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The TNF Receptor 1-Associated Protein TRADD Signals Cell Death and NF-κB Activation

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Summary

Many diverse activities of tumor necrosis factor (TNF) are signaled through TNF receptor 1 (TNFR1). We have identified a novel 34 kDa protein, designated TRADD, that specifically interacts with an intracellular domain of TNFR1 known to be essential for mediating programmed cell death. Overexpression of TRADD leads to two major TNF-induced responses, apoptosis and activation of NF-κB. The C-terminal 118 amino acids of TRADD are sufficient to trigger both of these activities and likewise sufficient for interaction with the death domain of TNFR1. TRADD-mediated cell death can be suppressed by the *crmA* gene, which encodes a specific inhibitor of the interleukin-1β-converting enzyme. However, NF-κB activation by TRADD is not inhibited by *crmA* expression, demonstrating that the signaling pathways for TNF-induced cell death and NF-κB activation are distinct.

Introduction

Tumor necrosis factor (TNF) is a pleiotropic cytokine whose varied biological activities are signaled through two distinct cell surface receptors (reviewed by Tartaglia and Goeddel, 1992). These receptors, termed TNFR1 and TNFR2, are of approximate masses 55 kDa and 75 kDa, respectively, and are expressed on most cell types (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990). The primary amino acid sequences of the intracellular domains bear no detectable similarity, leading to the prediction that the two receptors interact with different proteins and activate distinct signal transduction pathways (Lewis et al., 1991). Gene knockout experiments (Pfeffer et al., 1993; Rothe et al., 1993; Erickson et al., 1994) and studies with receptor-specific agonistic antibodies (Engelmann et al., 1990; Espevik et al., 1990; Tartaglia et al., 1991; Wong et al., 1992; Gehr et al., 1992) have confirmed this prediction and demonstrated that the two TNF receptors generate largely nonoverlapping signals. The majority of the known activities of TNF have been attributed to TNFR1. Direct signaling through TNFR2 occurs less extensively and appears to be mainly confined to cells of the immune system.

The two TNF receptors are members of the growing TNF receptor superfamily, which includes the Fas antigen and CD40 (reviewed by Smith et al., 1994). The binding to these receptors of their respective ligands induces receptor oligomerization and is thought to link receptors to downstream signaling pathways (reviewed by Tartaglia and Goeddel, 1992; Smith et al., 1994). However, direct coupling to such pathways has not yet been demonstrated. A potential breakthrough in this regard was the identification of two related proteins, TRAF1 and TRAF2 (for TNF receptor–associated factors 1 and 2, respectively), that form a heterodimeric complex and associate with the cytoplasmic domain of TNFR2 (Rothe et al., 1994). TRAF1 and TRAF2 share a novel region of homology, the TRAF domain. A third member of this protein family, designated CD40bp or TRAF3, has recently been shown to associate with the cytoplasmic domain of CD40 (Hu et al., 1994). However, the actual contributions of the TRAFs to signal transduction by TNFR2 and CD40 remain unresolved.

TNFR1 is responsible for most of the biological properties of TNF, including programmed cell death, antiviral activity, and activation of the transcription factor NF-κB in a wide variety of cell types (Engelmann et al., 1990; Espevik et al., 1990; Tartaglia et al., 1991; Wong et al., 1992; Tartaglia et al., 1993). It also plays an essential role in host defense against microorganisms and bacterial pathogens (Pfeffer et al., 1993; Rothe et al., 1993). Mutagenesis studies have identified a so-called death domain of approximately 80 amino acids near the C-terminus of TNFR1 that is required for signaling antiviral activity and cell death (Tartaglia et al., 1993) as well as for NF-κB activation (Y.-F. Hu and D. V. G., unpublished data). The death domain of TNFR1 also triggers activation of an endosomal acidic sphingomyelinase (Wiegmann et al., 1994). A homologous domain that can also initiate programmed cell death is found in the Fas antigen (Itoh and Nagata, 1993). The apoptosis induced by both TNF and Fas was recently shown to involve the activation of the interleukin-1β-converting enzyme (ICE) or an ICE-like cysteine protease (Tewari and Dixit, 1995).

The TNFR1-associated proteins involved in generating the various TNF-induced signals remain unknown, although coimmunoprecipitation experiments have revealed that three phosphoproteins and a serine protein kinase activity associate with TNFR1 following TNF treatment (VanArsdale and Ware, 1994). In this report, we describe the molecular cloning of TNFR1-associated death domain protein (TRADD), a novel protein that interacts specifically with the death domain of TNFR1. Overexpression of TRADD activates two major TNF signaling pathways, apoptosis, and NF-κB activation. Furthermore, the ICE inhibitor encoded by the cowpox virus *crmA* gene protects against TRADD-mediated cell death. However, crmA does not prevent TRADD-induced NF-κB activation, demonstrating that the two signaling pathways emanating from TRADD are distinct.

Results

Isolation of cDNA Clones Encoding TNFR1-Interacting Proteins

To identify proteins that directly interact with the intracellular region of human TNFR1, we used the yeast two-hybrid system (Fields and Song, 1989). From approximately 50

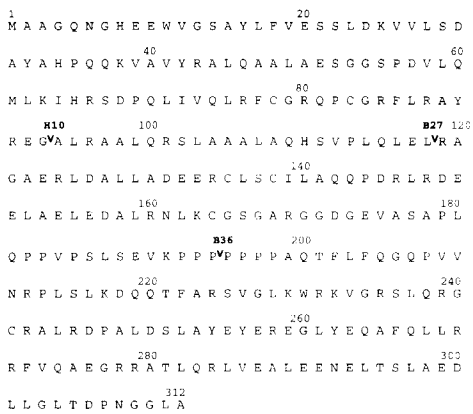

Figure 1. Predicted Amino Acid Sequence of TRADD

The amino acid sequence deduced from the sequence of two full-length *TRADD* cDNAs is shown. The three clones isolated by two-hybrid screening were fused to Gal4ad at the positions indicated by H10, B27, and B36.

million transformants, 48 positive clones, as determined by activation of *his* and *lacZ* reporter genes, were obtained. Of these clones, 41 encoded portions of the cytoplasmic region of TNFR1, indicating that the death domain (Tartaglia et al., 1993) of the receptor can self-associate. Similar findings using the yeast two-hybrid system were reported recently (Song et al., 1994; Boldin et al., 1995). Three of the remaining seven clones (H10 [219 amino acids], B27 [194 amino acids], and B36 [118 amino acids]) were partial-length cDNAs derived from the same gene. We screened human umbilical vein endothelial cell (HUVEC) and HeLa cDNA libraries by using the B27 cDNA as probe and obtained four cDNAs of approximately 1.5 kb. DNA sequence analysis of these clones revealed an open reading frame predicted to encode a protein of 312 amino acids (Figure 1) with a molecular mass of 34.2 kDa that we have designated TRADD. Database searches utilizing BLAST and FASTA programs failed to identify any proteins having significant sequence similarity to TRADD.

Detection of *TRADD* mRNA and Protein

Northern blot analysis indicated that low amounts of *TRADD* mRNA were expressed constitutively in all human tissues examined (Figure 2A). This result is consistent with TRADD involvement in TNFR1 signal transduction, as TNFR1 mRNA is also expressed ubiquitously (Loetscher et al., 1990; Schall et al., 1990; Lewis et al., 1991). The ~1.4 kb size of the *TRADD* transcript confirms that the cDNA clones represent full-length copies of *TRADD* mRNA.

Polyclonal antibodies against TRADD were generated by expressing a glutathione S-transferase (GST)–TRADD fusion protein in Escherichia coli and using the purified GST–TRADD chimera as immunogen. Rabbit anti-TRADD antiserum specifically recognized two proteins of approximately 34 kDa when tested by Western blot analysis using lysates prepared from 293 cells transiently transfected with a *TRADD* expression vector (Figure 2B). The weak upper band of the doublet may be a posttranslationally modified TRADD. Protein bands of the same size, corresponding to endogenous TRADD, could be detected in lysates from ECV304, HeLa, and HepG2 cells (Figure 2B) prepared with 50-fold more cells.

TRADD Specifically Interacts with TNFR1 and Self-Associates

To confirm that full-length TRADD interacts specifically with TNFR1, a protein consisting of the GAL4 activation domain fused to full-length TRADD (GAL4ad–TRADD) was coexpressed with the GAL4bd–TNF–R1icd fusion protein in yeast strain SFY526. The GAL4ad–TRADD chimera interacted with the GAL4bd–TNF–R1icd fusion protein, but not with the GAL4 DNA-binding domain alone (Table 1). We also tested the ability of the GAL4ad–TRADD fusion protein to interact with receptors that are related to TNFR1 either in terms of their structure (TNFR2 and Fas antigen) or the signals they generate (Fas antigen and the type I interleukin-1 receptor [IL-1R1]). GAL4ad–TRADD failed to interact with the cytoplasmic domains of TNFR2, IL-1R1, or Fas antigen expressed as GAL4bd chimeras (Table 1).

To examine further the specificity of the interaction between TRADD and TNFR1, the GAL4bd was fused to two different C-terminal deletion mutants of TNF–R1icd as well as to the cytoplasmic domain of murine TNFR1. GAL4ad–TRADD interacted strongly with an active receptor mutant (Δ413–426) lacking 14 amino acids, yet interacted weakly with an inactive mutant (Δ407–426) lacking 20 amino acids (Table 1). These results indicate that amino acids 407–412 of TNFR1, which are required for the signaling of cell death (Tartaglia et al., 1993), contribute to, but are not required for, its TRADD interaction. TRADD also interacted with mouse TNFR1 in this assay system, but more weakly than with the homologous human TNFR1.

We performed in vitro biochemical assays to confirm the specific interaction of TNFR1 with TRADD observed in the two-hybrid system. GST fusion proteins containing the cytoplasmic domains of TNFR1, TNFR2, IL-1R1, and Fas antigen were tested for interaction with ^{35}S-labeled TRADD prepared by in vitro transcription and translation. TRADD associated only with the GST–TNFR1 fusion protein (Figure 3A). Furthermore, TRADD did not associate with a GST–TNFR1(–20) fusion protein derived from the inactive TNFR1 Δ407–426 mutant.

To determine whether TRADD exists as a monomer, we prepared a reverse two-hybrid construct in which TRADD was fused to the GAL4bd. The results of a yeast cotransformation experiment using this construct and the GAL4ad–TRADD construct indicated that these two proteins interact with each other (Table 1). The simplest interpretation of this result is that TRADD is an oligomeric protein.

TRADD–TNFR1 Interaction in Human Cells

Extensive mutational analysis of TNFR1 has led to the identification of several residues in its death domain that are important for signaling cell death (Tartaglia et al., 1993). To determine whether a correlation exists between

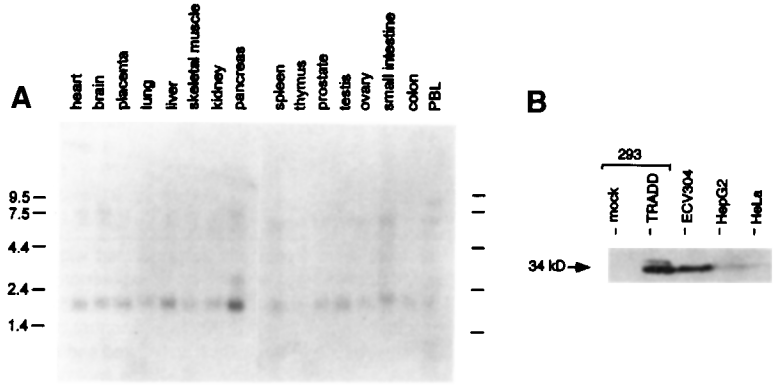

Figure 2. Identification of *TRADD* mRNA and Protein

(A) Northern blot analysis of *TRADD* mRNA in multiple human tissues. *TRADD* mRNA is the band at ~1.4–1.5 kb. The weakly hybridizing band at ~5 kb is from nonspecific hybridization to 28S rRNA.

(B) Western blot analysis of TRADD protein in mock- and pRK–TRADD-transfected 293 cells, and in nontransfected ECV304, HepG2, and HeLa cells. Lysates were prepared from 2 × 10^4 293 cells (lanes 1–2) or 10^6 ECV304, HepG2, and HeLa cells (lanes 3–5) and proteins separated by SDS–PAGE on a 10% gel. Western blotting was performed with rabbit anti-TRADD antiserum. Positions of molecular weight standards (in kilodaltons) are shown on the left. An equivalent Western blot using preimmune serum failed to detect any proteins in the 34 kDa range (data not shown).

the ability of a TNFR1 mutant to deliver a cytotoxic signal and to interact with TRADD, we utilized a mammalian cell coimmunoprecipitation assay. An expression vector that directs the synthesis of TRADD containing an N-terminal Myc epitope tag was cotransfected with various TNFR1 constructs into human embryonic kidney 293 cells. Cell extracts were immunoprecipitated by using polyclonal antibodies against the extracellular domain of TNFR1, and coprecipitating TRADD was detected by Western blotting with an anti-Myc monoclonal antibody. As determined by this assay, TRADD specifically associates with TNFR1 (Figure 3B). Five different deletion and point mutants of TNFR1 were also examined by this method. The two active mutants (Δ413–426 and Δ212–308) were able to coprecipitate TRADD, whereas two of the three inactive mutants (Δ212–340 and K343, F345, R347) failed to do so. The third inactive mutant (Δ407–426) coprecipitated TRADD weakly in some experiments (Figure 3B) and not at all in others (data not shown). Accordingly, it appears that residues throughout the ~80 amino acid death domain of TNFR1 are critical for TRADD interaction.

Overexpression of TRADD Induces Apoptosis

One of the major activities signaled by TNF through TNFR1 is programmed cell death or apoptosis. To investigate a possible role for TRADD in TNF-mediated apoptosis, 293 cells were transiently transfected with a *TRADD* expression vector and examined by phase contrast microscopy 24 hr later. Those 293 cells that overexpressed TRADD had obvious morphological differences from those transfected with a control vector (Figure 4A). The TRADD-expressing cells displayed the typical characteristics of adherent cells undergoing apoptosis, becoming rounded and condensed (average diameter ~15 μm) and detaching from the dish. Cells transfected with a control vector remained flat, with an average length of ~40–50 μm.

One biochemical hallmark of apoptosis is the internucleosomal fragmentation of nuclear DNA, which results in a distinct laddering pattern when analyzed by gel electrophoresis (Tomei and Cope, 1991). We examined nuclear DNA from 293 cells transfected with either a contol vector or with the *TRADD* expression vector. DNA isolated from

Table 1. Interactions between TRADD and TNFR1

DNA-Binding Hybrid	Activation Hybrid	Colony Color	Relative β-Galactosidase Activity
GAL4bd–TRADD	GAL4ad	White	<1
GAL4bd	GAL4ad–TRADD	White	<1
GAL4bd–TRADD	GAL4ad–TRADD	Blue	69
GAL4bd–TNFR1	GAL4ad–TRADD	Blue	47
GAL4bd–TNFR1(–14)	GAL4ad–TRADD	Blue	76
GAL4bd–TNFR1(–20)	GAL4ad–TRADD	Light blue	12
GAL4bd–muTNFR1	GAL4bd–TRADD	Light blue	27
GAL4bd–IL1R1	GAL4ad–TRADD	White	<1
GAL4bd–Fas	GAL4ad–TRADD	White	<1
GAL4bd–TNFR2	GAL4ad–TRADD	White	<1

Yeast SFY526 cells were cotransformed with expression vectors encoding various GAL4 DNA-binding domain (GAL4bd) and GAL4 transcription activation domain (GAL4ad) fusion proteins. Each transformation mixture was plated on two synthetic dextrose plates lacking tryptophan and leucine. One plate was used to perform filter assays for colony color. Colonies on the second plate from each transformation were combined (at least 100 colonies per plate) and grown in liquid culture. β-Galactosidase activity was determined on the pooled colonies by the CPRG assay (Iwabuchi et al., 1993).

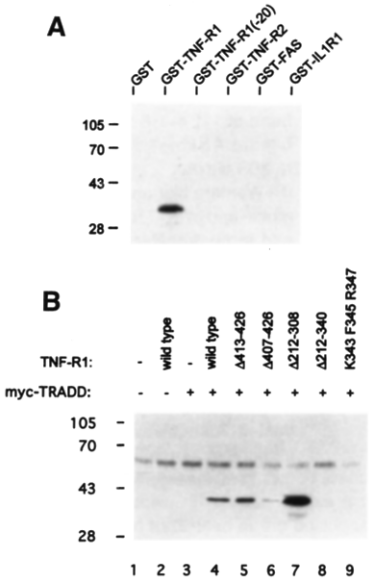

Figure 3. Interaction of TRADD with TNFR1

(A) In vitro interaction of ^{35}S–TRADD with the intracellular regions of several receptors expressed as GST fusion proteins. ^{35}S–TRADD was incubated with purified GST fusion proteins and processed as described in Experimental Procedures. Positions of molecular weight standards (in kilodaltons) are shown.

(B) In vivo interaction of TRADD with TNFR1 mutants. 293 cells (2 × 10^5) were transiently transfected with the Myc epitope–tagged *TRADD* expression vector (1 μg) and the indicated pRK–TNFR1 constructs (1 μg). After 24 hr, extracts were prepared and immunoprecipitated with polyclonal antibody to TNFR1. Coprecipitating Myc–TRADD was detected by immunoblot analysis using the anti-Myc monoclonal antibody.

Table 2. Cell Death Induced by TRADD Overexpression

Expression Vector	Number of Blue Cells per Well		
	HeLa	HepG2	NIH 3T3
pRK control	2110 ± 195	214 ± 16	608 ± 34
TRADD	25 ± 3	1 ± 1	1 ± 1
TRADD, crmA	2083 ± 61	66 ± 20	625 ± 17
TRADD, Bcl-2	1151 ± 74	0 ± 0	21 ± 3
TRADD, E1B	75 ± 9	0 ± 0	5 ± 2

The indicated cell lines were transiently cotransfected with pRK-TRADD (1 μg), pCMV-βgal (0.5 μg), and 3.5 μg of expression vector for *crmA*, *Bcl-2*, or E1B into the various cell lines (2 × 10^5 cells/well). Samples were supplemented with the pRK5 vector control to bring total DNA for each transfection to 5 μg. Cells were fixed and stained with X-Gal 36 hr after transfection. Data (± SEM) are shown as the number of blue cells per 35 mm dish for at least three independent transfections.

TRADD-expressing cells displayed a profile characteristic of apoptosis and closely resembled the DNA from untransfected 293 cells that were treated with TNF (Figure 4B).

It was recently shown (Tewari and Dixit, 1995) that TNF-induced apoptosis can be inhibited by the ICE-specific serpin inhibitor encoded by the cowpox *crmA* gene (Ray et al., 1992). This led us to examine the effects of crmA protein and other known inhibitors of apoptosis (Bcl-2 and adenovirus 19K E1B proteins) on TRADD-induced cell death. The appearance of 293 cells cotransfected with expression vectors encoding both TRADD and crmA was indistinguishable from cells transfected with a control vector alone (Figure 4A). Conversely, coexpression of either the *Bcl-2* or *E1B* gene product did not counteract the apoptotic effect of TRADD expression on 293 cells. Furthermore, crmA coexpression also blocked the generation of TRADD-induced DNA laddering (Figure 4B). These results are consistent with the interpretation that TRADD-induced apoptosis involves activation of ICE or a related protease.

To ensure that the induction of apoptosis by TRADD was not a peculiarity of 293 cells, we also examined the effects of TRADD overexpression on HeLa, HepG2, and murine NIH 3T3 cells. In these cases, a β-galactosidase cotransfection assay (Kumar et al., 1994) was used to examine cell viability. Cells were transiently transfected with a β-galactosidase expression plasmid and the various expression vectors described above. After 36 hr, cells were stained with X-Gal and positive blue cells visualized and

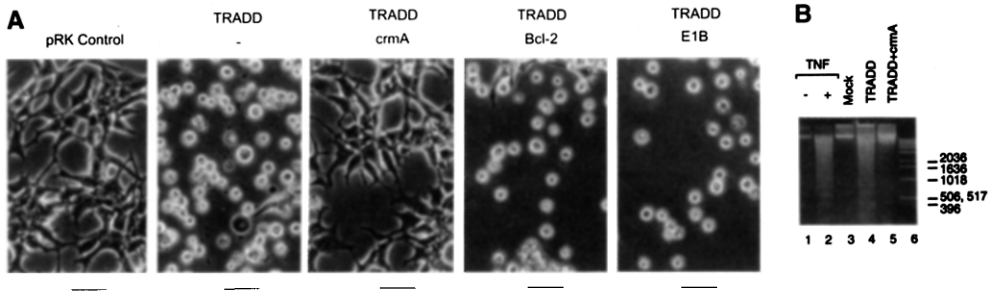

Figure 4. Apoptosis Induced by TRADD Overexpression and Its Inhibition by crmA Expression

(A) Morphology of 293 cells transiently overexpressing TRADD. Cells (2 × 10^5) were transiently transfected with the indicated expression vectors (1 μg) and analyzed 24 hr later by phase contrast microscopy for signs of apoptosis. Scale bar, 50 μm.

(B) DNA fragmentation in transfected 293 cells and TNF-treated 293 cells. Untransfected 293 cells (2 × 10^6) were treated with 320 nM actinomycin D in the presence or absense of TNF (100 ng/ml) for 18 hr before preparation of genomic DNA. Large-scale transfections of 2 × 10^6 293 cells were performed with the control vector pRK5 (20 μg); pRK–TRADD (5 μg) plus pRK5 (15 μg); or pRK–TRADD (5 μg) plus pRK–crmA (15 μg). After 24 hr, DNA was isolated and analyzed as described in Experimental Procedures. The positions of the size markers (in base pairs) are shown on the right.

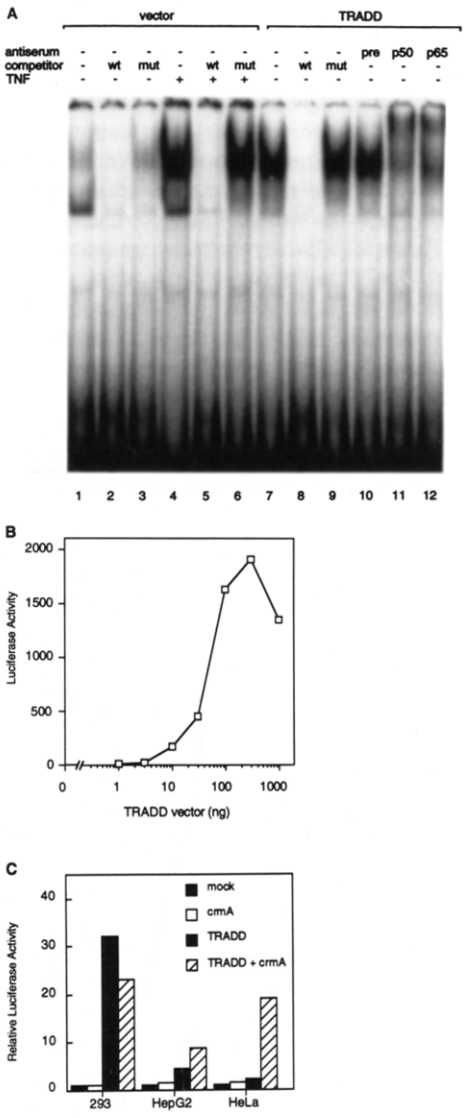

Figure 5. Transcription Factor NF-κB is Activated by TRADD Overexpression

(A) EMSA of NF-κB activation in 293 cells. 293 cells (10^6) were transfected with 10 μg of pRK5 control (lanes 1–6) or pRK–TRADD (lanes 7–12) DNA. Cells were treated with 100 ng/ml TNF for 3 hr prior to harvest (lanes 4–6). Nuclear extracts were prepared 24 hr after transfection, and 10 μg aliquots were combined with the ^{32}P-labeled NF-κB oligonucleotide probe. Individual reactions were supplemented with a 50-fold excess of cold competitor oligonucleotide containing either a wild-type (lanes 2, 5, 8) or mutated (lanes 3, 6, 9) NF-κB sequence. Reaction mixtures were incubated with 1 μl of preimmune serum (lane 10), anti-p50 serum (lane 11), or anti-p65 serum (lane 12) for 10 min prior to addition of probe.

(B) Effect of TRADD expression on NF-κB activity in 293 cells determined by an E-selectin promoter–luciferase gene reporter assay. 293 cells (2×10^5) were transfected with 1 μg of pELAM-luc reporter plasmid, 0.5 μg of pRSV-βgal, indicated amounts of pRK–TRADD, and enough pRK5 control plasmid to give 2.5 μg of total DNA. Luciferase activities were determined 24 hrs after transfection and normalized on the basis of β-galactosidase expression levels. Values shown are averages for an experiment in which each transfection was performed in duplicate.

(C) NF-κB activation by TRADD overexpression in 293, HepG2, and HeLa cells. Cells (2×10^5) were transfected with 4 μg of total DNA as follows. Each transfection received 1 μg of pELAM-luc, 0.5 μg of pRSV-βgal, and one of each of the following: 2.5 μg of pRK5 control vector (solid black bars); 2 μg of pRK–crmA, 0.5 μg of pRK5 (solid white bars); 0.5 μg of pRK–TRADD, 2 μg of pRK5 (dark shading); or 0.5 μg of pRK–TRADD, 2 μg of pRK–crmA (light shading). Cells were harvested 24 hr after transfection and luciferase levels determined. The fold induction in luciferase activity compared with the control pRK5 transfection is indicated.

counted. In all cell lines, a dramatic (~100-fold) reduction in the number of β-galactosidase-positive cells was observed for the *TRADD* vector compared with the control vector (Table 2). When crmA and TRADD were coexpressed, the numbers of blue HeLa and NIH 3T3 cells were the same as in the vector controls. Coexpression of crmA protected about one-third of transfected HepG2 cells from TRADD-induced cell death. As was seen in 293 cells, neither Bcl-2 nor E1B expression exerted a protective effect on TRADD-mediated apoptosis in HeLa or NIH 3T3 cells. Bcl-2 did provide partial protection for HeLa cells from the effect of TRADD overexpression (Table 2).

TRADD Overexpression Activates NF-κB

Another important activity of TNF signaled by TNFR1 is activation of the transcription factor NF-κB. To examine a possible role for TRADD in this process, we performed electrophoretic mobility shift assays (EMSAs) on nuclear extracts from transfected 293 cells. TRADD-expressing 293 cells were found to contain a significant amount of activated NF-κB even in the absense of exogenous TNF. In contrast, specific NF-κB complexes were detected only after TNF treatment in 293 cells transfected with an empty expression vector (Figure 5A). Supershift experiments performed with antibodies demonstrate that the major component of the activated NF-κB complex appears to be the p65–p50 heterodimer.

Dose–response experiments were performed to determine whether TRADD expression might lead to activation of a NF-κB-dependent reporter gene. An E-selectin–luciferase reporter construct was cotransfected with increasing amounts of the *TRADD* expression vector into 293 cells. TRADD expression potently activated the reporter gene, with maximal luciferase activity (approximately 400-fold induction) occurring at a 0.32 μg dose of the *TRADD* expression vector (Figure 5B). These levels of reporter gene induction are greater than the ~20-fold induction observed when 293 cells are treated with TNF alone (data not shown). Further increases in *TRADD* vector resulted in diminished levels of reporter activity, probably owing to induction of cell death (see below).

CrmA Does Not Block TRADD-Induced NF-κB Activation

Since crmA is a potent inhibitor of apoptosis induced by either TNF treatment or TRADD expression, we examined its effect on NF-κB activation. CrmA expression had little or no effect on TRADD-induced NF-κB activation in 293 cells as determined either by EMSA (data not shown) or by the luciferase reporter gene assay (Figure 5C). HeLa and HepG2 cells were also examined for NF-κB activation following transient transfection with the *TRADD* expres-

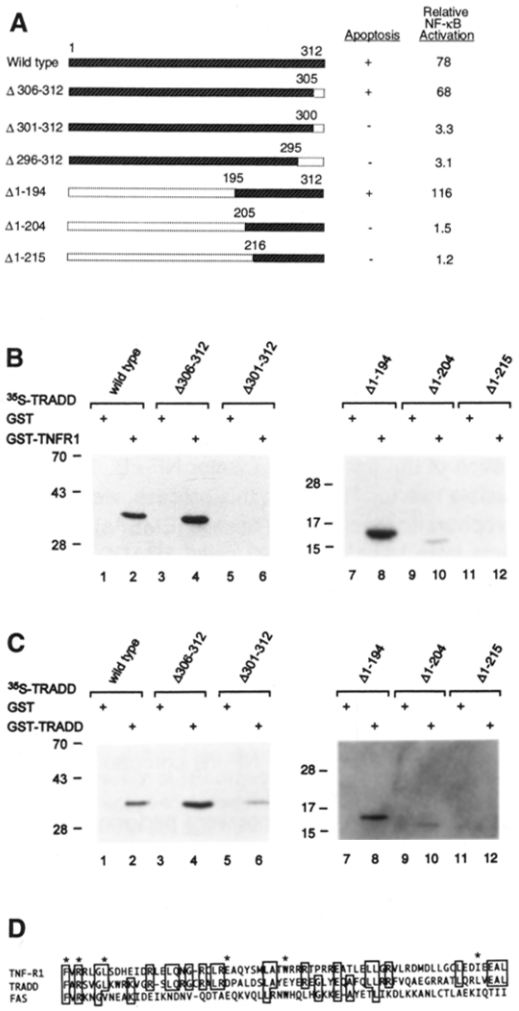

Figure 6. Analysis of TRADD Deletion Mutants

(A) Apoptosis and activation of NF-κB mediated by TRADD deletion mutants. The horizontal bars represent the sequence of TRADD, with shaded regions corresponding to intact sequences and dotted regions indicating deleted sequences. Apoptosis assays were performed 24 hr after transfection of 293 cells with the indicated Myc-tagged TRADD constructs. Plus indicates that the majority of cells became condensed, rounded, and detached (as in Figure 4A), while minus indicates that no morphological changes were observed. NF-κB activation assays were performed following cotransfection of 293 cells with TRADD and the NF-κB reporter plasmid pELAM-luc as described in Experimental Procedures. Data are shown as fold increase in luciferase levels relative to levels in 293 cells transfected with the control vector pRK5.

(B) Interaction of ^{35}S-TRADD deletion mutants with GST-TNFR1. The various ^{35}S-labeled TRADD deletion mutants (equivalent counts per minute) were incubated with purified GST or GST-TNFR1 bound to glutathione-Sepharose beads and processed as described in Experimental Procedures. C-terminal (lanes 1–6) and N-terminal (lanes 7–12) deletion mutants were fractionated on 10% and 15% SDS-PAGE, respectively, and exposed to X-ray film for 8 hr.

(C) In vitro interaction of ^{35}S-TRADD deletion mutants with GST-TRADD. Experiments were performed as described above but using GST-TRADD instead of GST-TNFR1. Dried gels were exposed to X-ray film for 5 days.

(D) Alignment of TRADD, TNFR1, and Fas death domains. Amino acids 222–289 of TRADD are compared with amino acids 345–412 of TNFR1 and 232–298 of Fas antigen. Boxes indicate identities between TRADD and either TNFR1 or Fas. Asterisks above the sequence indicate the six individual residues where mutation to alanine has been shown to inactivate TNFR1 signaling (Tartaglia et al., 1993).

sion vector alone. In most experiments, no activation was seen in these cell lines, a result potentially attributable to rapid induction of cell death (see Table 2). To determine whether NF-κB could be activated by TRADD if cell death were inhibited, these cell lines were cotransfected with *crmA* and *TRADD* expression vectors. While crmA expression alone had no effect, substantial activation of NF-κB was observed in the *crmA–TRADD* cotransfection experiments (Figure 5C). These results demonstrate an inherent ability of TRADD to activate NF-κB, which can be more readily observed if the death pathway is blockaded by crmA expression.

Deletion Mutagenesis of TRADD

The experiments described thus far have identified four distinct properties of TRADD: first, interaction with TNFR1; second, self-association; third, induction of apoptosis; and fourth, activation of NF-κB. To ascertain whether these properties reside in common or distinct domains of TRADD, we constructed a series of N- and C-terminal deletion mutants (Figure 6A). The ability of each mutant to induce apoptosis was determined in the 293 transient assays described above. A TRADD mutant (Δ1–194) containing only 118 C-terminal amino acids was able to trigger cell death, whereas a mutant (Δ1–204) ten amino acids shorter did not. Deleting from the C-terminus, a mutant (Δ301–312) lacking only 12 residues was inactive, but a mutant (Δ306–312) lacking seven amino acids retained the ability to induce apoptosis of 293 cells. This analysis localized the apoptosis activation function (death domain) of TRADD to a 111 residue region extending from amino acid 195 to amino acid 305 (Figure 6A). NF-κB activation for each TRADD mutant was determined in the NF-κB reporter cotransfection assay. According to this analysis, the region of TRADD required for NF-κB activation was strictly concordant with that required for apoptosis (Figure 6A).

The ^{35}S-labeled TRADD deletion mutants were assayed for association with a GST–TNFR1 fusion protein (Figure 6B). Two mutants (Δ306–312 and Δ1–194) bound as well as wild-type TRADD to TNFR1. These same two mutants were biologically active, as determined by the apoptosis and NF-κB activation assays. TRADD mutants that were inactive in these biological assays bound TNFR1 poorly or not at all.

Similarly, the ability of the TRADD deletion mutants to self-oligomerize was assessed by use of a GST–TRADD fusion protein and the ^{35}S-labeled TRADD mutants (Figure 6C). The same mutants that interacted with GST–TNFR1 were able to bind GST–TRADD, although by this assay, self-association was much weaker than interaction with TNFR1. The one difference observed was that the biologically inactive Δ301–312 mutant, which does not interact with TNFR1, was still able to self-associate. These results demonstrate that a 111 amino acid domain (amino acids 195–305) of TRADD is capable of oligomerization, TNFR1 interaction, stimulation of programmed cell death, and NF-κB activation.

Death Domains of TRADD and TNFR1 Share Sequence Similarity

Database screens failed to identify any proteins bearing significant similarity to the primary amino acid sequence of TRADD. However, since the 111 amino acid death domain of TRADD shares many properties with the ~80 amino acid death domain of TNFR1, these two sequences were directly compared. Alignment of TRADD residues 196–302 relative to residues 319–425 of TNFR1 results in 25 identities over 107 amino acids (23%). The most obvious sequence similarity was found in the stretch of 68 amino acids shown in Figure 6D. If a single gap is introduced into both sequences, 22 identities (32%) and 21 conservative changes are found. Whereas the majority of the identities are leucines and arginines, three observations indicate that the sequence similarity may be functionally significant. First, six amino acids have been identified in the death domain of TNFR1 that, when mutated to alanine, abolish signaling (Tartaglia et al., 1993). Five of these six amino acids, which extend to both ends of the aligned sequence (Figure 6D), are identical or highly conserved in TRADD. Second, the sequence similarity between TRADD and TNFR1 over this region is roughly the same as that between the functionally similar death domains of TNFR1 and Fas antigen. Third, TRADD and Fas antigen, which appear not to interact with each other, share only nine identities (13%) over these 68 residues.

Discussion

TRADD Activates TNFR1 Signaling Pathways

The signal transduction events initiated by ligand binding to receptor tyrosine kinases (Schlessinger and Ullrich, 1992) and hematopoietic cytokine receptors (Kishimoto et al., 1994) are now relatively well understood. In contrast, little is known at the molecular level about the postreceptor signaling mechanisms utilized by the TNF receptor superfamily. There are now twelve known members of this family, yet unambiguous evidence for direct receptor coupling with intracellular proteins exists only for TNFR2 and CD40. In the former instance, a heterodimer consisting of the two related proteins TRAF1 and TRAF2 associates via TRAF2 with sequences in the TNFR2 cytoplasmic domain that are necessary for signal transduction (Rothe et al., 1994). In the latter case, another TRAF domain protein, CD40bp or TRAF3, is found to associate with CD40 (Hu et al., 1994).

Most of the known pleiotropic activities of TNF are a consequence of TNFR1 activation (Tartaglia and Goeddel, 1992). An ~80 amino acid death domain located near the C-terminus of TNFR1 is sufficient to initiate signals for apoptosis, antiviral activity, and NF-κB activation (Tartaglia et al., 1993; Y.-F. Hu and D. V. G., unpublished data). To identify candidate proteins for evaluation as TNFR1-associated signaling molecules, we utilized the yeast two-hybrid cloning approach of Fields and Song (1989). Consistent with the work of others (Song et al., 1994; Boldin et al., 1995), we found that the cDNAs isolated most frequently were those encoding the death domain of TNFR1. We also identified TRADD, a 34 kDa protein bearing little resemblance to previously described proteins.

Overexpression of TRADD in a variety of cell lines was found to be a potent inducer of programmed cell death, mimicking the effect of TNF treatment in the presence of actinomycin D. TRADD-mediated apoptosis is effectively blocked by coexpression of the cowpox virus *crmA* gene, which encodes a protease inhibitor of the serpin class (Pickup et al., 1986). CrmA is a specific inhibitor of ICE (Ray et al., 1992), a cysteine protease involved in IL-1β processing (Thornberry et al., 1992) and in some types of programmed cell death (Miura et al., 1993; Gagliardini et al., 1994). Significantly, crmA was recently shown to block the apoptosis triggered by both TNFR1 and Fas antigen (Tewari and Dixit, 1995), suggesting ICE involvement in these signaling pathways as well. Therefore, it is not surprising that TRADD-mediated cell death involves ICE, or an ICE-related protease; this result might be expected if TRADD overexpression were to activate a latent TNFR1 apoptotic pathway.

The Fas antigen signals cell death through a portion of its cytoplasmic domain that is 28% identical to the death domain of TNFR1 (Itoh and Nagata, 1993). Interestingly, TRADD does not appear to interact with Fas antigen. This observation suggests that the receptor-proximal apoptotic signaling pathways activated by TNFR1 and Fas antigen are distinct, but converge downstream at some point before ICE activation. Alternatively, the two pathways may be parallel and activate distinct crmA-inhibitable, ICE-related proteases. In either case, it is possible that a TRADD-related protein will be discovered that associates with the death domain of Fas antigen. Furthermore, both alternatives are consistent with reports that TNF and Fas activate programs of cell death that are at least partially distinct (Wong and Goeddel, 1994; Schulze-Osthoff et al., 1994).

Both TNF and IL-1 induce the transcriptional activation of a large set of genes involved in acute phase and inflammatory responses by activating the transcription factor NF-κB. In general, the proinflammatory activity of TNF is signaled through TNFR1 (Kruppa et al., 1992; Pfeffer et al., 1993), although there are some cell lines in which TNFR2 can activate NF-κB (Rothe et al., 1994; Lægreid et al., 1994). Transient overexpression of TRADD potently activated NF-κB in several TNF-responsive cell lines. However, in several instances this activation could be seen only when the cell death pathway was inhibited by crmA expression. Our failure to observe any interaction between TRADD and either IL-1R1 or TNFR2 suggests that TRADD is not involved in NF-κB activation initiated by these two receptors and may be exclusively dedicated to TNFR1 signal transduction.

Deletion mutagenesis experiments identified a region of 111 amino acids near the C-terminus of TRADD that is sufficient to trigger both apoptosis and NF-κB activation. This death domain is also sufficient for interaction with TNFR1 and self-association. Furthermore, this region is related in primary sequence to the death domain of TNFR1, but bears little similarity to the Fas antigen death domain. This raises the possibility that the greater se-

quence identity between TRADD and TNFR1 defines a structural framework that accounts for their ability to interact specifically.

To date, we have been unable to identify a TRADD mutant that interacts with TNFR1 but fails to signal. Such a mutant might be expected to act as a dominant negative inhibitor of TNF signaling, thereby demonstrating an essential role for TRADD in TNFR1 signal transduction. However, strong correlative evidence for such a role comes from several observations. First, no active TRADD deletion mutants were found that failed to interact with TNFR1. Second, mutants of TNFR1 that are incapable of signaling cell death or NF-κB activation do not interact with TRADD. Third, two-hybrid screening using murine TNFR1 as bait resulted in the isolation of a cDNA for murine TRADD (M. Pan and D. V. G., unpublished data). Finally, human TRADD is able to interact with the cytoplasmic domains of murine and human TNFR1, which do not exhibit species specificity, yet share only 59% sequence identity (Lewis et al., 1991).

Mechanisms of TRADD-Induced Signal Transduction

The disparate effects of crmA expression on TRADD-induced apoptosis and NF-κB activation are significant, as they suggest divergence of the apoptotic and NF-κB pathways mediated by TNFR1. Furthermore, the failure of crmA expression to block NF-κB activation indicates that this aspect of TRADD activity is not an artifact of sick or dying cells. Apparently, the interaction of the death domain of TNFR1 with the C-terminal region of TRADD activates either two separate signaling cascades or one primary signal that subsequently splits into two (or more) pathways before ICE is encountered on the apoptotic pathway.

Just how TRADD is able to transmit signals downstream from TNFR1 remains a mystery at this time. Western blot analyses indicate that TRADD is normally expressed in very low amounts. On the basis of cDNA cloning results (approximately one positive in 10^6 clones screened) and Northern blot analyses, we estimate that *TRADD* mRNA levels are typically less than 5 molecules per cell. Perhaps TRADD has a higher affinity for aggregated than for monomeric TNFR1, such that under normal physiological conditions, interaction can only occur following TNF-induced receptor aggregation. Another possibility is that a TRADD or TNFR1-associated inhibitory protein prevents interaction of TRADD with TNFR1 prior to receptor aggregation. Both possibilities might be expected to represent delicately balanced signaling systems that could be overridden by enforced expression of TRADD. Similarly, overexpression of TNFR1 has been shown to trigger cell death, IL-8 gene induction (Boldin et al., 1995), and NF-κB activation (H. H. and D. V. G., unpublished data). In this case, ligand-independent aggregation of TNFR1 is observed, which might result in recruitment of TRADD to the receptor complex.

The identification and characterization of TRADD described herein provides an initial step in deciphering the intracellular signaling pathways activated by TNF binding to TNFR1. However, many important questions remain unresolved: first, what role does TRADD play in other TNFR1-transduced signals, such as activation of sphingomyelinases or the TNFR1-associated serine kinase activity? Second, what is the function of the ~200 N-terminal amino acids of TRADD that are not required for induction of apoptosis and NF-κB activation? Third, how does TRADD connect to downstream signaling events? Fourth, do other members of the TNF receptor superfamily interact with TRADD or related molecules to initiate their signaling cascades? Finally, do TRADD-like proteins participate in other (non-TNF receptor superfamily–mediated) pathways of programmed cell death? We hope that the molecular reagents and observations reported herein will help stimulate a resolution to these and other questions.

Experimental Procedures

Reagents and Cell Lines

Recombinant human TNF was provided by Genentech, Incorporated. The rabbit anti-TNFR1 polyclonal antibody was described previously (Tartaglia et al., 1991). The rabbit anti-TRADD antiserum was raised against a GST–TRADD fusion protein by BabCo (Richmond, California). The monoclonal antibody against the Myc epitope (S-M-E-Q-K-L-I-S-E-E-D-L-N) was provided by R. Schreiber. Rabbit anti-p50 and anti-p65 polyclonal antibodies were purchased from Santa Cruz Biotechnology. The HtTA-1 (for HeLa expressing a tetracycline-controlled transactivator; Dr. H. Bujard), 293 (R. Tjian), HepG2 (American Type Culture Collection [ATCC]), ECV304 (ATCC), and NIH 3T3 (S. McKnight) cell lines were obtained from the indicated sources.

Expression Vectors

The *TRADD* cDNA was cloned as a 1.4 kb EcoRI fragment into pRK5 under the transcriptional control of the cytomegalovirus (CMV) immediate-early promoter–enhancer (Schall et al., 1990). The resulting plasmid, pRK-TRADD, was used for mammalian cell expression and for in vitro transcription and translation using the SP6 promoter. Myc epitope tag constucts were made by replacing the eight N-terminal codons of *TRADD* with DNA encoding the sequence M-A-S-M-E-Q-K-L-I-S-E-E-D-L. C-terminal deletion mutants of TRADD were generated by replacement of sequences between the XhoI site in *TRADD* and the HindIII site in pRK5 with synthetic DNA containing the appropriate coding sequence and in-frame stop codons. N-terminal deletion mutants of TRADD were generated by polymerase chain reaction (PCR). The various mutant *TNFR1* expression vectors were described previously (Tartaglia et al., 1993). A plasmid containing the cowpox virus *crmA* gene (Pickup et al., 1986) was obtained from G. Palumbo. A 1.0 kb *crmA* fragment was generated by PCR and inserted into the pRK5 vector to give the plasmid pRK–crmA. The *Bcl-2* expression vector pSFFV–Bcl-2, based on the long terminal repeat of the splenic focus-forming virus, was provided by S. Korsmeyer. The CMV-based expression vector pCMV19K for the adenovirus 19 kDa E1B protein was provided by J. Fraser. The NF-κB–luciferase reporter plasmid pELAM-luc, containing E-selectin promoter sequences from position −730 to position +52 (Schindler and Baichwal, 1994), was provided by U. Schindler.

Yeast Two-Hybrid Cloning

DNA encoding the intracellular domain (amino acids 214–426) of TNFR1 was cloned into the yeast GAL4 DNA-binding domain vector pGBT9. The resulting plasmid, pGAL4bd–TNF–R1icd, was used as bait in two-hybrid screens of HeLa and B cell cDNA libraries (Clontech) following the Matchmaker Two-Hybrid System Protocol (Clontech). Positive yeast clones were selected by prototrophy for histidine and expression of β-galactosidase. Yeast DNA was recovered and transformed into E. coli. Plasmids containing cDNA clones were identified by restriction mapping and further characterized by DNA sequencing. Subsequent two-hybrid interaction analyses were carried out by co-transformation of plasmids containing the GAL4 DNA-binding (pGBT9)

and -activation (pGAD424) domains into Saccharomyces cerevisiae strain SFY526.

cDNA Cloning and Northern Blot Hybridization

The cDNA insert of approximately 1 kb from two-hybrid clone B27 was used as probe to screen human HeLa and HUVEC cDNA libraries in λgt11 (provided by Dr. Z. Cao; complexity of >2 × 10^6 clones each) by standard methods (Sambrook et al., 1989). Two independent positive clones were obtained from each library. Following subcloning into pBluescript KS (Stratagene), DNA sequencing was performed on an Applied Biosystems, Incorporated model 373A automated DNA sequencer by use of the Prism Dye Terminator Cycle sequencing kit (Applied Biosystems). Northern blot analysis of the human multiple tissue blot (Clontech) was performed according to the instructions of the manufacturer, by use of an ~400 bp EcoRI–NarI fragment from the 5′ end of *TRADD* cDNA as probe.

Cell Culture, Transfections, and Reporter Assays

293 and NIH 3T3 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 μg/ml penicillin G, and 100 μg/ml streptomycin (GIBCO). HtTA-1 (HeLa) cells were grown in the same medium containing 400 μg/ml G418. HepG2 cells were maintained in DMEM/F12 (1:1) medium with the same additives. For reporter assays, coimmunoprecipitations, and cell killing assays, ~2 × 10^5 cells/well were seeded on 6-well (35 mm) dishes and grown in 5% CO_2 at 37°C. Cells were transfected the next day by the calcium phosphate precipitation method (Ausubel et al., 1994). After an incubation of 24–36 hr, cells were washed twice with phosphate-buffered saline and then lysed with 200 μl of lysis buffer (25 mM Tris–phosphate [pH 7.8], 2 mM dithiothreitol [DTT], 2 mM 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid [CDTA], 10% glycerol, 1% Triton X-100). Aliquots of cell lysates [20 μl] were mixed with 100 μl of luciferase assay reagent (Promega) and the luciferase activity determined using a Model 20e luminometer (Turner Designs). β-Galactosidase activity was determined in a mixture containing 10 μl of cell lysate, 10 μl of 50 mM chlorophenol red β-D-galactopyranoside (CPRG), and 80 μl of Z buffer (60 mM Na_2HPO_4, 10 mM KCl, 1 mM β-mercaptoethanol [pH 7.0]). Samples were incubated at 37°C until red color developed, and absorbance was determined at 574 nm. These values were used to normalize transfection efficiencies. For DNA laddering and EMSA experiments, transfections were performed with 100 mm dishes seeded with ~10^6 cells. At 24–36 hr after transfection, DNA or nuclear extracts were prepared.

Coimmunoprecipitations and Western Blot Analysis

Western blot analysis to detect TRADD was performed with the anti-TRADD antiserum and horseradish peroxidase–coupled goat anti-rabbit IgG (Amersham) using enhanced chemiluminescence according to the protocol of the manufacturer. For immunoprecipitation assays, 50 μl aliquots of lysates from transfected cells were incubated with 1 μl of the anti-TNFR1 antibody and 450 μl of E1A buffer (50 mM HEPES [pH 7.6], 250 mM NaCl, 0.1% NP-40, 5 mM EDTA). The mixture was incubated at 4°C for 1 hr, then mixed with 20 μl of a 1:1 slurry of protein A–Sepharose (Pharmacia) and incubated for another hour. The beads were washed twice with 1 ml of E1A buffer, twice with 1 ml of high salt (1 M NaCl) E1A buffer, and twice again with E1A buffer. The precipitates were fractionated on 10% SDS–polyacrylamide gels and transferred to Immobilon P membrane (Millipore). The blot was subjected to Western blot analysis with anti-Myc monoclonal antibody and horseradish peroxidase–coupled rabbit anti-mouse immunoglobulin.

Generation of GST Fusion Proteins and In Vitro Binding Assays

TRADD and the cytoplasmic regions of TNFR1, TNFR1(−20), Fas antigen, IL-1R1, and TNFR2 were expressed individually as GST (glutathione S-transferase) fusion proteins by use of pGEX vectors (Pharmacia). Expression and purification of the GST fusion proteins were performed as described (Smith and Johnson, 1988). ^{35}S-labeled proteins were generated with the TNT SP6 Coupled Reticulocyte Lysate System (Promega) and the various *TRADD* expression constructs in pRK5. For each in vitro binding assay, 10 μl of glutathione–Sepharose beads (Pharmacia) bound to the appropriate GST fusion protein (~5 μg) was incubated with ^{35}S-labeled polypeptides in 1 ml of E1A buffer at 4°C for 1 hr. The beads were then washed six times with E1A buffer. Proteins on the beads were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) and exposed to Kodak X-ray film.

Apoptosis Assays

DNA fragmentation assays were performed as described (Hermann et al., 1994), using approximately 10^7 transfected or nontransfected cells. DNA aliquots (150 ng) were fractionated by electrophoresis in a 1.6% agarose gel.

β-Galactosidase cotransfection assays for determination of cell death were performed as described by Kumar et al. (1994). Cells were observed microscopically, and the number of blue cells per 35 mm well was determined by counting.

EMSAs

Nuclear extracts were prepared as described by Osborn et al. (1989). Double-stranded oligonucleotides (5′-GATGCCATTGGGGATTTCCTCTTTACTG) containing an NF-κB-binding site were ^{32}P-labeled using polynucleotide kinase. Each gel shift assay was performed in a 30 μl reaction mixture containing 10 μg of nuclear extracts, 0.4 ng of radiolabeled oligonucleotide probe, 1 μg of sonicated E. coli DNA, 6 μl of 5× EMSA buffer (100 mM HEPES [pH 7.6], 250 mM KCl, 5 mM DTT, 5 mM EDTA, 25% glycerol), and where necessary, 20 μg of cold competitor oligonucleotides. The mutant competitor oligonucleotides have the underlined NF-κB site changed to GGaagcTTCC. Mixtures were incubated at room temperature for 10 min and then subjected to electrophoretic fractionation on a 5% polyacrylamide gel at 4°C.

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GenBank Accession Number

The accession number for the human TRADD sequence reported in this paper is L41690.