α/β -dependent signaling cascade and is independent of MEKK1.

Our studies indicate that TRAIL-R1, TRAIL-R2, and TRAIL-R4 are also capable of inducing JNK kinase activity

(Fig. 4). TRAIL-R1-induced JNK activation can be inhibited by dominant negative mutants of TRAF2, MEKK1, and MKK4 but not by those of NIK and IKK β . Therefore, TRAIL-R1-induced JNK activation is mediated by a TRAF2-MEKK1-MKK4 dependent pathway and is independent of the NIK-IKK kinase cascade. These data suggest that TRAIL-R1-induced NF- κ B and JNK activation pathways bifurcate at TRAF2. Because TRAF2 does not directly interact with TRAIL-R1, TRAIL-R2, and TRAIL-R4, whereas FADD is not involved in TRAIL receptor-mediated NF- κ B activation pathway, we believe that unidentified adapter proteins other than TRADD and FADD are required for recruiting TRAF2 to TRAIL-R1, TRAIL-R2, and TRAIL-R4 signaling complexes.

In addition to NF- κ B and JNK activation, TRAIL-R1 can potently induce apoptosis. The pathway leading to TRAIL-R1-induced apoptosis, however, remains controversial. We screened yeast two-hybrid libraries with the intracellular domain of TRAIL-R1 as bait and identified FADD as a protein that specifically interacted with TRAIL-R1.² Furthermore, a FADD dominant negative mutant significantly inhibited TRAIL-R1-induced apoptosis in 293 cells (Fig. 5A). Our data support the hypothesis that FADD is involved in TRAIL-R1-induced apoptosis pathway.

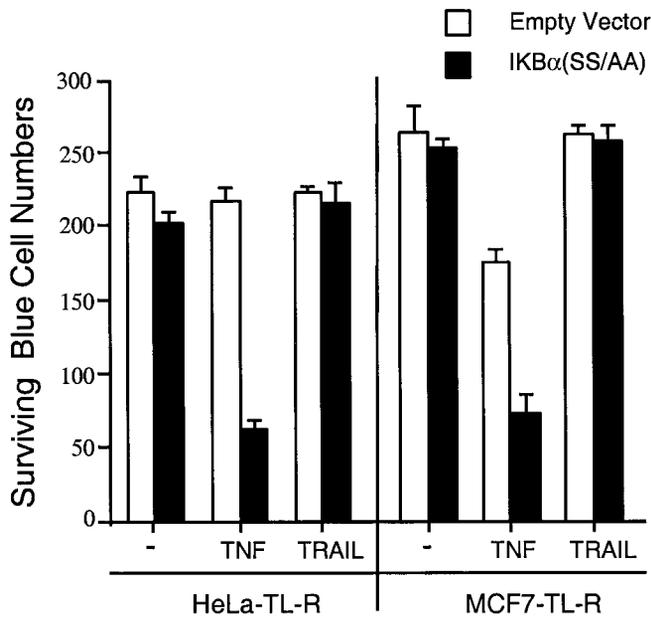


FIG. 6. Overexpression of IκBα(S32A/S36A) potentiates TNF- but not TRAIL-induced apoptosis. HeLa-TL-R and MCF7-TL-R cells (2×10^5) were transfected with 2 μg of IκBα(S32A/S36A) (black bars) expression plasmid or 2 μg of empty control plasmid (white bars) together with 0.5 μg of pCMV-β-galactosidase plasmid. Fourteen hours after transfection, transfected cells were treated with 20 ng/ml TNF, 200 ng/ml TRAIL, or left untreated for 10 h and then stained with X-gal. Surviving blue cells in each sample were counted under a microscope. Data shown are averages and standard deviations for a representative experiment in which each transfection was performed in duplicate.

Previously, it has been shown that overexpression of TRAIL-R1 can induce apoptosis in FADD(-/-) embryonic fibroblasts (22), suggesting that FADD is dispensable for TRAIL-R1-induced apoptosis. However, these experiments can not exclude the possibility that FADD is required for apoptosis induced by ligation of TRAIL-R1 with TRAIL in untransfected cells. For example, the death domain containing TRAIL-R1, when overexpressed, may artificially interact with other death domain-containing proteins, such as RIP, and therefore induce apoptosis in FADD(-/-) cells. Alternatively, a FADD-like molecule, which may have higher affinity with TRAIL-R1, can also transduce the death signal from TRAIL-R1 to the downstream caspase cascades.

In our studies, we also found that TRAF2(87-501), NIK(K429A/K430A), IKKα(K44A), IKKβ(K44A), MEKK1(K1255M), and IκBα(SS/AA), the mutants which blocked TRAIL receptor-induced NF-κB and/or JNK activation (Figs. 2 and 4), did not inhibit TRAIL-R1-induced apoptosis (Fig. 5). These data suggest that TRAIL-R1 induces apoptosis, NF-κB, and JNK activation through distinct pathways.

Previous studies indicate that TRAIL-R4 can protect cells from TRAIL-induced apoptosis. At least two hypotheses have been proposed to explain this observation. First, TRAIL-R4 may function as a decoy receptor for TRAIL-induced apoptosis, for example, by competing with TRAIL-R1 and TRAIL-R2 for TRAIL binding. This hypothesis, however, cannot explain the observations that some TRAIL-sensitive cells express both TRAIL-R3 and TRAIL-R4, whereas some TRAIL resistant cells do not have detectable TRAIL-R3 and TRAIL-R4 (3). Second, TRAIL-R4 may activate a protective signal to inhibit TRAIL-induced apoptosis, for example, by activating NF-κB. Previously, it has been shown that activation of NF-κB can inhibit TNF-induced apoptosis, probably through transcriptional induction of apoptosis inhibitory genes (20). However, this hypothesis is complicated by the fact that stimulation of

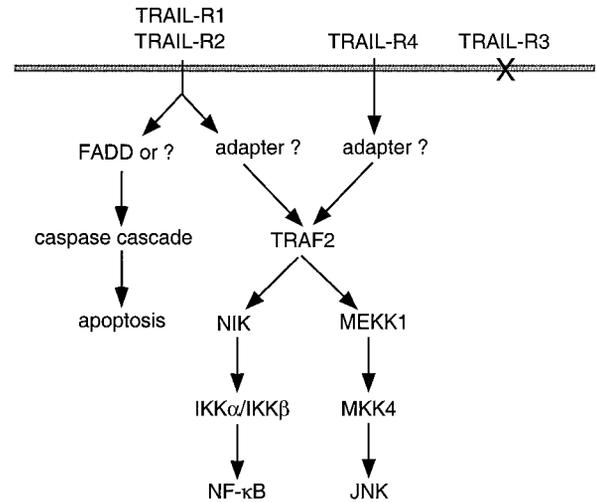


FIG. 7. A model for TRAIL receptor-mediated signaling pathways. See text for details.

TRAIL-R1 and TRAIL-R2 simultaneously activates NF-κB and induces apoptosis. One can argue that NF-κB activity induced by TRAIL-R1 and TRAIL-2 is too low to antagonize the dominant apoptotic effect induced by TRAIL-R1 and TRAIL-R2. In this study, we found that overexpression of TRAIL-R4 did not protect cells from apoptosis induced by TRAIL-R1. In addition, a dramatic up-regulation of NF-κB activity by overexpressing NIK and IKKβ had no significant effect on TRAIL-R1-induced apoptosis (Fig. 5). Moreover, inhibition of NF-κB activation by an IκBα mutant, IκBα(S32A/S36A), sensitized cells to TNF- but not TRAIL-induced apoptosis (Fig. 6). These data suggest that activation of NF-κB is not sufficient for protecting cells from TRAIL-induced apoptosis, and an alternative mechanism other than NF-κB activation may account for the protective role of TRAIL-R4 on TRAIL-induced apoptosis.

In conclusion, our data indicate that TRAIL induces apoptosis, NF-κB activation, and JNK activation through distinct pathways (Fig. 7). We also conclude that NF-κB activation is not sufficient for protecting cells from TRAIL-induced apoptosis.

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