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I-TRAF is a novel TRAF-interacting protein that regulates TRAF-mediated signal transduction

(tumor necrosis factor/tumor necrosis factor receptors/NF-κB/TRAF2)

MIKE ROTHE*, JESSIE XIONG*, HONG-BING SHU*, KEITH WILLIAMSON*, AUDREY GODDARD†, AND DAVID V. GOEDDEL*‡

*Tularik, Inc., Two Corporate Drive, South San Francisco, CA 94080; and †Department of Molecular Biology, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080

Contributed by David V. Goeddel, May 6, 1996

ABSTRACT **Tumor necrosis factor (TNF) receptor-associated factor (TRAF) proteins associate with and transduce signals from TNF receptor 2, CD40, and presumably other members of the TNF receptor superfamily. TRAF2 is required for CD40- and TNF-mediated activation of the transcription factor NF-κB. Here we describe the isolation and characterization of a novel TRAF-interacting protein, I-TRAF, that binds to the conserved TRAF-C domain of the three known TRAFs. Overexpression of I-TRAF inhibits TRAF2-mediated NF-κB activation signaled by CD40 and both TNF receptors. Thus, I-TRAF appears as a natural regulator of TRAF function that may act by maintaining TRAFs in a latent state.**

The tumor necrosis factor (TNF) receptor-associated factor (TRAF) family of proteins is involved in transducing signals from various members of the TNF receptor (TNF-R) superfamily (1). TRAF1 and TRAF2 were originally purified as TNF-R2-associated proteins of 45 and 56 kDa, respectively (2). TRAF3 was identified by two-hybrid interaction cloning as a CD40-associating protein of 64 kDa (3–6).

The three known members of the TRAF family are composed of distinct structural domains. TRAF1 and TRAF2 share a conserved C-terminal "TRAF domain" of ≈230 aa that is involved in homo- and heterooligomerization and receptor association (2). Inspection of this region in TRAF3 identified two subdomains, the TRAF-C domain, comprising the C-terminal 150 aa, and the TRAF-N domain, which consists of a putative coiled-coil structure (4). Whereas the TRAF-C domain is highly conserved among the three known TRAFs, the TRAF-N domain is diverged in TRAF3. In addition, TRAF2 and TRAF3 contain an N-terminal RING finger and five zinc finger structures of weak sequence similarity (2–7).

TRAF1 and TRAF2 exist in a multimeric complex that interacts via TRAF2 with the signaling domains of both TNF-R2 and CD40 (2, 8). In addition, TRAF2 was recently found to associate with the TNF-R1-associated death domain protein (TRADD; ref. 9), a TNF-R1-interacting signaling protein (10). TRADD can simultaneously bind TNF-R1 and TRAF2 via distinct domains, thereby recruiting TRAF2 to the TNF-R1 signaling complex (9).

The two TNF receptors and CD40 can independently generate signals that lead to the activation of the transcription factor NF-κB (2, 11–14). Functional analysis demonstrated that TRAF2 is a common signal transducer for TNF-R2 and CD40 that mediates activation of NF-κB (8). This effector function of TRAF2 requires its N-terminal RING finger domain (8). TRAF2 also appears to be involved in NF-κB activation by TNF-R1, because overexpression of a dominant negative version of TRAF2 abolished NF-κB activation signaled by this receptor (9). The role of TRAF3 in signal transduction is less well defined, but it has been implicated in CD40-mediated induction of CD23 (4).

Another family of TRAF-interacting proteins was recently identified through the purification of additional proteins that associate with TNF-R2 (15). c-IAP1 and c-IAP2 are closely related cellular members of the inhibitor of apoptosis protein (IAP) family originally characterized in baculoviruses (16). The viral IAPs and c-IAPs contain N-terminal baculovirus IAP repeat motifs and a C-terminal RING finger. The c-IAPs do not directly contact TNF-R2 but rather associate with TRAF1 and TRAF2 through their N-terminal baculovirus IAP repeat motif-comprising domain. The recruitment of c-IAP1 or c-IAP2 to the TNF-R2 signaling complex requires a TRAF2–TRAF1 heterocomplex (15). Neither c-IAP1 nor c-IAP2 is involved in activation of NF-κB by TRAF2 (15).

In an effort to understand how TRAF2 signals downstream responses such as NF-κB, we initiated a search for TRAF2-interacting proteins. We isolated I-TRAF, a novel protein that binds to the TRAF-C domain of the three known TRAFs. When overexpressed, I-TRAF prevents association of TRAF2 with TNF-R2 and inhibits activation of NF-κB induced by TNF-R2 and CD40. Our analysis suggests that I-TRAF regulates TRAF function by maintaining TRAFs in a latent state.

MATERIALS AND METHODS

Cell Culture and Biological Reagents. Human embryonic kidney 293 cells were maintained as described (17). Polyclonal antibody against the N-terminal portion of human TRAF2 was raised against a 34-mer peptide (HEGIYEEGISILESSSAFP-DNAARREVESLPAVK) by BabCo (Richmond, CA). The rabbit anti-I-TRAF antiserum was raised against a glutathione *S*-transferase (GST)–I-TRAF fusion protein. The monoclonal antibody 9E10 against the Myc epitope was provided by R. Schreiber (Washington University). The anti-FLAG epitope monoclonal antibody M2 was purchased from Eastman Kodak.

Yeast Two-Hybrid Cloning. Yeast two-hybrid cloning using full-length TRAF2 in the vector pPC97 as bait (2) was performed following the Matchmaker Two-Hybrid System Protocol (CLONTECH). The cDNA libraries screened were prepared from murine fetal liver stromal cell line 7-4 RNA (2) and murine peripheral lymph node RNA (provided by P. Young, D. Dowbenko, and L. Lasky, Genentech). Subsequent two-hybrid interaction analysis between bait- and prey-

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Abbreviations: TNF, tumor necrosis factor; TNF-R, TNF receptor; TRAF, TNF-R-associated factor; TRADD, TNF-R1-associated death domain protein; IAP, inhibitor of apoptosis protein; c-IAP, cellular IAP; I-TRAF, TRAF-interacting protein; GST, glutathione *S*-transferase; IL-1, interleukin 1.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U59863 and U59864).

‡To whom reprint requests should be addressed.

encoded fusion proteins was carried out in *Saccharomyces cerevisiae* Y190 or HF7c cells as described (2, 8, 15).

cDNA Cloning. The cDNA insert from a murine I-TRAF two-hybrid clone was used as a probe to screen murine CT6 and human HeLa cDNA libraries (2, 10) under standard high and reduced stringency conditions, respectively (18). The cDNA inserts of positive phage clones were subcloned into pBluescript KS (Stratagene) and sequenced on both strands with Sequenase (United States Biochemical).

Transfections, Reporter Gene Assays, and Electrophoretic Mobility Shift Assay. For expression in mammalian cells, cDNAs encoding full-length human I-TRAFα and murine I-TRAFγ were cloned into pRK5 under the transcriptional control of the cytomegalovirus immediate-early promotor-enhancer (19). DNA fragments encoding truncated variants of human I-TRAFα were amplified by PCR. Transient transfection of 293 cells and reporter gene assays were performed as described (10, 15).

Induction of NF-κB DNA-binding activity was analyzed by electrophoretic mobility shift assay, with nuclear extracts prepared from TNF-stimulated or unstimulated 293 cells as described (2, 8).

Immunoprecipitation and Immunoblotting. Lysates from transiently transfected 293 cells were prepared and immunoprecipitated with anti-FLAG epitope monoclonal antibody or mouse IgG as control as described (15). Bound proteins were fractionated by SDS/8% PAGE and transferred to a nitrocellulose membrane. Immunoblot analysis was performed with rabbit polyclonal anti-I-TRAF antiserum as described (15).

For reimmunoprecipitation analysis, 293 cells were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine as described (2), and cell lysates incubated with 10 μl of polyclonal anti-serum and 40 μl of protein G beads. The initial immune complex was dissociated by boiling for 5 min in 50 mM Tris·HCl, pH 7.9/0.5% SDS, diluted 20-fold in RIPA buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS), and subjected to a second round of immunoprecipitation. Bound proteins were analyzed by SDS/10% PAGE and autoradiography.

Coprecipitation experiments with GST fusion proteins were performed essentially as described (15). Briefly, aliquots of lysates from transfected 293 cells (0.5 ml) were incubated for 15 min at 4°C with 10 μl of GST–TNF-R2 fusion protein (2) bound to glutathione agarose beads. The beads were washed and bound proteins analyzed by SDS/PAGE and immunoblotting using anti-FLAG monoclonal antibody. In all cases, expression of transfected constructs was verified by immunoblotting of aliquots of cell lysates.

Generation of GST Fusion Proteins and *in Vitro* Binding Assays. I-TRAF was expressed as a GST fusion protein using the pGEX3X vector (Pharmacia) and purified as described (20). ^{35}S-labeled proteins were generated with the TNT-coupled reticulocyte lysate system (Promega) and the various cDNAs cloned in pBluescript KS (Stratagene) or pRK5. For each binding assay, 0.5 μg of GST–I-TRAF or 0.5 μg of GST bound to glutathione Sepharose beads was incubated with equivalent cpm of the individual ^{35}S-labeled proteins in 1 ml of binding buffer (see above) at 4°C for 1 h. Beads were washed 6 times with binding buffer and precipitates were fractionated by SDS/10% PAGE. The gel was dried and exposed to Kodak x-ray film.

RESULTS

Identification of I-TRAF as a TRAF2-Interacting Protein. Previous analysis had implicated TRAF2 in TNF-R2- and CD40-mediated activation of NF-κB (8). To identify potential downstream components of the TNF-R2/CD40-TRAF2 signaling pathway, we used the yeast two-hybrid system (21) to screen cDNA libraries for TRAF2-interacting proteins. Multiple cDNA clones encoding several distinct proteins were obtained from fetal liver stromal cell and peripheral lymph node cDNA libraries. Restriction mapping of positive clones indicated that most were derived from the same gene. Four fetal liver and two peripheral lymph node cDNA clones were sequenced. The 5′-ends of six additional murine cDNA clones isolated by screening a CT6 cDNA library were also sequenced.

Analysis of the obtained DNA sequences revealed cDNAs corresponding to several distinct transcripts of a murine gene that was designated TRAF-interacting protein (I-TRAF). Due to alternative splicing, these transcripts have the potential to use two different translation initiation codons. The two major forms of murine I-TRAF mRNA are predicted to encode proteins of 413 and 447 aa that we have termed I-TRAFα and I-TRAFβ, respectively (Fig. 1). In addition, several splice variants resulting in premature termination of I-TRAFα were identified. The shortest of these truncated proteins, I-TRAFγ, comprises the amino terminal 202 amino acids of I-TRAFα (Fig. 1).

Using a murine I-TRAF hybridization probe, we also isolated several human I-TRAF cDNA clones from a HUVEC cDNA library, all of which encode a 425-aa protein with a predicted molecular weight of 48,000 that is 82% identical to murine I-TRAFα (Fig. 1). Data base searches failed to reveal any proteins having significant sequence similarity to I-TRAF. Northern blot analysis using mouse tissues indicated that the ≈2.4 kb I-TRAF mRNA is ubiquitously expressed (data not shown).

I-TRAF Interacts with the TRAF-C Domains of TRAF1, TRAF2, and TRAF3. I-TRAF was identified in a yeast two-hybrid screening using full-length TRAF2 as a bait. To delineate a region in TRAF2 that is required for I-TRAF binding, we examined the interaction of I-TRAF with various truncation mutants of TRAF2 in two-hybrid assays. N-terminal deletion mutants of TRAF2 lacking the RING finger or both

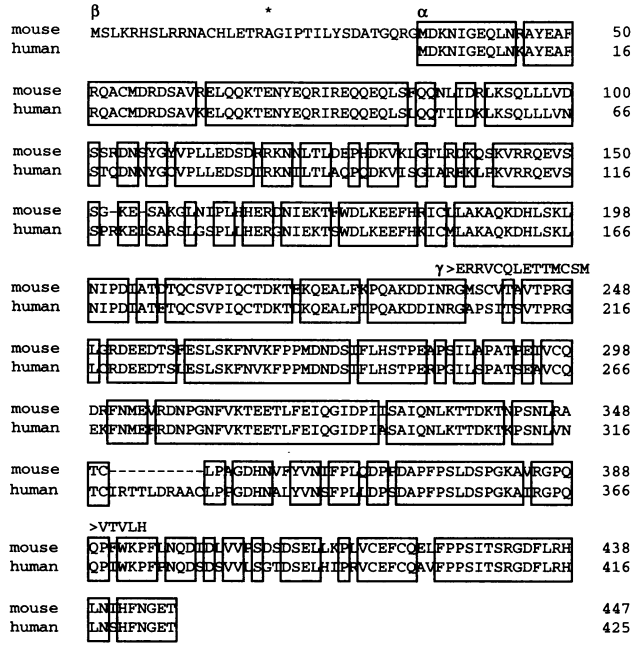

FIG. 1. Homology between murine and human I-TRAF. An optimized alignment of the protein sequences of murine and human I-TRAF is shown. Identical amino acids are boxed. The translation initiation codons of the murine I-TRAFα and I-TRAFβ splice variants are indicated by α and β, respectively. The splice junction is marked by an asterisk (*). All of the isolated human I-TRAF cDNA clones correspond to murine I-TRAFα. The initiator methionine of human I-TRAFα is preceded by an upstream in-frame stop codon not present in the murine I-TRAFα cDNA clones. The amino acid sequences of two additional alternative splice variants of mouse I-TRAFα, ending in premature termination codons, are also listed. The splice junction of the shortest splice variant, murine I-TRAFγ, is indicated by γ.

the RING finger and the five zinc finger structures were still able to bind I-TRAF (Table 1). No interaction could be detected between I-TRAF and a C-terminal deletion mutant of TRAF2 lacking the TRAF-C domain (Table 1). These findings indicate that I-TRAF binds to the conserved TRAF-C domain (amino acids 359–501) of TRAF2. Furthermore, both TRAF1 and TRAF3 also associated with I-TRAF (Table 1). The interaction of I-TRAF with TRAF1, TRAF2, and TRAF3 was confirmed by binding experiments in which human I-TRAF expressed as a GST fusion protein was found to interact specifically with ^{35}S-labeled TRAF proteins (Fig. 2*A*). Results from both two-hybrid and *in vitro* binding experiments showed that I-TRAF interacts more strongly with TRAF1 and TRAF2 than with TRAF3 (Table 1; Fig. 2*A*).

Inspection of the various I-TRAF cDNAs obtained by two-hybrid screening indicated that I-TRAFγ, consisting of the N-terminal portion of murine I-TRAF (amino acids 35–236), is sufficient for interaction with TRAF2 (Table 1). To further investigate the interaction of TRAF2 with I-TRAF, we used a transfection-based coimmunoprecipitation assay. An expression vector encoding FLAG epitope-tagged TRAF2 was transfected alone or with expression vectors encoding full-length or deletion mutants of human I-TRAFα into embryonic kidney 293 cells. Cell lysates were immunoprecipitated using a monoclonal antibody against the FLAG epitope, and coprecipitating I-TRAF was detected by immunoblotting with polyclonal anti-I-TRAF antibodies. Consistent with the results obtained by two-hybrid analysis, TRAF2 coprecipitated both full-length I-TRAFα and mutant I-TRAFα comprising the N-terminal 212 aa [I-TRAFα(1–212)] (Fig. 2*B*). Furthermore, TRAF2 was also able to specifically coprecipitate the C-terminal half of human I-TRAFα [I-TRAFα(213–425)] (Fig. 2*B*). These findings suggest that multiple regions within I-TRAF may mediate its association with TRAF2.

Endogenous Association of I-TRAF and TRAF2 in Mammalian Cells. To confirm the interaction of I-TRAF and TRAF2 in a native system endogenous TRAF2 was immunoprecipitated from lysates of untreated or TNF-treated 293 cells that were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine. The immune complex was dissociated and subjected to a second round of immunoprecipitation with anti-I-TRAF

Table 1. Interactions between I-TRAF and TRAFs

DNA-binding domain hybrid	Direct expression	Activation domain hybrid	Interaction
Vector	None	I-TRAFα	−
TRAF2	None	I-TRAFα	++
TRAF2	None	I-TRAFβ	++
TRAF2	None	I-TRAFγ	++
TRAF2(87–501)	None	I-TRAFα	++
TRAF2(264–501)	None	I-TRAFα	++
TRAF2(1–358)	None	I-TRAFα	−
TRAF1	None	I-TRAFα	++
TRAF3	None	I-TRAFα	+
TNF-R2	None	I-TRAFα	−*
TNF-R2	TRAF2	I-TRAFα	−*
TNF-R2	None	TRAF1	−*
TNF-R2	TRAF2	TRAF1	+*

Yeast Y190 cells were cotransformed with expression vectors encoding Gal4 activation domain–I-TRAF fusion proteins and Gal4 DNA-binding domain expression vectors as indicated. Each transformation mixture was plated on a synthetic dextrose plate lacking leucine and tryptophan. Filter assays for β-galactosidase activity were performed to detect interaction between fusion proteins. Double plus and plus signs indicate strong blue color development within 30 min and 1 h of the assay, respectively. Minus sign indicates no development of color within 24 h. "Three-hybrid" interaction analysis was performed in yeast HF7c cells as described (2).

*Plus and minus signs indicate growth or lack of growth, respectively, of transformed yeast colonies on plates lacking tryptophan, leucine and histidine. Control transformations with empty Gal4 vectors were negative and are not listed.

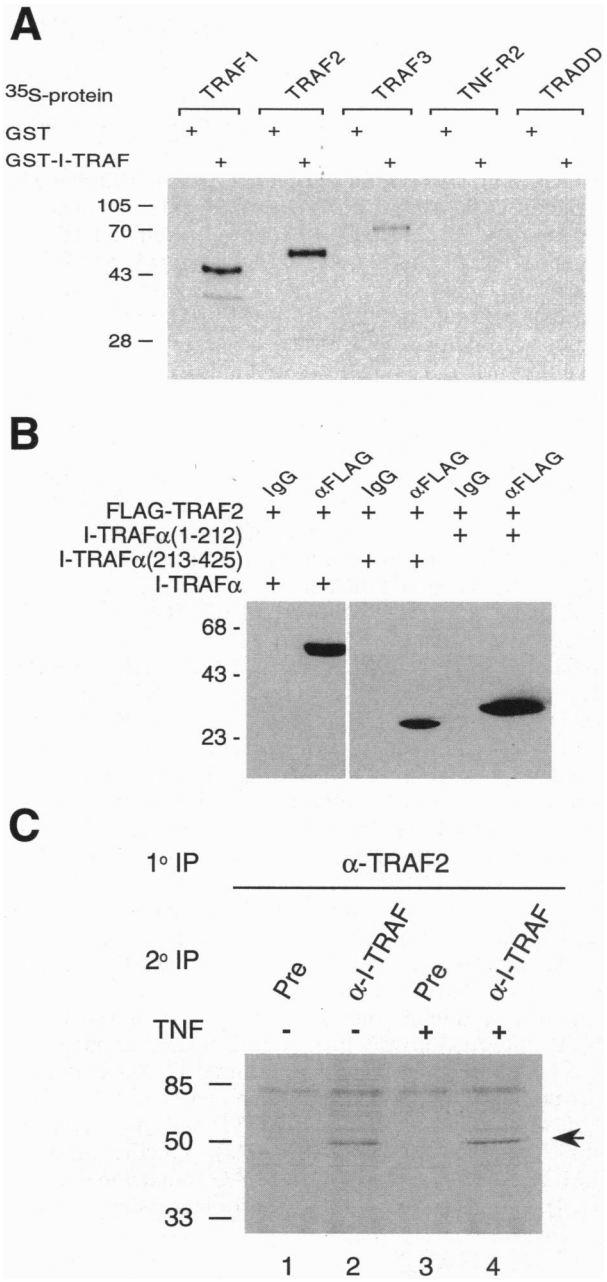

FIG. 2. Interaction of I-TRAF with TRAFs. (*A*) Association of I-TRAF with TRAF1, TRAF2, and TRAF3. The interactions of ^{35}S-labeled TRAF1, TRAF2, TRAF3, TNF-R2, and TRADD with GST-I-TRAF and control GST protein were examined as described. (*B*) Interaction of TRAF2 with I-TRAF mutants. 293 cells were transiently transfected with an expression vector (2.5 μg) encoding FLAG epitope-tagged TRAF2 and I-TRAFα expression constructs (2.5 μg), as indicated by plus signs. After 24 h, cell extracts were immunoprecipitated with anti-FLAG monoclonal antibody (αFLAG) or control IgG (IgG). Coprecipitating I-TRAF was detected by immunoblotting with polyclonal anti-I-TRAF antibodies. (*C*) Endogenous association of I-TRAF and TRAF2 in mammalian cells. 293 cells were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine and left untreated (lanes 1 and 2) or stimulated with human TNF (100 ng/ml) for 15 min (lanes 3 and 4). Cell lysates were immunoprecipitated with anti-TRAF2 antibodies (lanes 1–4), and the initial immune complex was dissociated and subjected to a second round of immunoprecipitation with anti-I-TRAF antibodies (lanes 2 and 4) or preimmune serum (lanes 1 and 3). The position of coprecipitated I-TRAF is marked by an arrow. Positions of molecular mass standards (in kDa) are shown on the left in all panels.

antibodies or preimmune serum as control. The anti-I-TRAF antibodies specifically precipitated a labeled protein of ≈48 kDa (Fig. 2*C*), corresponding in size to overexpressed I-TRAF (see above). In 293 cells, this association of I-TRAF and TRAF2 was observed independent of TNF stimulation (Fig. 2*C*; see below).

I-TRAF and c-IAP Bind to Distinct Sites Within the TRAF Domain of TRAF2. Next we examined the interaction between I-TRAF and the TRAF2-associating protein c-IAP1 (15). Human I-TRAF and FLAG epitope-tagged c-IAP1 were transiently coexpressed in 293 cells, cell lysates were immunoprecipitated with monoclonal anti-FLAG epitope antibody, and coprecipitating I-TRAF was detected by immunoblotting with polyclonal anti-I-TRAF antibodies. In this assay, I-TRAF did not directly associate with c-IAP1 (Fig. 3). However, when I-TRAF and c-IAP1 were coexpressed in the presence of overproduced TRAF1, TRAF2, or both TRAF proteins, strong coprecipitation of I-TRAF and c-IAP1 was observed (Fig. 3; data not shown). Thus, I-TRAF and c-IAP1 bind to nonoverlapping sites within the TRAF domains of TRAF1 and TRAF2. This is in agreement with results from two-hybrid analysis, which mapped the binding of I-TRAF and c-IAP1 to the TRAF-C and TRAF-N domains of TRAF2, respectively (see above and ref. 15).

I-TRAF Prevents Association of TRAF2 with TNF-R2. I-TRAF does not directly interact with TNF-R2 (Table 1; Fig. 2*A*). However, as TRAF1, TRAF2, and c-IAPs can form a complex with TNF-R2, it was important to ask if I-TRAF can indirectly associate with TNF-R2 via TRAFs. A three-hybrid interaction test was performed to address this question. Whereas TRAF2 can bind simultaneously to TRAF1 and TNF-R2 in this assay (2), it was not able to mediate I-TRAF interaction with TNF-R2 (Table 1). In fact, I-TRAF expression in yeast was found to inhibit the association of TRAF2 with TNF-R2 (data not shown). This result is consistent with I-TRAF and TNF-R2 both binding to the TRAF-C domain of TRAF2 (see above and ref. 15).

The observed inhibitory effect of I-TRAF on the TRAF2–TNF-R2 interaction was further investigated in mammalian cells. TRAF2 containing an N-terminal FLAG epitope was expressed in human 293 cells. When cell extracts were incubated with a fusion protein of GST and the cytoplasmic domain of TNF-R2 (GST–TNF-R2), specific binding of TRAF2 was detected using anti-FLAG monoclonal antibody (Fig. 4). Coexpression of TRAF2 with increasing amounts of

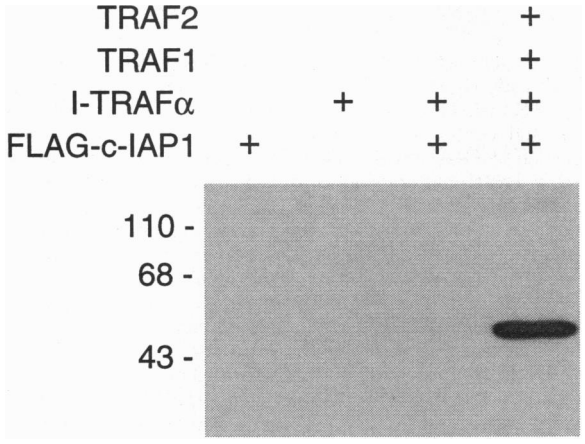

FIG. 3. Interaction of I-TRAF with c-IAP1. 293 cells were transiently transfected with I-TRAFα, TRAF2, TRAF1 and FLAG epitope-tagged c-IAP1 expression vectors (2.5 μg) as indicated by plus signs. After 36 h, cell lysates were immunoprecipitated with anti-FLAG monoclonal antibody. Coprecipitating I-TRAFα was detected by immunoblot analysis using polyclonal anti-I-TRAF antibodies. The positions of molecular mass markers (in kDa) are shown on the left.

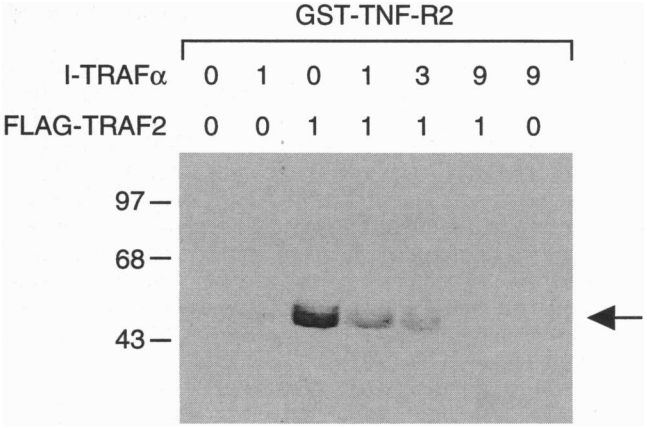

FIG. 4. I-TRAF blocks association of TRAF2 with TNF-R2. 293 cells were transiently transfected with the indicated amounts (in μg) of I-TRAFα and FLAG epitope-tagged TRAF2 expression vectors. After 24 h, cell lysates were incubated with GST–TNF-R2 fusion protein bound to glutathione agarose beads as described. Coprecipitating TRAF2 was detected by immunoblotting with monoclonal anti-FLAG antibody. The interaction of TRAF2 (arrow) with the GST–TNF-R2 fusion protein is inhibited by increased coexpression of I-TRAF. The positions of molecular mass markers (in kDa) are shown on the left.

I-TRAF effectively blocked the TRAF2–TNF-R2 interaction (Fig. 4). Furthermore, no I-TRAF was precipitated by the TRAF2–GST–TNF-R2 complex (data not shown). Nor could I-TRAF be coimmunoprecipitated from 293 cells with the TNF-R2–TRAF1/2 complex (data not shown). These results show that TRAF2 is not able to bind TNF-R2 and I-TRAF simultaneously, possibly due to equivalent or overlapping binding sites in the TRAF-C domain. Since 293 cells do not possess endogenous TNF-R2 capable of recruiting TRAF2 into the receptor signaling complex (8, 9), the observed association of TRAF2 and I-TRAF in these cells is constitutive (see above; Fig. 2*C*).

I-TRAF Overexpression Inhibits TRAF2-Mediated NF-κB Activation. The ability of I-TRAF to bind the three known TRAF family members raised the possibility that I-TRAF functions as a general regulator of TRAF-mediated signaling events. In particular, the observed inhibitory effect of I-TRAF on TRAF2–TNF-R2 interaction suggested that I-TRAF expression might negatively influence TRAF2 signal transduction. Consequently, we measured the effect of I-TRAF expression on TRAF2-mediated NF-κB activation. Electrophoretic mobility shift assay showed that transient expression of TRAF2 in 293 cells potently induces NF-κB DNA-binding activity (8), whereas overexpression of I-TRAFα alone had no such effect (Fig. 5*A*). When overexpressed, I-TRAFα dramatically inhibited TRAF2-mediated NF-κB activation (Fig. 5*A*) in a manner similar to overexpression of a mutant TRAF protein (TRAF2(87–501)) that exerts a dominant negative effect on TRAF2 signaling (ref. 8; Fig. 5*A*). The inhibitory effect of I-TRAF on TRAF2-mediated NF-κB activation was further investigated using a NF-κB-dependent reporter gene (22). In this assay, overexpression of I-TRAFα inhibited TRAF2-induced reporter gene activation in a dose-dependent manner (Fig. 5B).

We next examined the effect of I-TRAF overexpression on NF-κB activation triggered by the TRAF2-interacting receptors CD40 and TNF-R2. Transient expression of these receptors has been shown to induce ligand-independent receptor aggregation, which activates NF-κB in a TRAF2-dependent process (8). As observed above for TRAF2, NF-κB activation through both TNF-R2 (Fig. 5*C*) and CD40 (Fig. 5*D*) was effectively blocked by increased expression of I-TRAFα.

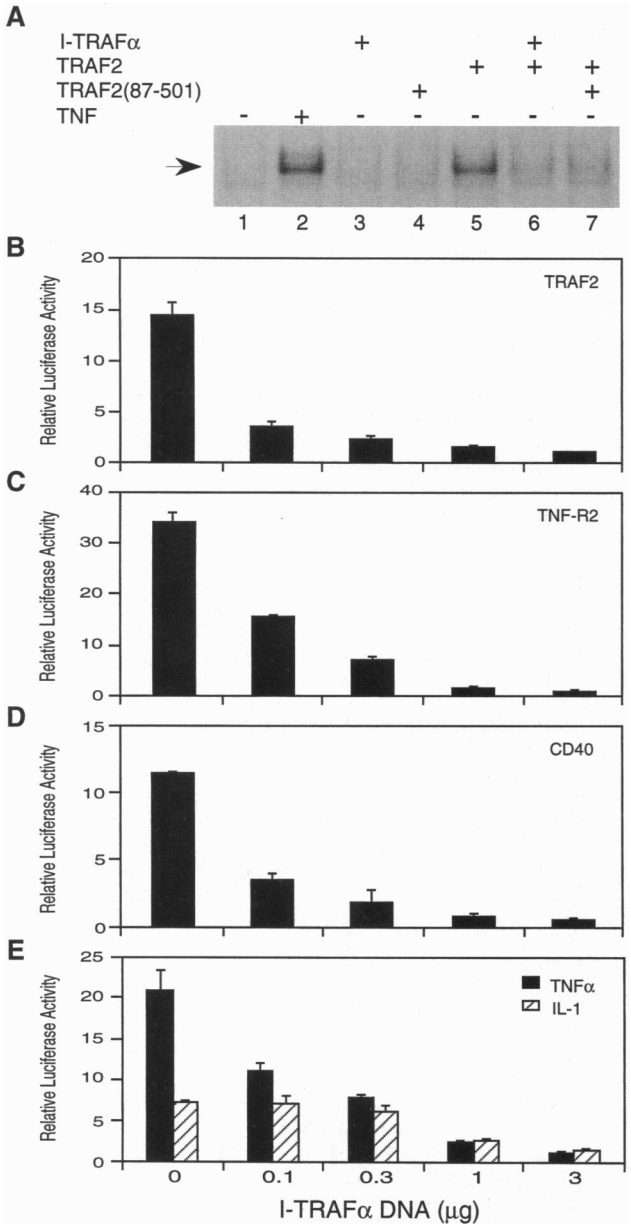

FIG. 5. Inhibitory effect of I-TRAF overexpression on NF-κB activation signaled by TRAF2. (*A*) Inhibition of Induction of NF-κB DNA-binding activity by I-TRAF. 293 cells were transiently transfected with pRK control vector (lanes 1 and 2) or expression vectors for TRAF2 (3 μg; lanes 5–7), I-TRAF (12 μg; lanes 3 and 6), and TRAF2(87–501) (12 μg; lanes 4 and 7). Cells were stimulated with human TNF (100 ng/ml) (lane 2) for 1 h before harvest or left untreated (lanes 1 and 3–7). Nuclear extracts were prepared 24 h after transfection and analyzed for NF-κB DNA-binding activity by electrophoretic mobility shift assay with a radiolabeled double-stranded oligonucleotide containing two NF-kB sites. *B* refers to oligonucleotide probe in a complex with protein. (*B–E*) Inhibition of NF-κB-dependent reporter gene activation by I-TRAF. 293 cells were transiently transfected with an E-selectin promotor-luciferase reporter gene plasmid (0.5 μg; *B–E*) and increasing amounts (in μg) of expression vectors for I-TRAFα (*B–E*), in the presence of TRAF2 expression vector (0.5 μg; *B*) or expression vectors for murine TNF-R2 (0.1 μg; *C*) or CD40 (1 μg; *D*). Each transfection also contained 1 μg of pRSV-βgal. After 24 h (*B–D*), luciferase activities were determined and normalized on the basis of β-galactosidase expression. In *E*, cells were harvested 42 h after transfection following stimulation for 6 h with 20 ng/ml of either TNF (closed bars) or IL-1 (hatched bars). Values relative to control transfections containing the reporter gene plasmid and empty vectors are shown as mean ± SEM for one representative experiment in which each transfection was performed in duplicate.

Effect of I-TRAF on NF-κB Activation Signaled by TNF-R1 and Interleukin 1 (IL-1). Since TRAF2 is also required for NF-κB activation signaled by TNF-R1 (9), we investigated the effect of I-TRAF overproduction on NF-κB-dependent reporter gene activation triggered by this receptor. Overexpression of I-TRAF inhibited TNF-induced NF-κB activation in 293 cells (Fig. 5*E*), which is exclusively mediated through endogenous TNF-R1 (8). This finding is consistent with the involvement of TRAF2 in TNF-R1-mediated NF-κB activation (9) and with our observation that I-TRAF overexpression can negatively regulate TRAF2 function.

Whereas TRAF2 is required for TNF-induced NF-κB activation, overexpression of dominant negative TRAF2 has no effect on NF-κB activation induced by IL-1 in 293 cells (9). Surprisingly, when expressed at high levels, I-TRAF abolished IL-1-mediated NF-κB activation in 293 cells (Fig. 5*E*). Thus, I-TRAF appears to possess a general ability to inhibit NF-κB activation triggered by diverse stimuli. To exclude that I-TRAF nonspecifically suppresses reporter gene activation in 293 cells, we examined its effect on an interleukin-4-responsive reporter gene (U. Schindler, personal communication). In this assay, overexpression of I-TRAF did not inhibit interleukin 4-induced reporter gene activation (data not shown).

DISCUSSION

The recent identification of distinct classes of receptor-associated signal transducers provided insights into how members of the TNF-R superfamily initiate downstream signaling events (reviewed in ref. 1). Signaling proteins containing TRAF domains were initially characterized based on their direct association with the cytoplasmic domains of TNF-R2 and CD40, whereas death domain-containing proteins were found to interact with TNF-R1 and the Fas antigen. More recently, it became apparent that both TRAF domain proteins as well as death domain proteins function as adaptors that recruit additional signaling proteins into the cognate receptor complexes. For example, TRAFs mediate the indirect association of TNF-R2 with members of the IAP family, c-IAP1 and c-IAP2 (15). Also, TRADD interacts with both TRAFs and other death domain proteins, thereby enabling them to bind to the TNF-R1 signaling complex (9).

Recognizing the adaptor function of TRAF2, we embarked on a yeast two-hybrid screen for TRAF2-interacting proteins to identify candidate downstream components of the TNF-R2/CD40-TRAF2 signaling pathway. We found that the cDNAs isolated most frequently encoded a novel protein we termed I-TRAF. I-TRAF does not interact with the RING finger or zinc finger domains of TRAF2 that have been implicated in TRAF2-dependent NF-κB activation (ref. 8; M. Takeuchi, M.R., and D.V.G., unpublished data), but rather it binds to the conserved TRAF-C domains of the three known TRAFs. Based on these findings it appears unlikely that I-TRAF is specifically involved in mediating TRAF2-induced NF-κB activation. This conclusion is supported by the failure of I-TRAFα overexpression to induce activation of NF-κB in mammalian cells. Therefore, we conclude that I-TRAF may be a general regulator of TRAF protein function.

A unique property of I-TRAF that distinguishes it from TRAF2-interacting proteins such as c-IAPs is that I-TRAF cannot be recruited into the TNF-R2 signaling complex. This is because both I-TRAF and TNF-R2 bind to the same or overlapping sites in the TRAF-C domain of TRAF2. In fact, I-TRAF was found to prevent association of TRAF2 with TNF-R2. Similarly, I-TRAF overexpression inhibited TRAF2-dependent NF-κB activation induced by overexpression of TRAF2, TNF-R2, and CD40. This inhibitory activity of I-TRAF localizes in its C-terminal domain (J.X., M.R., and D.V.G., unpublished results).

Our analysis of I-TRAF is consistent with a model in which I-TRAF functions as a general regulator of TRAF-mediated signaling events (Fig. 6). In particular, we speculate that I-TRAF may be a natural inhibitor of TRAF function that regulates TRAF protein activity by sequestering TRAFs in a latent state in the cytoplasm. By binding TRAFs in the absence of ligand (CD40L or TNF), I-TRAF may prevent continuous signaling by inhibiting spontaneous TRAF aggregation. This hypothesis is in agreement with the finding that overexpression of TRAF2 in the absence of receptor clustering is sufficient to trigger NF-κB activation (8). Ligand-induced aggregation of CD40 or TNF-R2 might then be expected to release TRAFs from I-TRAF inhibition by providing a new, higher affinity TRAF binding site. Dissociation of I-TRAF would allow TRAFs to translocate to the cytoplasmic membrane, where they initiate specific signaling cascades (Fig. 6). In this model, various TRAF-interacting signaling proteins such as c-IAPs are recruited to the TNF-R2 signaling complex by virtue of their association with TRAFs. Although ligand-dependent association of TRAFs with their cognate receptors has not yet been demonstrated, binding of the death domain proteins TRADD and FADD to TNF-R1 (9) and the Fas antigen (23), respectively, has been shown to be induced by ligand stimulation. Similarly, the indirect recruitment of TRAF2 into the TNF-R1 signaling complex via TRADD occurs in a ligand-dependent manner (H.-B.S. and D.V.G., unpublished data).

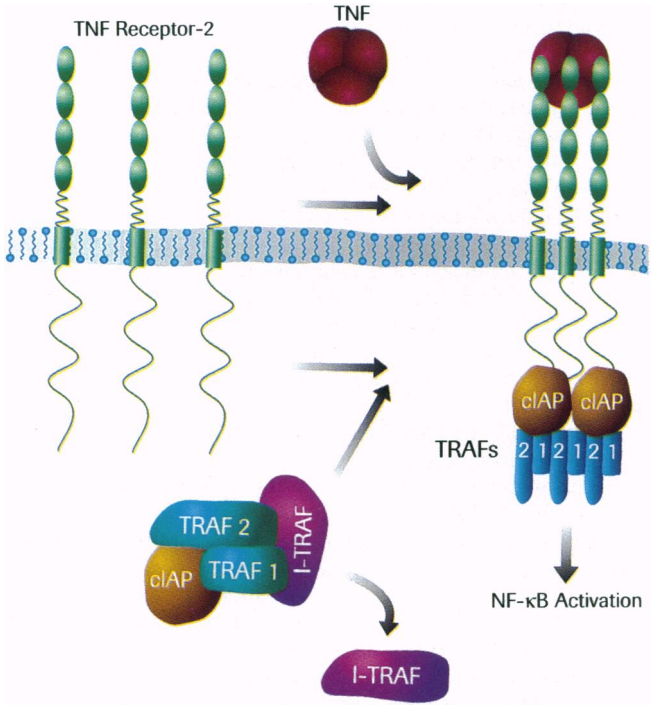

FIG. 6. A model for the activation of the NF-κB signal transduction pathway by the TNF-R2–TRAF signaling complex

Alternatively to our proposed model, it is possible that I-TRAF may act to turn off or reset TRAF2 activation signals after completion of receptor-mediated signaling. I-TRAF may also play a role in regulating TRAF protein stability in that its association with TRAFs could either exert a stabilizing effect or target TRAFs for degradation. A detailed characterization of I-TRAF's role in TNF receptor signaling will have to await targeted disruption of its encoding gene in mice.

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