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TRADD–TRAF2 and TRADD–FADD Interactions Define Two Distinct TNF Receptor 1 Signal Transduction Pathways

Hailing Hsu, Hong-Bing Shu, Ming-Gui Pan, and David V. Goeddel
Tularik, Incorporated
270 East Grand Avenue
South San Francisco, California 94080

Summary

Tumor necrosis factor (TNF) can induce apoptosis and activate NF-κB through signaling cascades emanating from TNF receptor 1 (TNFR1). TRADD is a TNFR1-associated signal transducer that is involved in activating both pathways. Here we show that TRADD directly interacts with TRAF2 and FADD, signal transducers that activate NF-κB and induce apoptosis, respectively. A TRAF2 mutant lacking its N-terminal RING finger domain is a dominant-negative inhibitor of TNF-mediated NF-κB activation, but does not affect TNF-induced apoptosis. Conversely, a FADD mutant lacking its N-terminal 79 amino acids is a dominant-negative inhibitor of TNF-induced apoptosis, but does not inhibit NF-κB activation. Thus, these two TNFR1–TRADD signaling cascades appear to bifurcate at TRADD.

Introduction

Tumor necrosis factor (TNF) is a proinflammatory cytokine whose pleiotropic biological properties are signaled through two distinct cell surface receptors (reviewed by Tartaglia and Goeddel, 1992; Rothe et al., 1992). TNF receptor 1 (TNFR1; 55–60 kDa) and TNFR2 (75–80 kDa) are expressed on most cell types and are ~30% identical in their extracellular, cysteine-rich, ligand-binding regions (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990). This extracellular domain architecture is characteristic of the larger TNFR superfamily, whose members include the Fas antigen, CD27, CD30, CD40, and several other receptors (reviewed by Smith et al., 1994). With the exception of TNFR1 and Fas, both of which can induce programmed cell death through a shared ~80 amino acid "death domain" of 28% identity (Tartaglia et al., 1993a; Itoh and Nagata, 1993), the cytoplasmic domains of receptors in this superfamily share no obvious sequence similarities.

Until recently, the molecular mechanisms utilized by members of the TNFR family to generate cellular responses have remained largely undefined. However, during the past year an improved understanding of these mechanisms has begun to emerge with the identification of two distinct classes of receptor-associated proteins, both of which appear to couple these receptors to downstream signaling cascades.

Three intracellular proteins that contain death domains were identified through yeast two-hybrid interaction cloning by virtue of their interactions with the death domains of TNFR1 and Fas. TRADD is a 34 kDa protein that interacts specifically with TNFR1 (Hsu et al., 1995), whereas the 23 kDa FADD (Boldin et al., 1995a; Chinnaiyan et al., 1995) and the 74 kDa RIP (Stanger et al., 1995) interact with Fas. In fact, death domains now appear to define interaction domains that are capable of both homotypic and heterotypic associations (Song et al., 1994; Boldin et al., 1995a, 1995b; Hsu et al., 1995; Chinnaiyan et al., 1995; Stanger et al., 1995). These observations suggest that death domains may function as adaptors to couple some members of the TNFR superfamily (at least TNFR1 and Fas) to other signaling proteins.

TRADD contains an N-terminal region of unknown function and a C-terminal death domain that is 23% identical to the death domain of TNFR1. TRADD may play an obligatory role in TNFR1 responses, since its overexpression activates TNFR1-like signaling pathways for both apoptosis and activation of the transcription factor NF-κB (Hsu et al., 1995). Similarly, overexpression of FADD (Boldin et al., 1995a; Chinnaiyan et al., 1995) or RIP (Stanger et al., 1995) mimics Fas activation, leading to programmed cell death. However, the mechanisms of signaling by TRADD, FADD, and RIP are not identical. Whereas the induction of cell death by TRADD or RIP requires only their death domains, apoptosis induced by FADD overexpression occurs independently of its C-terminal death domain. This suggests that FADD contains a "death effector" domain at its N-terminus, which may bind downstream apoptosis-signaling molecules for recruitment to Fas via death domain interactions.

The second family of signal transducing proteins utilized by the TNFR superfamily are the TNFR-associated factors (TRAFs). TRAF1 and TRAF2 were biochemically purified as TNFR2-associated proteins of 45 and 56 kDa, respectively (Rothe et al., 1994). TRAF1 and TRAF2 exist in a multimeric complex that interacts via TRAF2 with the signaling domains of both TNFR2 and CD40 (Rothe et al., 1994, 1995). TRAF3 was identified by two-hybrid interaction cloning as a CD40-associated protein of 64 kDa (Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995). The three known TRAFs share a highly conserved C-terminal "TRAF domain" of about 150 amino acids, which is involved in oligomerization and receptor association (Rothe et al., 1994, 1995; Cheng et al., 1995; Sato et al., 1995). Overexpression of TRAF2, but not TRAF1 or TRAF3, activates NF-κB (Rothe et al., 1995). Furthermore, a truncated TRAF2 lacking its N-terminal RING finger acts as a dominant-negative inhibitor of NF-κB activation mediated by both TNFR2 and CD40 (Rothe et al., 1995). Therefore, TRAF2 is a common mediator of signal transduction by TNFR2 and CD40 and, perhaps, by other members of the TNFR superfamily.

Two of the most important activities of TNF, apoptosis and NF-κB activation, are signaled by TNFR1 following its oligomerization by the trimeric TNF (Tartaglia and Goeddel, 1992). We recently reported the molecular cloning and characterization of TRADD, a novel protein that specifically interacts with the death domain of TNFR1 and activates signaling pathways for both of these activities when overexpressed (Hsu et al., 1995).

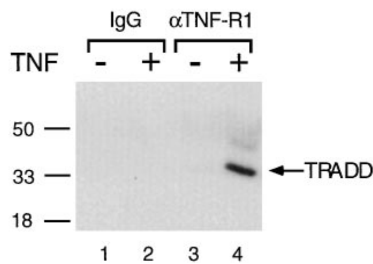

Figure 1. TNF Induces Association of TRADD with TNFR1

We treated U937 cells (5 × 10^7) with TNF for 15 min (lanes 2 and 4) or left them untreated (lanes 1 and 3). Cell lysates were immunoprecipitated with anti-TNFR1 monoclonal antibody 985 (lanes 3 and 4) or with mouse immunoglobulin G (IgG) control antibody (lanes 1 and 2). Coprecipitating TRADD was detected by immunoblot analysis using rabbit anti-TRADD antiserum. Positions of molecular mass standards (in kilodaltons) are shown.

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mFADD   MDPFLVLLHSLSGSLSGNDLMELKFLCRERVSKRKLERVQSGLDLFTVLL   50
hFADD   MDPFLVLLHSVSSSLSSSELTELKFLCLGRVGKRKLERVQSGLDLFSMLL   50

mFADD   EQNDLERGHTGLLRELLASLRRHDLLQRLDDFEAGTATAAPPGEADLQVA  100
hFADD   EQNDLEPGHTELLRELLASLRRHDLLRRVDDFEAGAAAGAAPGEEDLCAA  100

mFADD   FDI[VCDNVGRDWKRLARELKVSEAKMDGIEEKYPRSLSERVRESLKVWKN  150
hFADD   FNV[ICDNVGKDWRRLARQLKVSDTKIDSIEDRYPRNLTERVRESLRIWKN  150

mFADD   AEKKNASVAGLVKALRTFRLNLVADLV]EEAQES---VSKSENMSPVLRDS  197
hFADD   TEKENATVAHLVGALRSCQMNLVADLV]QEVQQARDLQNRSGAMSPMSWNS  200

mFADD   TVSSSETP                                           205
hFADD   DASTSEAS                                           208
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Figure 2. Predicted Amino Acid Sequence of Murine FADD

The amino acid sequences of murine and human FADD are aligned. Identical residues are stippled The death domain of FADD extends from amino acids 104–177, as indicated by the brackets.

Here we show that under normal conditions the association of TRADD with TNFR1 is TNF dependent. We also show that TRADD directly interacts with TRAF2 through its N-terminal half and with FADD through its C-terminal death domain. Furthermore, dominant-negative mutants of TRAF2 and FADD can block TNF-mediated NF-κB activation and cell death, respectively. Taken together, these data suggest that TNFR1 utilizes distinct TRADD-dependent mechanisms to activate signaling pathways for NF-κB activation and apoptosis.

Results

Interaction of TRADD with TNFR1 Is TNF Dependent

We previously demonstrated that TRADD specifically associates with TNFR1 when both proteins are overexpressed (Hsu et al., 1995). To determine whether the TRADD–TNFR1 interaction is physiologically relevant, we performed the following experiment using human U937 cells. Lysates from cells that had been treated with TNF for 15 min or left untreated were immunoprecipitated with a nonagonistic monoclonal antibody directed against the extracellular domain of TNFR1. Coprecipitating TRADD was detected by immuoblot analysis using a TRADD-specific polyclonal antiserum. TRADD coprecipitated with TNFR1 only in the TNF-treated cell lysates (Figure 1). Since TNF induces trimerization of TNFR1, this result suggests that the recruitment of TRADD to TNFR1 probably depends on a properly aggregated TNFR1 cytoplasmic domain.

Identification of TRAF2 and FADD as TRADD-Interacting Proteins

To identify potential downstream components of the TNFR1/TRADD signaling pathway, we used the yeast two-hybrid system (Fields and Song, 1989) to screen cDNA libraries for TRADD-interacting proteins. Multiple cDNA clones were obtained that encode several distinct proteins. As expected, two classes of clones encoded TRADD itself or portions of the intracellular region of TNFR1. Surprisingly, many of the cDNA clones were found to encode portions of TRAF2, a protein that interacts with TNFR2 and CD40 and transduces the signal for NF-κB activation by these two receptors (Rothe et al., 1994, 1995). The interaction of TRADD with TRAF2 was verified in yeast two-hybrid interaction assays using full-length TRAF2. TRADD was also found to interact strongly with TRAF1, and more weakly with TRAF3, in two-hybrid assays (data not shown).

The two-hybrid screen also yielded cDNA clones encoding full-length murine FADD and the C-terminal 126 amino acids of murine FADD. The 205 amino acid murine FADD shares 73% identity with human FADD throughout the region (amino acids 1–177) comprising the effector and death domains, whereas the C-terminal ∼30 amino acids are very poorly conserved (Figure 2). Since FADD was originally identified owing to its interaction with Fas (Boldin et al., 1995a; Chinnaiyan et al., 1995), we compared the relative strength of the TRADD–FADD and Fas–FADD interactions in two-hybrid assays. These experiments indicated that FADD consistently interacts 10- to 20-fold more strongly with TRADD than with Fas (data not shown).

Since TRAF1, TRAF2, and FADD interact strongly with TRADD in two-hybrid tests, we performed coimmunoprecipitation assays to test whether these proteins might interact in human cells. An expression vector encoding TRADD was transfected alone or with expression vectors encoding Flag epitope–tagged TRAF1, TRAF2, or FADD into human embryonic kidney 293 cells. Cell extracts were immunoprecipitated using a monoclonal antibody against the Flag epitope, and coprecipitating TRADD was detected by Western blotting with anti-TRADD polyclonal antibody. TRAF1, TRAF2, and FADD were individually able to coprecipitate TRADD (Figure 3).

Mapping of TRADD–TRAF2 and TRADD–FADD Interaction Domains

To determine the region(s) of TRADD responsible for interaction with TRAF2 and FADD, a series of N-terminal and C-terminal TRADD truncation mutants were examined in two-hybrid assays (Figure 4A). A C-terminal deletion mutant of TRADD lacking its entire death domain retained TRAF2-binding activity. This demonstrated that the TRAF2-binding domain resides in the N-terminal half (amino acids 1–169) of TRADD. A further C-terminal deletion mutant (TRADD[1–106]) failed to interact with TRAF2. In contrast, TRADD interacts with FADD through its C-terminal death domain (amino acids 195–312). This

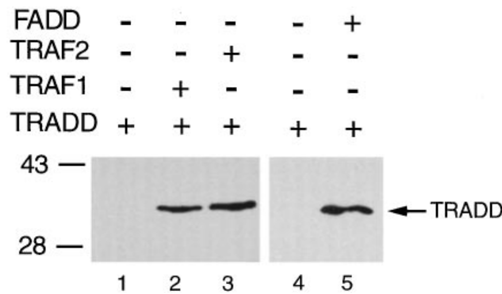

Figure 3. In Vivo Interaction of FADD and TRAF Proteins with TRADD

We transfected 293 cells (2×10^6) with the indicated combinations of expression vectors for TRADD and Flag epitope–tagged TRAF1, TRAF2, or FADD. After 24 hr, extracts were prepared and immunoprecipitated with an anti-Flag monoclonal antibody. Coprecipitating TRADD was detected by immunoblot analysis using polyclonal antibody against TRADD. Positions of molecular mass standards (in kilodaltons) are shown.

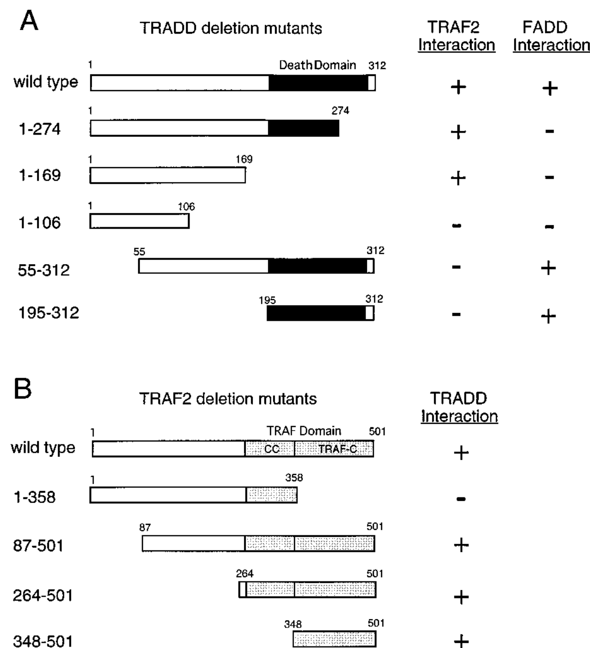

Figure 4. Mapping of TRADD–TRAF2 and TRADD–FADD Interaction Domains

(A) Interaction of TRAF2 and FADD with the N- and C-terminal domains of TRADD, respectively. Expression vectors encoding wild-type TRADD or the indicated deletion mutants fused to the GAL4 DNA-binding domain were cotransformed into yeast Y190 cells with a GAL4 activation domain–TRAF2 or GAL4 activation domain–FADD expression vector.

(B) The TRAF domain of TRAF2 interacts with TRADD. Expression vectors encoding wild-type TRAF2 or the indicated deletion mutants fused to GAL4 activation domain were cotransformed into yeast Y190 cells with a GAL4 DNA-binding domain–TRADD expression vector. The TRAF domain of TRAF2 can be subdivided into two parts, a coiled-coil (CC) region and a C-terminal (TRAF-C) region (Rothe et al., 1994). Transformation mixes were plated on synthetic dextrose plates lacking tryptophan and leucine. Filter assays were performed for β-galactosidase activity. Plus signs represent blue color development; minus signs indicate no color development.

is the same region of TRADD that is required for interaction with the death domain of TNFR1 (Hsu et al., 1995).

Deletion studies on TRAF2 indicate that its TRAF-C domain (amino acids 348–501) was sufficient for TRADD binding (Figure 4B). Previous studies had shown that the entire TRAF domain (amino acids 264–501) of TRAF2 can self-associate and interact with TRAF1, TNFR2, and CD40 (Rothe et al., 1994, 1995).

TRADD Recruits TRAF Proteins to TNFR1

The interaction of TRADD with TNFR1 and TRAFs occurs via its C-terminal death domain and N-terminal TRAF-binding domain, respectively. Therefore, TRADD might be able to bind simultaneously both TNFR1 and TRAFs, thereby recruiting TRAFs to the TNFR1 complex. To explore this possibility, we cotransfected 293 cells with plasmids that direct the synthesis of TNFR1, TRADD, and Flag-tagged TRAF1 or TRAF2. Cell extracts were immunoprecipitated with agonistic polyclonal antibodies against the extracellular domain of TNFR1, and coprecipitating TRAF proteins were detected by Western blotting with an anti-Flag monoclonal antibody. In lysates prepared from cells programmed for ectopic expression of TNFR1 and TRAF1 or TRAF2, antibody against TNFR1 failed to coprecipitate TRAF proteins (Figure 5A). This result, which suggests that no direct interaction occurs between the TNFR1 and TRAF1 or TRAF2, is in agreement with results obtained previously by yeast two-hybrid interaction assays (Rothe et al., 1995). However, when the same experiments were performed on lysates from cells that also expressed TRADD, both TRAF1 and TRAF2 were coimmunoprecipitated with TNFR1 (Figure 5A). These results provide evidence that TRADD can serve as an adaptor protein and recruit TRAF1, TRAF2, or both to TNFR1.

Since TRAF2 is capable of interacting with both TNFR2 and TRADD, we also considered the possibility that TRAF2 could recruit TRADD to the TNFR2 receptor complex. This was examined by cotransfection and immunoprecipitation experiments similar to those described above for TNFR1. However, no ternary TNFR2–TRADD–TRAF2 complex could be detected (data not shown), demonstrating that TRAF2 cannot simultaneously bind to both TNFR2 and TRADD. This finding suggests that TNFR2 and TRADD, both of which interact with the C-terminal TRAF domain of TRAF2 (see Figure 4B; Rothe et al., 1994), compete for the same, or an overlapping, binding site on TRAF2.

TRADD Enhances Association of FADD with TNFR1

The strong interaction between TRADD and FADD occurs via their death domains. TRADD also associates with TNFR1 through death domain interactions. To determine whether TRADD can simultaneously interact with both FADD and TNFR1 to recruit FADD to TNFR1, we performed coimmunoprecipitation experiments. FADD coprecipitated with Fas, consistent with previous results (Chinnaiyan et al., 1995), whereas no detectable FADD coprecipitated with TNFR1 (Figure 5B). In contrast, when TNFR1 and FADD were expressed in the

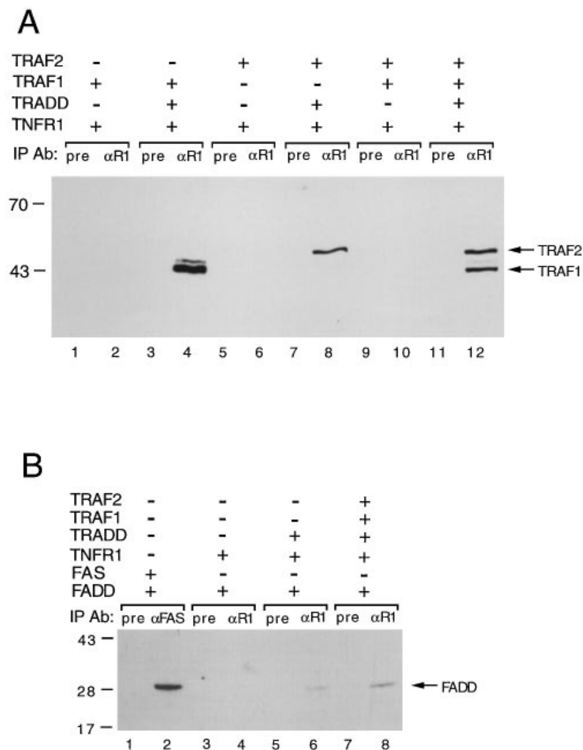

Figure 5. TRADD Recruits TRAF1, TRAF2, and FADD to TNFR1

(A) Coimmunoprecipitation of TNFR1–TRADD–TRAF complexes. We transfected 293 cells (2×10^6 per 100 mm plate) with the indicated combinations of expression vectors for TNFR1, TRADD, Flag–TRAF1, or Flag–TRAF2. After 24 hr, extracts were prepared and immunoprecipitated with preimmune serum (odd-numbered lanes) or with polyclonal antibody against the extracellular domain of TNFR1 (even-numbered lanes). Coprecipitating Flag–TRAF1 and Flag–TRAF2 were detected by immunoblot analysis with anti-Flag monoclonal antibody.

(B) Coimmunoprecipitation of TNFR1–TRADD–FADD complexes. We transfected 293 cells with the indicated combinations of expression vectors for Fas, TNFR1, TRADD, Flag–FADD, TRAF1, and TRAF2. Western blotting analysis indicated that Flag–FADD expression levels were similar in all samples (data not shown). Lysates were immunoprecipitated with preimmune (lanes 1, 3, 5, and 7), anti-Fas (lane 2), or anti-TNFR1 (lanes 4, 6, and 8) antiserum. Coprecipitating Flag–FADD was detected with anti-Flag monoclonal antibody.

presence of TRADD, FADD could be detected in the anti-TNFR1 immune complex. However, even under these conditions, the amount of FADD that coprecipitated with TNFR1 was less than with Fas. The addition of TRAF1 and TRAF2 did not inhibit, but rather slightly enhanced, the ability of TRADD to recruit FADD to TNFR1 (Figure 5B). This result shows that TRADD can simultaneously recruit TRAF proteins and FADD to TNFR1. Furthermore, these experiments suggest that, whereas TRADD–FADD and TRADD–TNFR1 complexes are relatively stable, trimeric TNFR1–TRADD–FADD complexes may exist only transiently or may require other proteins for their stabilization.

NF-κB Activation by TNFR1 Requires TRAF2

The activation of NF-κB by TNFR2 and CD40 is mediated by TRAF2 (Rothe et al., 1995). The presence of TRAF2 in the TNFR1 complex suggests that it may also be involved in signaling TNFR1 responses. However, our previous experiments using a dominant-negative mutation of TRAF2 lacking the N-terminal 86 amino acids that comprise the RING finger domain (TRAF2[87–501]) failed to demonstrate a role for TRAF2 in TNFR1-initiated NF-κB activation (Rothe et al., 1995). To examine further a potential role for TRAF2 in NF-κB activation by TNFR1, we selected the 293 cell line, since electrophoretic mobility shift assays have shown that TNF activates NF-κB exclusively through endogenous TNFR1 in these cells (Rothe et al., 1995).

We reasoned that our earlier failure to see an effect of TRAF2(87–501) on signaling through endogenous TNFR1 might be related to the stability of the TNFR1–TRADD–TRAF complex. In those experiments, TNF stimulation had been performed shortly after transfection, perhaps before the newly synthesized TRAF2(87–501) could displace endogenous TRAF1/TRAF2 from the complex. Therefore, we performed a time course experiment in which TNF was added at various times following TRAF2(87–501) transfection (Figure 6). We found that 293 cells transfected with a control expression vector responded to TNF with an approximately 20-fold increase in NF-κB reporter gene activity. When TNF stimulation was performed 16 hr after transfection with the TRAF2(87–501) expression vector, NF-κB activation was only partially compromised (approximately 7-fold activation). However, the inhibitory effect of TRAF2(87–501) increased with time. TRAF2(87–501) expression completely blocked TNF-induced NF-κB activation (1.2 ± 0.2-fold activation) when TNF treatment was performed 48 hr after transfection (Figure 6). Similar results were obtained using HeLa cells (data not shown).

TNF and interleukin-1 (IL-1) elicit many similar biological activities, including NF-κB activation in 293 cells. To determine the specificity of the observed TNF-inhibitory effect of TRAF2(87–501), we compared the effect of TRAF2(87–501) overexpression on NF-κB activation by these two cytokines. In a dose-response experiment, low levels (0.1 μg) of TRAF2(87–501) expression vector were sufficient to inhibit TNF-induced NF-κB-dependent reporter activity significantly, and high levels (3.2 μg) completely blocked activation. In contrast, overexpression of TRAF2(87–501) had no effect on IL-1-mediated NF-κB activation (Figure 7). These results provide strong evidence that the TRAF2(87–501) dominant-negative mutant is inhibiting NF-κB activation in a specific manner and that TRAF2 plays an important role in TNFR1 signaling.

The Apoptotic Pathway Activated by TNFR1 Does Not Require TRAF2

Two important activities of TNF signaled through TNFR1 are activation of NF-κB and induction of apoptosis. Overexpression of the TNFR1-associated protein TRADD can trigger both activities by activating two distinct signaling pathways (Hsu et al., 1995). However, overexpression of TRAF2 activates NF-κB (Rothe et al., 1995), but does not induce cell death (H. H. and D. V. G., unpublished data), suggesting that TRAF2 may not be an essential component of the TNFR1–TRADD apoptotic signaling cascade. To examine further whether TRAF2

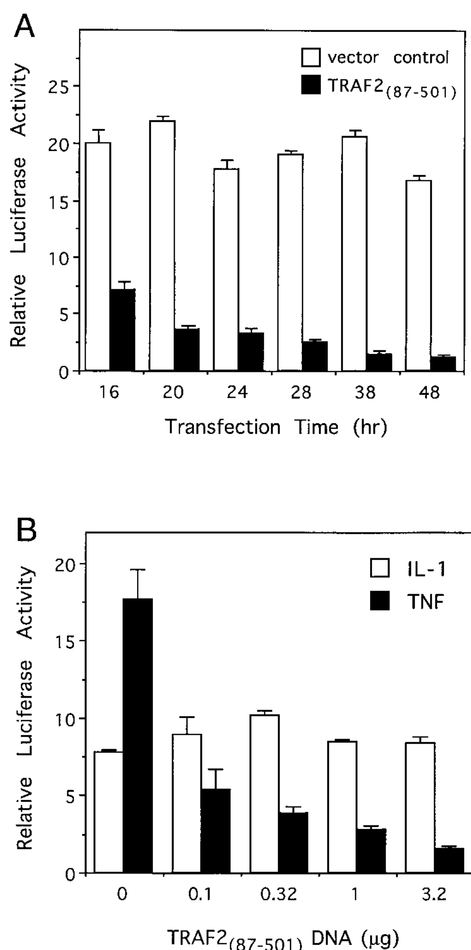

Figure 6. The TRAF2(87–501) Dominant-Negative Mutant Specifically Inhibits TNF-Induced NF-κB Activation

(A) Time-dependent effect of TRAF2(87–501) expression on TNF-induced NF-κB activation. We transfected 293 cells (2×10^5) with 1 μg of TRAF2(87–501) expression vector or 1 μg of pRK vector control, together with 1 μg of pELAM-luc reporter and 0.5 μg of pRSV-βgal plasmids. At the indicated times following transfection, cells were either treated with TNF (20 ng/ml) or left untreated for an additional 6 hr. The values indicated (shown as mean ± SEM) represent luciferase activities for TNF-treated cells relative to untreated cells for an experiment performed in duplicate.

(B) Dose response effect of TRAF2(87–501) expression on TNF-induced NF-κB activation. We transfected 293 cells (2×10^5) with 1 μg of pELAM-luc reporter, 0.5 μg of pRSV-βgal, and the indicated amounts of TRAF2(87–501) expression vector and supplemented them with pRK control vector for a total of 4.7 μg of DNA; 36 hr after transfection, cells were treated for 6 hr with 20 ng/ml of either TNF or IL-1. Values represent luciferase activities relative to the same cells without cytokine treatment and are shown as mean ± SEM for representative experiments performed in duplicate.

contributes to apoptosis, we studied the effect of the TRAF2 dominant-negative mutant on TNF-mediated killing of HeLa cells, a process which is signaled by TNFR1 (Tartaglia et al., 1993b). In this assay (Hsu et al., 1995), cells are cotransfected with a β-galactosidase expression plasmid. The cells are treated 36 hr later with TNF for 12 hr and then scored for cell death. Overexpression of TRAF2(87–501) had no protective effect in these assays, whereas CrmA, a known inhibitor of TNF-mediated apoptosis (Tewari and Dixit, 1995; Enari et al., 1995) completely blocked cell death (Table 1). Taken together, these observations strongly support a model for signaling by the TNFR1 complex in which TRAF2 is dispensable for apoptosis, but essential for NF-κB activation.

The Death Domain of FADD Inhibits TNF-Induced Apoptosis

Overexpression of FADD has been shown to induce programmed cell death (Boldin et al., 1995a; Chinnaiyan et al., 1995). This activity requires only amino acids 1–117 of FADD (Chinnaiyan et al., 1995). Futhermore, a FADD deletion mutant lacking amino acids 1–79 acts as a dominant-negative inhibitor of Fas-mediated apoptosis, demonstrating that the Fas apoptotic pathway requires FADD (V. M. Dixit, personal communication). Since FADD can directly associate with TRADD, we considered the possibility that FADD may also participate in TNF-induced cell death. We examined the effect of a deletion mutant of FADD lacking its 79 N-terminal amino acids (FADD[80–205]) on TNF-mediated killing of HeLa cells. Overexpression of wild-type FADD, but not FADD(80–205), was able to trigger cell death in these cells, in accordance with previous findings (Chinnaiyan et al., 1995). Importantly, FADD(80–205) was a potent inhibitor of TNF-mediated apoptosis, blocking cell death as effectively as CrmA (Table 1). Similar results were obtained using mouse NIH 3T3 cells (data not shown).

It has been shown previously that overexpression of either TRADD (Hsu et al., 1995) or TNFR1 (Boldin et al., 1995b) triggers cell death, presumably through an aggregation phenomenon that activates a TNFR1 signaling cascade. However, when 293 cells (Figure 7) or HeLa cells (data not shown) were cotransfected with the FADD(80–205) expression vector, neither TRADD nor TNFR1 overexpression induced cell death. The strong dominant-negative effect of FADD(80–205) on cell death induced by TNF, TNFR1, and TRADD suggests that FADD participates in the apoptotic pathway activated by TNF through TNFR1 and TRADD.

The Death Domain of FADD Does Not Inhibit TNF-Mediated NF-κB Activation

Since FADD(80–205) potently inhibits the TNFR1–TRADD pathway that triggers apoptosis, we asked whether it might also block the TNFR1–TRADD NF-κB activation pathway. In a dose-response experiment, FADD(80–205) overexpression failed to inhibit TNF-induced activation of an NF-κB-dependent reporter gene (Figure 8). In fact, when expressed at high levels, FADD(80–205) itself activates NF-κB. However, NF-κB activation mediated by TNF was still observed above these FADD(80–205)-induced levels. FADD(80–205) also failed to inhibit NF-κB activation triggered by overexpression of TRADD or TNFR1 (data not shown). These results suggest that FADD does not play a role in the TNFR1–TRADD signaling cascade leading to NF-κB activation.

Discussion

Recently, several novel proteins have been identified that interact with members of the TNFR superfamily to initiate intracellular signal transduction events (Rothe et

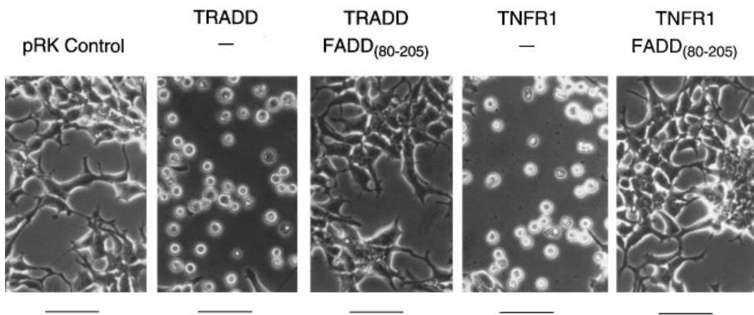

Figure 7. FADD(80–205) Inhibits Apoptosis Induced by Overexpression of TRADD and TNFR1

We transiently transfected 293 cells (2 × 10^5) with the indicated expression vectors (1 μg) and analyzed them 24 hr later by phase-contrast microscopy. Scale bar is 50 μm.

al., 1994; Hu et al., 1994; Boldin et al., 1995a; Cheng et al., 1995; Chinnaiyan et al., 1995; Hsu et al., 1995; Mosialos et al., 1995; Sato et al., 1995; Stanger et al., 1995). These signaling proteins fall into two structural classes, containing either death domains (TRADD, FADD, and RIP) or TRAF domains (TRAF1, TRAF2, and TRAF3). The death domain–containing proteins are involved in signaling by TNFR1 and Fas antigen, two receptors which themselves contain death domains. Therefore, death domains, in addition to their clear involvement in apoptotic signaling, appear to define sequences involved in protein–protein interactions. In contrast, the TRAF domain proteins interact with receptors (TNFR2 and CD40) that have no recognizable domains or motifs. The results of these initial cloning experiments seemed to confirm earlier predictions (Dembic et al., 1990; Lewis et al., 1991; Goodwin et al., 1991) that TNFR1 and TNFR2 would be found to activate independent and distinct signal transduction pathways.

TRADD interacts with the death domain of TNFR1 to initiate distinct signaling cascades for two of the most important biological activities of TNF, NF-κB activation and programmed cell death (Hsu et al., 1995). Our finding that this interaction is TNF dependent suggests that the death domains of TRADD and TNFR1 do not interact with each other as monomers. Instead, the trimeric TNF is likely to induce trimeric or perhaps higher order aggregates of TNFR1 (Banner et al., 1993) that are stabilized by the self-associating death domain of TNFR1 (Song et al., 1994; Boldin et al., 1995b). These aggregated TNFR1 death domains would then provide a high affinity binding site for TRADD, perhaps itself in a preassociated state (Figure 9).

A yeast two-hybrid screen was performed to identify TRADD-interacting proteins as candidate signal transducers for the NF-κB and apoptotic pathways. We isolated several clones encoding TRAF2, a 56 kDa protein originally purified through its association with TNFR2 (Rothe et al., 1994) and later shown to be a common mediator of NF-κB activation by TNFR2 and CD40 (Rothe et al., 1995). Thus, the two known classes of signal transducers for the TNFR family, TRAF domain and death domain proteins, are capable of direct interaction. We also isolated cDNAs encoding FADD, a death domain–containing protein previously shown to interact with Fas and to trigger cell death when overexpressed (Boldin et al., 1995a; Chinnaiyan et al., 1995).

Table 1. Inhibition of TNF-Induced Apoptosis of HeLa Cells by FADD(80–205), but Not by TRAF2(87–501)

	Number of Blue Cells per Well	
Expression Vector	Minus TNF	Plus TNF
pRK control	187 ± 25	0 ± 0
TRAF1	219 ± 12	0 ± 0
TRAF2	249 ± 4	0 ± 0
TRAF2(87–501)	226 ± 11	0 ± 0
FADD	9 ± 4	0 ± 0
FADD(80–205)	229 ± 22	237 ± 15
CrmA	266 ± 18	189 ± 39

HeLa cells (2 × 10^5 cells per well) were transiently cotransfected with pCMV-βgal (0.5 μg) and 2.5 μg of expression vector for TRAF1, TRAF2, TRAF2(87–501), FADD, FADD(80–205), or CrmA; 36 hr after transfection, cells were treated with 10 μg/ml cycloheximide with or without 20 μg/ml TNF for 12 hr. Data (± SEM) are shown as the number of blue cells per 35 mm dish for two independent transfections.

TRADD–TRAF2 Interaction and Activation of NF-κB

The identification of a TRADD–TRAF2 complex raised the possibility that TRAFs might associate indirectly with TNFR1 and TRADD might associate indirectly with

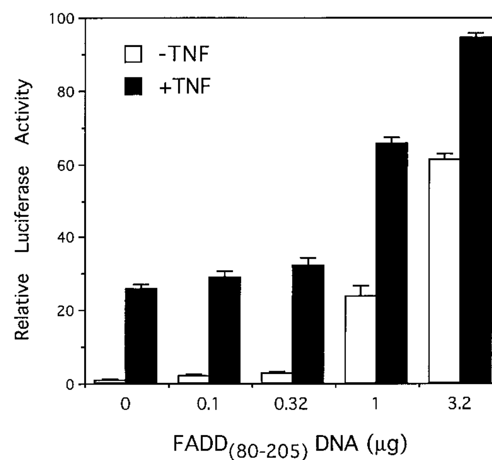

Figure 8. FADD(80–205) Is Not a Dominant-Negative Inhibitor of TNF-Induced NF-κB Activation

We transfected 293 cells (2 × 10^5) with 1 μg of pELAM-luc reporter, 0.5 μg of pRSV-βgal, and the indicated amounts of FADD(80–205) expression vector and supplemented them with pRK control vector for a total of 4.7 μg of DNA; 36 hr after transfection, cells were treated for 6 hr with 20 ng/ml TNF. Values represent luciferase activities relative to vector-transfected cells and are shown as mean ± SEM for representative experiments performed in duplicate.

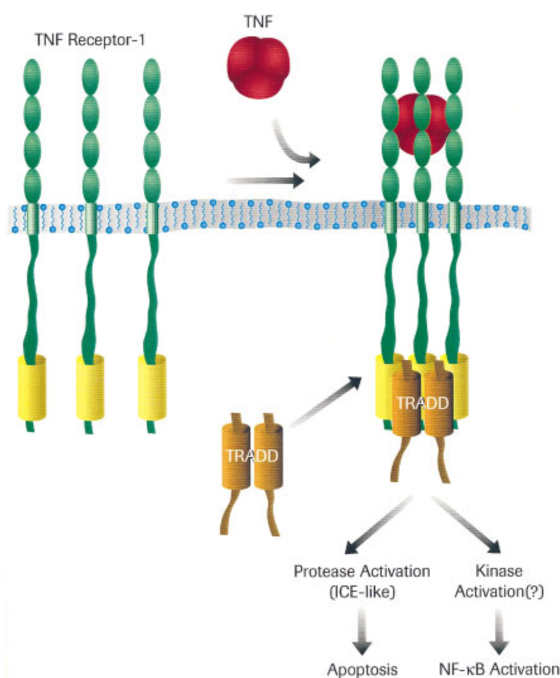

Figure 9. A Model for the Activation of Two Distinct TNFR1 Signal Transduction Pathways by TNF

The trimeric TNF induces TNFR1 aggregation, which is stabilized by homotypic interactions of the death domain of TNFR1. TRADD (perhaps as an oligomer) associates via its own death domain with the aggregated death domain of TNFR1 to initiate signaling cascades for both apoptosis and NF-κB activation. TRAF2 and FADD are TRADD-interacting proteins that define the NF-κB and cell death pathways, respectively (see text).

TNFR2. We found that TNFR1–TRAF–TRAF complexes can indeed exist. Conversely, TNFR2–TRADD–TRAF complexes cannot form, presumably because TNFR2 and TRADD compete for binding to similar sites in TRAF domains. These findings were conceptually appealing for two reasons. First, both TNFRs are independently capable of NF-κB activation (Wiegmann et al., 1992; L—greid et al., 1994; Rothe et al., 1994; Hsu et al., 1995) and association with TRAF2, a protein whose overexpression leads to NF-κB activation (Rothe et al., 1995). Second, of the two TNFRs, only TNFR1 interacts with the cell death–inducing protein TRADD (Hsu et al., 1995), and this receptor also exerts a far more dominant role in the signaling of apoptosis than does TNFR2 (Tartaglia et al., 1991, 1993b).

The role of TRAF2 in TNFR1 signaling was examined by expressing TRAF2(87–501), a truncated version of TRAF2 lacking its N-terminal RING finger domain. This dominant-negative TRAF2 mutant can inhibit NF-κB activation by TNFR2 and CD40 (Rothe et al., 1995). When overexpressed in 293 cells, TRAF2(87–501) blocked NF-κB activation by TNF, a process that is TNFR1 dependent. NF-κB activation by the cytokine IL-1 was unaffected by TRAF2(87–501), demonstrating that TRAF2 is not involved in IL-1 signaling. TRAF2(87–501) overexpression also had no effect on TNF-mediated apoptosis. This result is consistent with TRAF2 defining a point at which the two (or more) TNFR1–TRADD signal transduction pathways diverge. The TRAF2 pathway, which is shared with TNFR2, CD40, and probably other members of the TNFR superfamily, ultimately would result in NF-κB activation.

On the surface, the results described here would seem to contradict our earlier characterization of TRADD deletion mutants (Hsu et al., 1995). In those experiments we showed that a TRADD mutant (TRADD[195–312]) lacking amino acids 1–194 was capable of activating NF-κB when overexpressed. This result implied that the N-terminal 194 amino acids of TRADD, and by inference proteins such as TRAF2 that bind to this region of TRADD, would not be required for NF-κB activation. This apparent paradox can be explained by the following experimental result. We have found that the NF-κB activation pathway induced by TRADD(195–312) can be potently inhibited by overexpression of the TRAF2(87–501) dominant-negative mutant (data not shown) and is therefore TRAF2 dependent. To date, all TRAF2-dependent pathways for NF-κB activation appear to require TRAF2 aggregation (Rothe et al., 1995). Therefore, our current interpretation of these data is that TRADD(195–315) overexpression leads to aggregation of endogenous TRADD and its associated TRAF2.

Many details of the molecular mechanism(s) by which TRAF2 activates NF-κB remain a mystery. NF-κB consists of p50 and p65 subunits that are normally associated with the cytoplasmic inhibitor protein IκB (Liou and Baltimore, 1993; Beg and Baldwin, 1993). Activation of NF-κB by the proinflammatory cytokines IL-1 and TNF (Osborn et al., 1989) results in rapid serine phosphorylation and degradation of IκB, releasing NF-κB for translocation to the nucleus (Beg et al., 1993; Palombella et al., 1994). The phosphorylation of IκB is thought to be the cytokine-regulated step that targets IκB for degradation by the constitutive ubiquitin–proteasome pathway (Thanos and Maniatis, 1995). In this regard, TNF has been shown to activate a TNFR1-associated serine protein kinase activity (VanArsdale and Ware, 1994). If one assumes this kinase is required for NF-κB activation by TNF, then the essential role of the RING finger domain of TRAF2 in NF-κB activation might be to regulate kinase activity, recruit the kinase to the TNFR1 signaling complex, or both.

TRADD–FADD Interaction and Signaling of Apoptosis

The strong interaction observed between the death domains of TRADD and FADD suggested the possibility that TRADD might be able to recruit FADD to TNFR1. Indeed, low levels of FADD were found in the TNFR1 complex when FADD, TRADD, and TNFR1 were coexpressed, raising the question of whether FADD is involved in TNFR1-mediated signaling.

To address a possible role for FADD in signal transduction by TNFR1, we examined an N-terminal deletion mutant of murine FADD obtained in our two-hybrid screen. Expression of FADD(80–205) completely blocked cell death induced by TNF treatment, or by overexpression of TNFR1 or TRADD, without affecting NF-κB activation. The anti-apoptotic activity of FADD(80–205) in

these assays was as potent as that of CrmA and much more potent than Bcl-2 (Hsu et al., 1995). A similarly truncated human FADD acts as a dominant-negative inhibitor of Fas-mediated apoptosis (V. M. Dixit, personal communication). Therefore, FADD may be a component of the cell death pathways triggered by both TNFR1 and Fas. Alternatively, FADD(80–205) may exert its dominant-negative effect through titration of an unidentified component of the TNFR1 cell death pathway. However, high level expression of FADD(80–205) did not appear to interfere with TRADD binding to TNFR1 (data not shown).

The signaling of cell death by TNFR1 and Fas is not well understood, but considerable evidence suggests the apoptotic pathway requires activation of a protease cascade that ultimately leads to cleavage of cell death substrates (reviewed by Martin and Green, 1995). CrmA expression blocks this pathway by directly binding to cysteine proteases of the CED-3/interleukin-1β-converting enzyme (ICE) family (Tewari and Dixit, 1995; Enari et al., 1995; Los et al., 1995). Presumably, FADD(80–205) blocks the cell death pathway at a receptor-proximal step that precedes protease activation, either by displacing FADD or by tying up other components required to execute the death signal(s). As such, FADD(80–205) should be a useful reagent for dissecting apoptotic cascades initiated by other events.

Apoptosis mediated by TNFR1 and Fas are similar in that both signaling cascades are initiated by death domains and end in activation of ICE-like protease(s). However, clear differences in these two cell death pathways have been observed (Wong and Goeddel, 1994; Schulze-Osthoff et al., 1994). For example, Fas-mediated cell death occurs much more rapidly than that triggered by TNFR1 (Clement and Stamenkovic, 1994; Abreu-Martin et al., 1995). These differences might be due to differential contributions from other, non-FADD components of the receptor complexes. It is also possible that the greater affinity of FADD for Fas than for TNFR1–TRADD observed in our coimmunoprecipitation experiments might reflect affinity differences under physiological conditions. In turn, the amount of FADD present in the respective receptor complexes might correlate with potency of the death signal.

Conclusions

The demonstration that TRADD interacts with TRAF2 and FADD, and can recruit both to TNFR1, suggested that TRAF2 and FADD may be involved in TNFR1–TRADD-mediated signaling. That these interactions define two distinct signaling pathways emanating from TRADD (Figure 9) is supported by the ability of TRAF2 and FADD to activate NF-κB and induce apoptosis, respectively. This hypothesis is further strengthened by the effectiveness of dominant-negative mutants at selectively inhibiting one pathway or the other: TRAF2(87–501) blocks NF-κB activation by TNFR1 and FADD(80–205) blocks apoptosis. It will now be of great interest to identify downstream events in these signaling cascades that connect TRAF2 to IκB phosphorylation and FADD to cysteine protease activation.

Experimental Procedures

Reagents and Cell Lines

Recombinant human TNF and IL-1 were provided by Genentech. The rabbit anti-TNFR1, anti-Fas, and anti-TRADD antisera and the monoclonal antibody against the Myc epitope tag have been described previously (Tartaglia et al., 1991; Wong and Goeddel, 1994; Hsu et al., 1995). Monoclonal antibody 985 against the extracellular domain of TNFR1 was provided by Genentech. The monoclonal antibody against the Flag epitope was purchased from Kodak International Biotechnologies. The human 293 embryonic kidney (R. Tjian), human HeLa derivative HtTA-1 (H. Bujard), human U937 histiocytic lymphoma (G. Wong), and murine NIH 3T3 fibroblast (American Type Culture Collection) cell lines were obtained from the indicated sources.

Expression Vectors

Mammalian cell expression vectors encoding TNFR1, TRADD, TRAF1, TRAF2, TRAF2(87–501), and CrmA have been described previously (Hsu et al., 1995; Rothe et al., 1995). The expression vectors for Fas were provided by Dr. V. Dixit. The full-length FADD and FADD(80–205) expression plasmids was prepared by inserting a SalI–NotI fragment from the two-hybrid *FADD* cDNA clones in-frame with an N-terminal Flag epitope in the vector pRK5. The control expression plasmid pRK5, the NF-κB reporter plasmid pELAM-luc, and pCMV-βgal were also described previously (Hsu et al., 1995). Restriction sites in the *TRADD* cDNA (StuI, SmaI, and NotI) were used to obtain deletion mutants of TRADD (amino acids 1–274, 1–169, and 1–106, respectively). Wild-type and deletion mutants of TRADD were cloned into the yeast GAL4 DNA-binding domain vector pGBT9 (Clontech). Plasmids containing the GAL4 activation domain fused with TRAF2, TRAF2(87–501), and TRAF2(264–501) were as described previously (Rothe et al., 1994, 1995). TRAF2(348–501)/GAL4ad was generated by cloning the appropriate coding sequence into pPC86. TRAF2(1–358)/GAL4ad was provided by Dr. H. Y. Song.

Yeast Two-Hybrid Cloning

The plasmid GAL4bd–TRADD (Hsu et al., 1995), which encodes the GAL4 DNA-binding domain fused to full-length TRADD, was used as bait in two-hybrid screens of HeLa (Clontech) and mouse fetal liver stromal cell (provided by L. Lasky) cDNA libraries. The isolation of positive clones and subsequent two-hybrid interaction analyses were carried out as described elsewhere (Hsu et al., 1995). DNA sequencing was performed on an Applied Biosystems model 373A automated DNA sequencer.

Transfections and Reporter Assays

The 293, NIH 3T3, and HtTA-1(HeLa) cell lines were maintained in high glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 μg/ml penicillin G, and 100 μg/ml streptomycin (GIBCO). For reporter and apoptosis assays, $\sim 2 \times 10^5$ cells per well were seeded on 6-well (35 mm) dishes. For coimmunoprecipitations, $\sim 2 \times 10^6$ cells per well were seeded on 100 mm plates. Cells were transfected the following day by the calcium phosphate precipitation method (Ausubel et al., 1994). Luciferase reporter assays were performed as described elsewhere (Hsu et al., 1995).

Coimmunoprecipitation and Western Blot Analysis

We grew U937 cells (5×10^7) in RPMI medium containing 10% fetal calf serum and 100 μg/ml each of penicillin G and streptomycin, washed them in warm PBS, and incubated them for 15 min in the presence or absence of TNF (100 ng/ml). Cells were lysed in 1 ml of lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 30 mM NaF, 2 mM sodium pyrophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Lysates were incubated with 25 μg of monoclonal antibody 985 or mouse IgG for 2 hr at 4°C, then mixed with 25 μl of a 1:1 slurry of protein GammaBind G–Sepharose, and incubated for another 2 hr. The Sepharose beads were washed twice with 1 ml of lysis buffer, twice with 1 ml of high salt (1 M NaCl) lysis buffer, and twice more with lysis buffer. Transfected 293 cells from each 100 mm dish were lysed in 1 ml of E1A buffer (50 mM HEPES [pH 7.6], 250 mM NaCl, 0.1% NP-40, 5 mM EDTA). For each immunoprecipitation, 0.5 ml aliquots of lysates were incubated with

1 μl of anti-TNFR1 or anti-Fas antibody or with 2 μl of anti-Flag antibody at 4°C for at least 1 hr. The lysates were mixed with 20 μl of a 1:1 slurry of protein A– or protein G–Sepharose (Pharmacia) and incubated for another hour. The Sepharose 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–PAGE and transferred to Immobilon-P membrane (Millipore). Subsequent Western blotting analyses were performed as described elsewhere (Hsu et al., 1995).

Apoptosis Assays

Transfected HeLa cells were washed with PBS, fixed in PBS containing 2% paraformaldehyde, 0.2% glutaraldehyde for 5 min at 4°C, and washed again with PBS. Fixed cells were stained overnight with PBS containing 1 mg/ml X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.02% NP-40, 0.01% SDS. The number of blue-staining cells was determined microscopically. We analyzed 293 cells for apoptosis by phase-contrast microscopy 24 hr after transfection.

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