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TRAIL Receptors 1 (DR4) and 2 (DR5) Signal FADD-Dependent Apoptosis and Activate NF-κB

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Summary

TRAIL induces apoptosis through two closely related receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5). Here we show that TRAIL-R1 can associate with TRAIL-R2, suggesting that TRAIL may signal through heteroreceptor signaling complexes. Both TRAIL receptors bind the adaptor molecules FADD and TRADD, and both death signals are interrupted by a dominant negative form of FADD and by the FLICE-inhibitory protein FLIP. The recruitment of TRADD may explain the potent activation of NF-κB observed by TRAIL receptors. Thus, TRAIL receptors can signal both death and gene transcription, functions reminiscent of those of TNFR1 and TRAMP, two other members of the death receptor family.

Introduction

Some members of the tumor necrosis factor (TNF) receptor family (Smith et al., 1994; Nagata, 1997) efficiently transmit death signals via a cytoplasmic motif called the death domain. To date, five such death receptors have been analyzed: Fas (Dhein et al., 1995; Nagata, 1997), TNF receptor 1 (TNFR1) (Tartaglia et al., 1993), TRAMP (wsl/Apo-3/DR3) (Kitson et al., 1996; Marsters et al., 1996b; Yu et al., 1996; Bodmer et al., 1997), TRAIL-R1 (DR4) (Pan et al., 1997b), and TRAIL-R2 (DR5) (Pan et al., 1997a; Schneider et al., 1997b; Sheridan et al., 1997). The best-studied death signaling pathway is the one triggered by Fas ligand (FasL). FasL induces Fas clustering, followed by the binding of FADD to the receptor's death domain (Boldin et al., 1995; Chinnaiyan et al., 1995; Kischkel et al., 1995). FADD then recruits the caspase FLICE (MACH/caspase 8), facilitating the connection from death receptors to caspases (Nicholson and Thornberry, 1997).

TRAIL-R1 and TRAIL-R2 are the most recently identified death receptors. Their ligand TRAIL shows a broad tissue distribution (Wiley et al., 1995) and like FasL, induces rapid apoptosis of various cell lines (Wiley et al., 1995; Marsters et al., 1996a; Pitti et al., 1996). In contrast to FasL, however, TRAIL is not cytotoxic to tissues despite the widespread expression of TRAIL-R1 and -R2, suggesting that strong regulatory mechanisms control TRAIL receptor signals. TRAIL-R3 (DcR1/TRID) (Pan et al., 1997a; Schneider et al., 1997b; Sheridan et al., 1997), which lacks a cytoplasmic domain, can act as a decoy receptor to inhibit TRAIL signaling, possibly contributing to the cellular nonresponsiveness. Moreover, we and others have recently characterized a family of viral and mammalian proteins called FLICE-inhibitory proteins, or FLIPs (I-FLICE/FLAME/CASPER/CASH), which interfere with death receptor signaling pathways by binding to FADD and FLICE (Bertin et al., 1997; Hu et al., 1997a, 1997b; Irmler et al., 1997; Shu et al., 1997; Srinivasula et al., 1997; Thome et al., 1997). FLIPs block TRAIL receptor–mediated death signals even though it has been reported that TRAIL receptor signals are independent of FADD (Marsters et al., 1996a; Pan et al., 1997a, 1997b; Sheridan et al., 1997). We therefore studied the signaling pathways triggered by the two TRAIL receptors in more detail and report that in several cell lines tested, FADD appears to be implicated in TRAIL signal transmission.

Results and Discussion

The Cytoplasmic Domains of TRAIL-R1 and TRAIL-R2 Form Heteroaggregates

Death receptors typically initiate signaling through ligand-induced homotrimerization. However, the high overall structural homology between TRAIL-R1 and TRAIL-R2 led us to examine whether apoptosis induced by TRAIL could be transmitted via heterocomplexes of the two closely related receptors. Expression vectors coding for the cytoplasmic regions of TRAIL-R1 and TRAIL-R2 carrying two distinct tag epitopes (Myc or Flag) were cotransfected into 293T cells. Figure 1 shows that the cytoplasmic portion of TRAIL-R2 forms homoaggregates but, in addition, also coprecipitates with the cytoplasmic portion of TRAIL-R1. In contrast, we detected no association of the TRAIL receptors with the cytoplasmic domain of Fas. Thus, it is possible that TRAIL can bind either to a TRAIL-R1 trimer or to a TRAIL-R2 trimer, or to a combination of both receptors. The mixed receptor complexes may differ from TRAIL-R1 and TRAIL-R2 in their ligand-binding affinity or may recruit distinct intracellular adaptor proteins, possibly leading to the activation of specific signaling pathway(s).

TRAIL-R1 and TRAIL-R2 Signal Apoptosis via FADD

Both TRAIL-R1 and TRAIL-R2 efficiently induce apoptosis (Pan et al., 1997a; Schneider et al., 1997b; Sheridan et al., 1997). When 293T cells were transfected with equal quantities of TRAIL-R1 or TRAIL-R2 respectively, a similar extent of DNA degradation was observed (Figure 2A). In contrast, overexpression of TRAIL-R3 did not induce cytotoxic effects (Figure 2A). To define the proteins involved in TRAIL receptor signaling, 293T cells were cotransfected with known inhibitors of the Fas-signaling pathway. Viral E8-FLIP and the long form of

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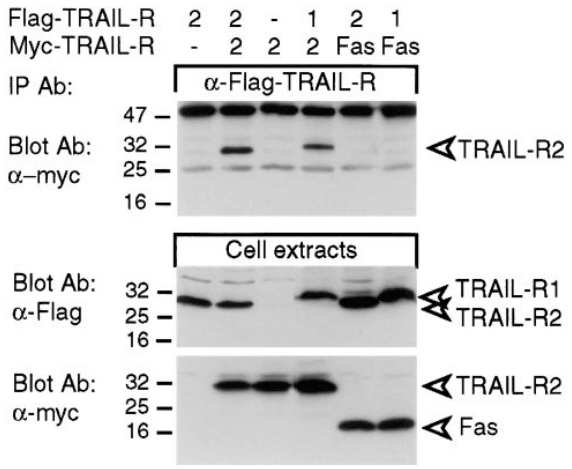

Figure 1. Association of TRAIL-R1 with TRAIL-R2

(Top) Immunoprecipitation of cell extracts from 293T cells, transfected with expression vectors for the cytoplasmic segments of TRAIL-R1 and TRAIL-R2, was performed using an anti-Flag antibody (precipitating Flag-TRAIL-R1 and Flag-TRAIL-R2, respectively). Samples were analyzed for coprecipitating Myc-TRAIL-R2 or Myc-Fas by Western blotting using anti-Myc antibodies. IP, immunoprecipitating.

(Middle and bottom) The expression levels of Flag-TRAIL-R1, Flag-TRAIL-R2, Myc-TRAIL-R2, and Myc-Fas in the corresponding cell extracts.

cellular FLIP ($FLIP_L$, which blocks FLICE recruitment and activation) (Irmler et al., 1997; Thome et al., 1997) and a FADD dominant negative version of the protein (FADD-DN, lacking the death effector domain) all reduced apoptosis mediated by both TRAIL-R1 and TRAIL-R2 (Figure 2A). We conclude that the apoptotic signaling pathways of the two receptors are similar and involve the intracellular proteins FADD and FLICE (caspase 8 or caspase 10).

To corroborate our findings, we investigated signaling in cells that had been stably transfected with TRAIL-R1 (Figure 2B). In contrast to the parental cell line, which shows little sensitivity to TRAIL despite the presence of TRAIL-R2 mRNA (Figure 2C), TRAIL-R1–transfected 293T cells (Figure 2C) are susceptible to TRAIL. When these cells were transfected with a FADD-DN expression vector, cells no longer responded to TRAIL, indicating that TRAIL-R1 signals triggered by the natural ligand are also FADD dependent. An essential role for FADD in TRAIL signaling was further supported by the observation that the stable expression of FADD-DN in the B lymphoma line BJAB rendered these cells insensitive to both TRAIL- and FasL-mediated killing (Figure 2D). The BJAB line expresses both TRAIL-R1 and TRAIL-R2 mRNA (Figure 2C), but only TRAIL-R2 mRNA was detected in TRAIL-sensitive Jurkat cells, which are rendered insensitive to TRAIL by viral and cellular FLIP (Irmler et al., 1997; Thome et al., 1997).

These results suggest that FADD is a constituent of both TRAIL-R1 and TRAIL-R2 signaling complexes. To analyze whether a physical association of FADD with the receptors does indeed occur, Flag-tagged cytoplasmic TRAIL-R1 or TRAIL-R2 were cotransfected with FADD expression vectors in 293T cells. Immunoprecipitation of the Flag-tagged TRAIL receptors revealed that FADD associated with both TRAIL-R1 and TRAIL-R2 (Figure 3A), although the association was weaker than that observed in parallel experiments using the Flag-tagged cytoplasmic region of Fas (data not shown). In addition to FADD, TRADD was detected in these immunoprecipitates. However, TRADD binding was not observed in the absence of coexpressed FADD, indicating that the association of TRAIL-R1 and TRAIL-R2 with TRADD may be mediated by FADD or may be dependent on the presence of FADD in the signaling complex. Similar results were obtained when the association of full-length TRAIL-R1 with FADD and TRADD was investigated (Figure 3B).

TRAIL-R1 and TRAIL-R2 Activate NF-κB

TRADD is an essential adaptor protein in the signal cascade of TNFR1 and TRAMP, leading to the activation of the transcription factor NF-κB. The presence of TRADD in the signaling complex of the two TRAIL receptors prompted us to assay NF-κB activity in cells transiently transfected with a TNF promoter–derived NF-κB–luciferase reporter gene together with increasing amounts of TRAIL-R1 and TRAIL-R2 expression vectors. Expression of both TRAIL receptors strongly induced the activity of the reporter gene in a dose-dependent fashion (Figure 4). An increase in luciferase activity of up to 10-fold was measured, comparable to levels induced by TRAMP (Bodmer et al., 1997).

Several lines of evidence indicate that FADD plays an essential role in the death-inducing signaling complex of TRAIL receptors. First, FADD-DN inhibits TRAIL-induced cell death in three different cell lines: BJAB, 293T, and CV-1 (Walczak et al., 1997); second, Jurkat cell lines expressing FLIP of viral or cellular origin become resistant to TRAIL-mediated apoptosis (Irmler et al., 1997; Thome et al., 1997); and third, FADD coimmunoprecipitates with TRAIL-R1 and TRAIL-R2. Our results are in agreement with a report published while this manuscript was under revision (Walczak et al., 1997) but do not support previous findings that TRAIL-R1 does not associate with FADD (Marsters et al., 1996a; Pan et al., 1997a, 1997b; Sheridan et al., 1997). This discrepancy is probably due to differences in the experimental conditions used.

TRAIL-R1 and TRAIL-R2 trigger at least two distinct signaling pathways, leading either to death or to NF-κB activation. In this respect, signals induced by TRAIL-R1 and TRAIL-R2 are similar to that induced by TNFR1 and TRAMP: all four receptors recruit FADD and TRADD upon overexpression, albeit with different efficiencies. However, the TRAIL receptors recruit these adaptors not very efficiently, suggesting that binding may be indirect and possibly mediated by yet an additional adaptor protein. Moreover, the molecular ordering of the adaptor proteins appears to be different. Whereas TRADD is upstream of FADD in the TNFR1 and TRAMP signaling, the converse is observed in the TRAIL-R1 and TRAIL-R2 signaling pathways. It will be useful to determine how this difference influences the receptors' decision either to activate NF-κB or to initiate the cell death program.

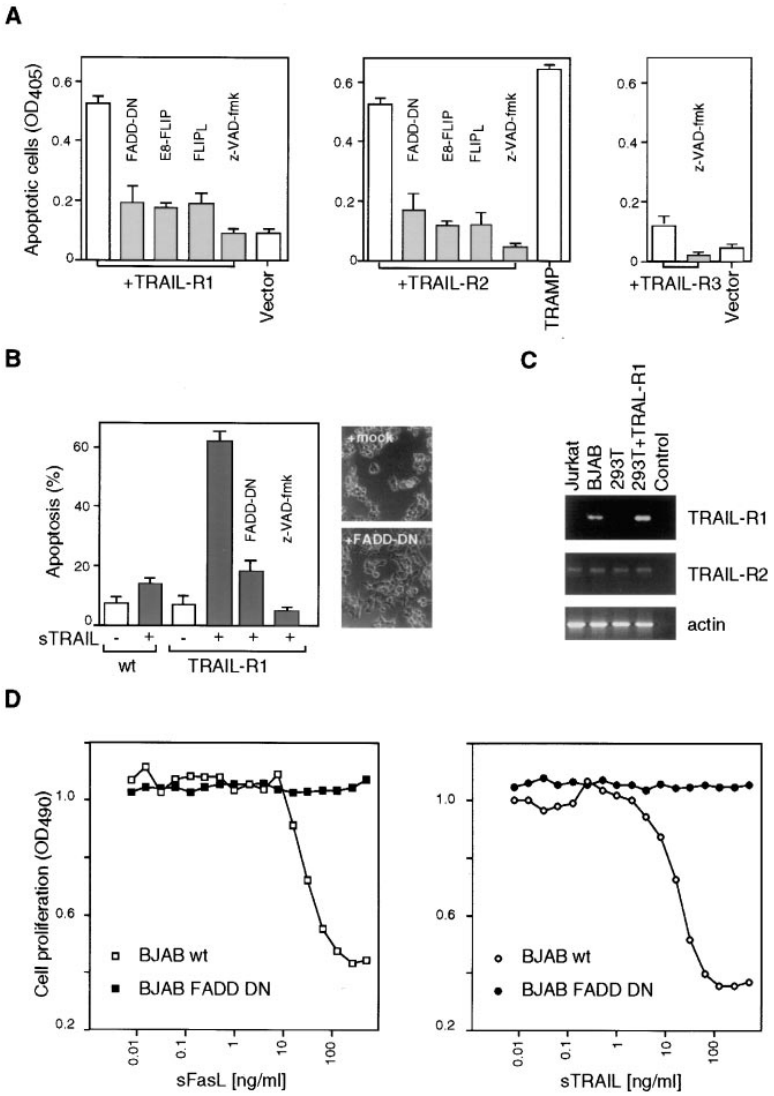

Figure 2. TRAIL-R1 and TRAIL-R2 (but Not TRAIL-R3) Induce Apoptosis That Can Be Inhibited by Proteins Competing with FADD Recruitment

(A) DNA fragmentation of 293T cells expressing TRAIL-R1, TRAIL-R2, or TRAIL-R3, respectively, was determined by analyzing cytoplasmic histone-containing DNA complexes. Full-length TRAIL receptors were transfected alone or in combination with expression vectors for equine herpes virus E8-FLIP, human $FLIP_L$, or FADD-DN. Where indicated, 50 μM z-VAD-fmk was added to the cells 8 hr after the onset of transfection. As a positive control, TRAMP expression vector (2 μg) was transfected. Data are the means ± SEM of triplicate determinations. OD, optical density.

(B) Apoptosis of 293T cells stably expressing TRAIL-R1. Parental (wt) and TRAIL-R1–expressing cells were mock transfected or transfected with an expression vector for FADD-DN for 10 hr and then treated with recombinant soluble TRAIL (sTRAIL) (solid bars, 500 ng/ml) for 14 hr. Cells were analyzed by phase contrast microscopy (right, sTRAIL-treated TRAIL-R1–expressing cells), and the percentage of apoptotic cells was determined (left). Controls shown are cells not exposed to sTRAIL (open bars) and treated with z-VAD-fmk. Data are the means ± SD of triplicate determinations, each consisting of 150 cells.

(C) Expression of TRAIL-R1 and TRAIL-R2 in various cell lines. Reverse transcriptase amplification of TRAIL-R1, TRAIL-R2, and actin from RNA of BJAB Burkitt lymphoma, 293T cells, 293T cells transfected with TRAIL-R1, and the Jurkat T lymphoma cell line. The control amplification was done in the absence of added cDNA.

(D) BJAB cells transfected with dominant negative FADD (BJAB FADD-DN) or control transfectants (BJAB wt) were incubated with soluble FasL (sFasL) or sTRAIL for 16 hr at the indicated concentrations, and cell survival was measured by a cell proliferation assay.

Experimental Procedures

Reagents, Expression Vectors, and Cell Lines

The 293T human embryonic kidney (HEK) cells, BJAB Burkitt lymphoma cells, and BJAB cells stably transfected with a dominant negative FADD (residues 80–208) (Chinnaiyan et al., 1996) were kindly provided by M. Peter (German Cancer Research Center, Heidelberg, Germany). For the generation of 293T cells stably expressing TRAIL-R1, a HindIII–BamHI fragment of TRAIL-R1 (Schneider et al., 1997b) was subcloned into pcDNA3.1/Hygro (+) vector (InVitrogen). Stable clones were obtained by selection in medium containing 200 μg/ml hygromycin B.

Flag-tagged recombinant soluble human FasL and TRAIL were used as described (Schneider et al., 1997a; Thome et al., 1997). Monoclonal antibodies (MAbs) used for immunoprecipitation and Western blotting included an anti-Flag M2 antibody and anti-Flag M2 agarose (Kodak International Biotechnologies), an anti-FADD antibody (Transduction Laboratories), and an antibody against the Myc epitope (9E10).

The full-length TRAIL receptors have been described previously (Schneider et al., 1997b). The cytoplasmic domains of TRAIL-R1 (amino acids 261–465) and TRAIL-R2 (amino acids 232–439) were amplified by polymerase chain reaction (PCR) using the proofreading Pwo polymerase (Boehringer, Mannheim) and were subcloned into the EcoRI and XhoI site of pCRIII-derived vectors, in frame with an N-terminal Flag or Myc epitope.

The Flag-TRAIL-R1 expression plasmid (Pan et al., 1997b) was kindly provided by V. M. Dixit (University of Michigan Medical School, Ann Arbor, MI) and the Myc-TRADD expression vector was a gift from D. V. Goeddel (Tularik, San Francisco, CA).

Cell Lysis, Coimmunoprecipitation, and Western Blot Analysis

For coimmunoprecipitation experiments, 293T cells were transfected in 10 cm dishes by the calcium phosphate procedure (Sambrook et al., 1989) for 8 hr with a total of 10 μg of plasmid. Control plasmid was added when necessary to keep the total amount of plasmid constant. Thirty to 36 hr after transfection, cells were lysed in 250 μl of lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl [pH 7.4], 150 mM NaCl) supplemented with complete protease inhibitor cocktail (Boehringer Mannheim). Postnuclear lysates were precleared for at least 1 hr on Sepharose 6B (Pharmacia) before precipitation for 2 hr or overnight with 3 μl of anti-Flag agarose. Precipitates were washed twice with lysis buffer and twice in lysis buffer containing 0.1% Nonidet P-40; boiled in sample buffer; and analyzed by SDS–polyacrylamide gel electrophoresis and Western blotting onto nitrocellulose (Hybond ECL, Amersham). Blots were saturated with 5% skim milk, 0.5% Tween-20 in phosphate-buffered saline,

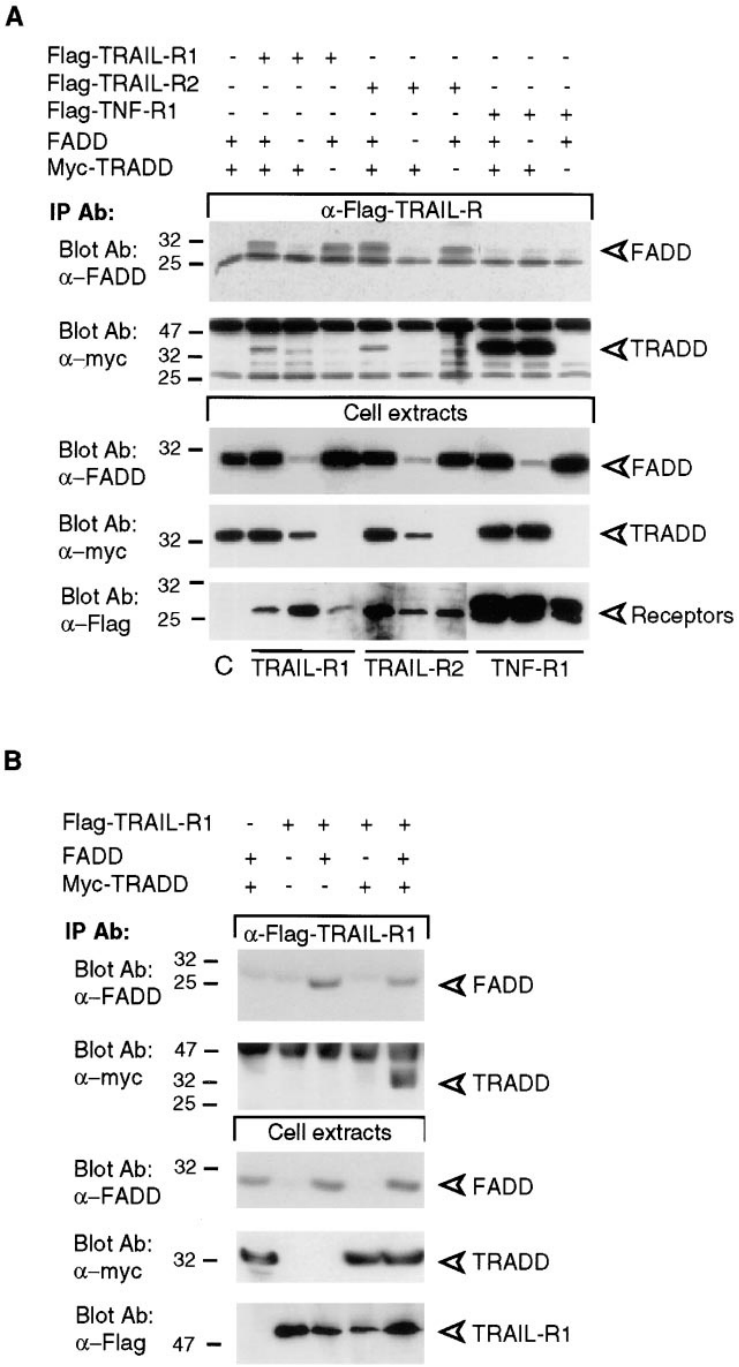

Figure 3. Interaction of TRAIL-R1 and TRAIL-R2 with FADD and TRADD

(A) (Top) 293T cells were transfected with the expression vectors coding for the cytoplasmic segment of the indicated receptors and an irrelevant plasmid to keep the total amount of plasmid constant. Lysates were immunoprecipitated with anti-Flag antibodies (precipitating the TRAIL receptors), and the presence of coprecipitating FADD and TRADD was assessed by Western blot analysis using anti-FADD and anti-Myc antibodies, respectively. IP, immunoprecipitating. (Bottom) The expression of the transfected proteins in the postnuclear cell extracts.

(B) Analysis as in (A), but using Flag-tagged full-length TRAIL-R1 to demonstrate the interaction with FADD and TRADD.

and revealed using either anti-Flag MAb at 5 μg/ml, anti-FADD MAb at 1 μg/ml, or anti-Myc MAb (9E10) at 2 μg/ml, followed by incubation with peroxidase-conjugated secondary antibody (Jackson Laboratories). Bound antibodies were detected using the enhanced chemiluminescence kit (Amersham International) according to the manufacturer's instructions.

Cell Death and Cell Proliferation Assays

293T cells (2×10^5) (wild-type or stably expressing TRAIL-R1) were transiently transfected in 6 cm plates with 2 μg of pCRIII expression vector encoding full-length TRAIL-R1, -R2, or -R3, together with expression vectors for v-FLIP (E8 of EHV-2, 0.5 μg) (Thome et al., 1997), human $FLIP_L$ (0.1 μg) (Irmler et al., 1997), or FADD-DN (2 μg) and irrelevant plasmid to keep the total amount of plasmid constant. Cells were washed and the caspase inhibitor z-VAD-fmk was added, where indicated, at a concentration of 50 μM, 8 hr after transfection. Thirty-two hours after transfection the extent of cell death was assessed by morphological inspection of the cells by phase contrast microscopy, and nuclear DNA degradation was determined in cell lysates using a kit from Boehringer (no. 1544 675) that detects cytoplasmic histone-associated DNA fragments.

BJAB cells (100 μl, 50,000 cells, in 96-well plates) were incubated at 37°C in the presence of soluble FasL or soluble TRAIL at the indicated concentrations and 1 μg/ml of M2 MAb. After 16 hr, 20 μl of a solution containing 2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tertrazolium, inner salt (MTS) reagent (Promega) and 50 μg/ml phenazine methosulfate was added to the cells. Following color development (3 hr), absorbance at 490 nm was measured with an enzyme-linked immunosorbent assay reader.

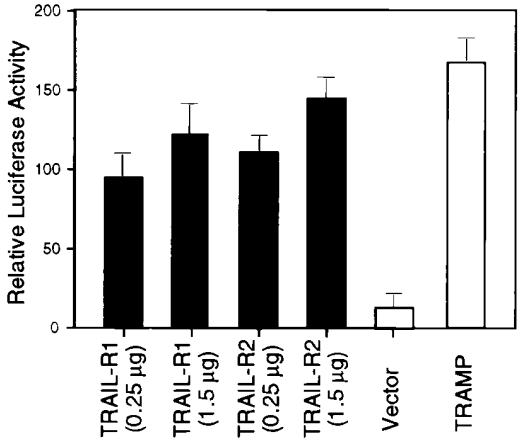

Figure 4. Transcription Factor NF-κB Is Activated by TRAIL-R1 and TRAIL-R2

Effect of increasing TRAIL receptor expression on NF-κB activity in 293T cells, determined by a TNF promoter–derived, NF-κB–luciferase gene reporter assay. The control shows NF-κB activity induced by TRAMP. Data are the means ± SD of triplicate determinations.

NF-κB Reporter Assay

Approximately 2 × 10^5 293T cells were seeded on 35 mm dishes and transfected the next day by the calcium phosphate precipitation method. Luciferase activity was determined and normalized as described (Bodmer et al., 1997).

Reverse Transcriptase PCR

Total RNA extracted from Jurkat, BJAB, 293T, and 293T cells transfected with TRAIL-R1 was reverse transcribed using the Ready to Go system (Pharmacia) according to the manufacturer's instructions. TRAIL-R1, TRAIL-R2, and β-actin cDNAs were detected by PCR amplification with Taq DNA polymerase (steps of 1 min each at 94°C, 55°C, and 72°C for 30 cycles) using specific oligonucleotides: for TRAIL-R1, JT834 5′-CAGAACGTCCTGGAGCCTGTAAC-3′ and JT835 5′-ATGTCCATTGCCTGATTCTTTGTG-3′; for TRAIL-R2, JT836 5′-GGGAAGAAGATTCTCCTGAGATGTG-3′ and JT837 5′-ACA TTGTCCTCAGCCCCAGGTCG-3′; and for β-actin, 5′GGCATCGT GATGGACTCCG3′ and 5′GCTGGAAGGTGGACAGCGA3′).

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