TRAF-interacting Protein (TRIP): A Novel Component of the Tumor Necrosis Factor Receptor (TNFR)- and CD30-TRAF Signaling Complexes That Inhibits TRAF2-mediated NF-κB Activation

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Summary

Through their interaction with the TNF receptor–associated factor (TRAF) family, members of the tumor necrosis factor receptor (TNFR) superfamily elicit a wide range of biological effects including differentiation, proliferation, activation, or cell death. We have identified and characterized a novel component of the receptor–TRAF signaling complex, designated TRIP (TRAF-interacting protein), which contains a RING finger motif and an extended coiled-coil domain. TRIP associates with the TNFR2 or CD30 signaling complex through its interaction with TRAF proteins. When associated, TRIP inhibits the TRAF2-mediated NF-κB activation that is required for cell activation and also for protection against apoptosis. Thus, TRIP acts as a receptor–proximal regulator that may influence signals responsible for cell activation/proliferation and cell death induced by members of the TNFR superfamily.

Members of the TNF receptor (TNFR)[1] superfamily play important roles in the induction of diverse signals leading to cell growth, activation, and apoptosis (1). Whether the signals induced by a given receptor leads to cell activation or death is, however, highly cell-type specific and tightly regulated during differentiation of cells. For example, the TNFRs can exert costimulatory signals for proliferation of naive lymphocytes but also induce death signals required for deletion of activated T lymphocytes (1). The cytoplasmic domains of these receptors lack intrinsic catalytic activity and also exhibit no significant homology to each other or to other known proteins. Exceptions to this include Fas(CD95) and TNFR1 that share a significant homology within an 80–amino acid region of their cytoplasmic tails (called the "death domain"; 2, 3). Therefore, it is suggested that the TNFR family members can initiate different signal transduction pathways by recruiting different types of intracellular signal transducers to the receptor complex (1).

Indeed, several types of intracellular signal transducers have been identified that initiate distinct signal transduction pathways when recruited to the members of TNFR superfamily (4–19). Recent biochemical and molecular studies showed that a class of signal-transducing molecules are recruited to Fas(CD95) or TNFR1 via interaction of the death domains (2, 3, 6, 12, 17, 20). For example, Fas(CD95) and TNFR1 recruit FADD(MORT1)/RIP or TRADD/FADD (MORT1)/RIP through the interactions of their respective death domains (2, 3, 6, 12, 17, 20, 21). Clustering of these signal transducers leads to the recruitment of FLICE/MACH, and subsequently, to cell death (13, 14).

The TNFR family members can also recruit a second class of signal transducers called TRAFs (TNFR-associated factor), some of which are responsible for the activation of NF-κB or JNK (9, 20, 22). TRAF proteins were identified by their biochemical ability to interact with TNFR2, CD40, CD30, or LT-βR (4, 5, 10, 11, 15, 23–27). These receptors interact directly with TRAFs via a short stretch of amino acids within their cytoplasmic tails, but do not interact with the death domain containing proteins (4, 5, 15, 24–27). To date, five members of the TRAF family have been identified as signaling components of the TNFR family members. All TRAF members contain a conserved TRAF domain, ~230 amino acids in length, that is used for either homo- or heterooligomerization among the TRAF family, for interactions with the cytoplasmic regions of the TNFR superfamily, or for interactions with downstream signal transducers (4, 5, 8, 10, 11, 19, 23–25, 28). In addition to the TRAF domain, most of the TRAF family members contain an NH_2-terminal RING finger and several zinc finger structures, which appear to be important for their effector functions (4, 5, 10, 11, 23–25).

[1]*Abbreviations used in this paper:* β-gal; β-galactosidase; c-IAP, cellular inhibitors of apoptosis protein; GST, glutathione-S-transferase; h, human; IL-1R, IL-1 receptor; m, mouse; TNFR, TNF receptor; TRAF, TNFR-associated factor; TRIP, TRAF-interacting protein.

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Several effector functions of TRAFs were revealed by recent experiments based on a transfection system. TRAF2, first identified by its interaction with TNFR2 (4), was subsequently shown to mediate NF-κB activation induced by two TNF receptors, CD40 and CD30 (9, 28–30). TRAF5 was also implicated in NF-κB activation mediated by LT-βR (10), whereas TRAF3 (also known as CRAF1, CD40bp, or LAP1; references 5, 11, 24, and 25) was shown to be involved in the regulation of CD40-mediated CD23 upregulation in B cells (5). The role of other TRAF members in the TNFR family–mediated signal transduction is not clear. They may possess some effector functions as yet to be revealed, or work as adapter proteins to recruit different downstream signal transducers to the receptor complex. For example, TRAF1 is required for the recruitment of members of the cellular inhibitor of apoptosis protein (c-IAP) family to the TNFR2-signaling complex (7). In addition to the signal transduction by the TNFR family members, TRAFs may regulate other receptor-mediated signaling pathways. For example, TRAF6 is a component of IL-1 receptor (IL-1R)–signaling complex, in which it mediates the activation of NF-κB by IL-1R (31). Since TRAFs form homo- or heterooligomers, it is suggested that the repertoire of TRAF members in a given cell type may differentially affect the intracellular signals triggered by these receptors. This may be accomplished by the selective interaction of TRAFs with a specific set of downstream signal transducers. Although many aspects of TRAF-mediated effector functions leading to cellular activation have been defined, it needs to be determined whether TRAF proteins will also mediate the apoptotic signals induced by the "death-domain-less" members of the TNFR superfamily (1, 27, 32–36).

Here we report the isolation and characterization of a novel component of the TNFR superfamily/TRAFs signaling complex, named TRIP (TRAF-interacting protein). TRIP associates with the receptor/TRAF signaling complex, and inhibits the TRAF2-mediated NF-κB activation. Biochemical studies indicate that TRIP associates with the TNFR2 or CD30 receptor complex via its interaction with TRAF proteins, suggesting a model which can explain why the ligation of these receptors can promote different cell fates: proliferation or death.

Materials and Methods

Yeast Two-Hybrid Screening. A bait plasmid pEG202-TRAF1 (27), which encodes the LexA-DNA binding domain fused to TRAF1(183-409), was used for a yeast two-hybrid screening of a mouse thymocyte cDNA library (provided by F. Alt, Harvard Medical School, Cambridge, MA). The plasmids and yeast strains for the two-hybrid system were provided by R. Brent (Harvard Medical School). The isolation of positive clones and subsequent analyses were carried out as previously described (27). The interaction of proteins in the two-hybrid assay was scored by the β-galactosidase activity of yeast transformants containing both activators and baits upon galactose induction as previously described (30). In brief, yeast cells were permeabilized with 0.0025% SDS and 5% chloroform, and the cell debris was removed by centrifugation. The β-galactosidase assay was performed at 25°C and OD420 was measured.

cDNA Cloning and Northern Blot Hybridization. The *TRIP* cDNA insert of ~1.0 kb isolated by two-hybrid screening was used as probe to screen mouse thymocytes and T cell hybridoma cDNA libraries in λZAP (Strategene Corp., La Jolla, CA) as previously described (37). A human thymocyte cDNA library in λgt10 (Clontech, Palo Alto, CA) was similarly screened using full-length mouse *TRIP* (*mTRIP*) cDNA. For sequence analysis of *mTRIP* and human *TRIP* (*hTRIP*), several cDNA clones were sequenced using the Sequenase Kit (United States Biochemical Corp., Cleveland, OH). Northern analysis of mouse tissue RNA was performed as described (37).

Reverse Transcriptase-PCR Assay. For the stimulation of lymphocytes, lymph node cells were isolated from BALB/c mice (4–6 wk old) and cultured on plates coated with anti-TCR Ab (10 μg/ml) and anti-CD28 Ab (1 μg/ml) for 48 h as described (37). Total RNA was prepared from unstimulated and stimulated lymph node cells (total RNA isolation kit; Strategene Corp.). First-strand cDNA was synthesized from 10 μg of total RNA using M-MLV reverse transcriptase and random hexanucleotides following the protocols provided by the supplier (GIBCO BRL, Gaithersburg, MD). Quantitative PCR was performed in the linear phase of amplification by testing PCR products from different dilutions of first-strand cDNA products. PCR amplification was performed for 35 cycles using 1 of 1,000 of the first-strand cDNA synthesized above. PCR products were then electrophoresed in a 2% agarose gel and subjected to Southern blot analysis as described previously (37). The following primers used for quantitative PCR analysis were: *TRAF1* (sense), 5′-AACGAATTCATGGCCTC-CAGCTCAGCCCCT-3′; *TRAF1* (antisense), 5′-CTTGGAT-CCCTACTGAGCCAGCAGCTTCTCCTT-3′; *c-IAP1* (sense), 5′-GGCGAATTCATGGACAAAACTGTCTCCCAG-3′; *c-IAP1* (antisense), 5′-TAGCTGCAGGGATCCATCCTTGAT-3′; *TRIP* (sense), 5′-AGTGAATTCATCATGCCTATCCTCTCTCT-GTG-3′; *TRIP* (antisense), 5′-CTGGGATCCTCACATGTCT-CGAATCATCTCCTC-3′; *actin* (sense), 5′-ATGAA GATCCT-GACCGAGCG-3′; *actin* (antisense), 5′-TACTTGCGCTGAG-GAGGAGC-3′.

Reagents and Cell Lines. Rabbit polyclonal antisera recognizing mTRIP were prepared by Animal Pharm Services, Inc. (Healdsburg, CA) using bacteria-produced glutathione-S-transferase (GST)–TRIP fusion proteins. Polyclonal antisera were negatively selected with purified glutathione-S-transferase (GST) proteins before use. Anti-TRAF1 and anti-TRAF2 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal Ab against the HA epitope (12CA5) was purchased from BabCo (Berkeley, CA). 293 cells were obtained from E. Spanopoulou (Mount Sinai School of Medicine, NY). Recombinant human TNF and IL-1 were purchased from R & D Sys. Inc. (Minneapolis, MN).

Recombinant Plasmids. Eukaryotic expression vectors for wild-type or mutant forms of TRAF1 and TRAF2, CD8-TNFR2, CD8-CD30, GST-TNFR2, and GST-CD30 have been described (27, 30). Expression vectors for TRADD was made by cloning the PCR-amplified murine *TRADD* cDNA into pHβApr-1-neo. An epitope tagged TRIP was made by PCR with the 5′ primer (5′-AGTGAATTCATCATGCCTATCCTCTCTCTG-3′) and the 3′ primer (5′-GGAGTTAACTGACATAAGAAGGTATC-CAGC-3′), which was subsequently cloned into 5′-EcoRI-HpaI-3′ sites in the pBlue-HA vector carrying nucleotides coding for the sequence LTGGGSGFYPYDVPDYA* as described previously (37). The HA epitope is underlined and * indicates the stop

codon. Epitope-tagged *mTRIP* cDNA was cloned into pcDNA3.1 (Invitrogen, San Diego, CA). Deletion mutants of TRIP was similarly generated by PCR as described (27, 30). The reporter constructs, p(κB)$_3$-IFN-LUC and pCMV–β-galactosidase (βgal) (provided by K. Saksela [Rockefeller University, NY] and E. Spanopoulou [Mount Sinai School of Medicine, NY]), were previously described (28, 30). To generate eukaryotic expression vectors for GST–wild-type TRIP or mutant TRIPs, various TRIP cDNAs were generated by PCR and in-frame cloned into 5′-BamHI-NotI-3′ sites in pEBG vector as described (27).

Transfection and Reporter Assays. Transfection of 293 cells were performed in 6-cm dishes by calcium phosphate precipitation as described previously (27). Each transfection maintained an equal amount of total DNA by adding appropriate amount of the control vector, pcDNA3.1 (Invitrogen). 48 h after transfection, luciferase activity was determined and normalized relative to β-galactosidase activity as described previously (30).

Precipitation of GST Fusion Proteins and Western Blot Analysis. 293 cells were transfected with various combinations of expression vectors as indicated. 36 h after transfection, cells were harvested in phosphate-buffered saline/1 mM phenylmethylsulfonyl fluoride, pelleted, and lysed in lysis buffer (20 mM Hepes [pH 7.9], 100 mM KCl, 300 mM NaCl, 10 mM EDTA, 0.1% NP-40, plus protease inhibitors) as described (30). After lysis, aliquots of cell lysates were incubated with glutathione-Sepharose (Pharmacia, Piscataway, NJ) for 2 h at 4°C. The beads were then washed five times with the lysis buffer, followed by an additional wash with the lysis buffer lacking NP-40. The proteins were then recovered by boiling in SDS-PAGE sample buffer. The eluted proteins were separated on 10% SDS-PAGE and transferred to Immobilon P (Millipore Corp., Bedford, MA). The blot was subjected to Western analysis using enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL) as described (30).

Results

Isolation of TRIP as a TRAF-interacting Protein. Previous experiments have suggested that TRAF1 may function as an adapter protein to recruit additional signaling proteins such as c-IAPs to the receptor complex (7), or that TRAF1 may initiate an as yet uncovered signaling pathway. To determine the potential role of TRAF1 in the receptor signaling complex, we searched for additional TRAF1-interacting proteins using the yeast two-hybrid system. By screening cDNA libraries derived from mouse thymocytes, multiple cDNA clones representing several distinct proteins were isolated (data not shown). Among these, one set of cDNAs encoded proteins which could interact with both TRAF1 and TRAF2 in the two-hybrid assay.

Analysis of the DNA sequence of the TRAF1- and TRAF2-interacting cDNA clones revealed that they were derived from a single novel gene named *TRIP*. Since TRIP interacted strongly with both TRAF1 and TRAF2 in the two-hybrid assay, we tested whether these proteins interacted in mammalian cells. Expression vectors encoding TRAF1 or TRAF2 were coexpressed in 293 cells in the presence of an expression vector encoding either GST alone or GST-TRIP fusion protein. Cell lysates were precipitated with glutathione–Sepharose beads, and analyzed by Western blot analysis with anti-TRAF1 or anti-TRAF2 antibodies. Consistent with the yeast two-hybrid assay, GST-TRIP coprecipitated both TRAF1 and TRAF2, demonstrating that TRIP can interact directly with TRAF1 and TRAF2 in human cells (Fig. 1).

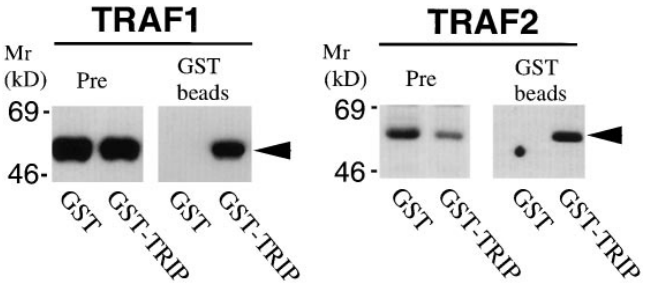

Figure 1. Interaction of TRIP with TRAF1 and TRAF2. An expression vector encoding TRAF1 or TRAF2 was co-transfected into 293 cells with an expression vector encoding either GST alone or GST–TRIP fusion proteins. After 36 h, cell lysates were prepared and subjected to purification with glutathione–Sepharose beads. Proteins coprecipitated with GST fusion proteins were analyzed by Western blot analysis with anti-TRAF1 or anti-TRAF2 polyclonal antibodies. (*Pre*) Cell lysates before precipitation with glutathione beads were analyzed by Western blot analysis to show that similar amounts of TRAF1 or TRAF2 are present in each sample. (*GST beads*) Proteins coprecipitated with GST-fusion proteins were analyzed. TRAF1 or TRAF2 are indicated with arrows.

Full-length sequence of *TRIP* was derived from sequence analysis of multiple cDNA clones from both thymocyte and T cell cDNA libraries (Fig. 2 *A*). *TRIP* mRNA is predicted to encode proteins of 470 amino acids. Using a murine *TRIP* as a hybridization probe, we also isolated several human *TRIP* cDNA clones from a human thymocyte cDNA library. Human *TRIP* encodes a 469–amino acid protein that is overall 76% identical to murine TRIP (Fig. 2 *A*). The amino acid sequence identity between the NH_2-terminal half of mTRIP and hTRIP (residues 1–270) is even higher (87% identical). A homology search of the TRIP amino acid sequence revealed that TRIP is a novel protein with an NH_2-terminal RING finger sequence motif (Fig. 2, *A* and *B*) (38). The NH_2-terminal RING finger motif of TRIP is followed by an extended putative coiled-coil domain (Fig. 2 *A*). The putative coiled-coil domain of TRIP can be further devided into the NH_2-terminal coiled-coil structure, similar to the rod-like tails of myosin heavy chains (residues 56–150; reference 39) and the COOH-terminal leucine zipper-like coils (residues 221–260; reference 40), both of which are implicated in protein–protein interactions. A helical representation of the putative leucine zipper (Fig. 2 *C*) shows that the position next to the zipper is always hydrophobic or uncharged, whereas other sides of the wheel contain many charged but few hydrophobic residues, suggesting an amphipathic structure that can interact with another helix.

To characterize *TRIP* further, its expression pattern was examined. Northern blot analysis of various mouse tissue RNA samples revealed that *TRIP*-specific probes detected a ~2.1-kb mRNA species present in various tissues, but most abundant in testes, thymus, and spleen (Fig. 3 *A*). To characterize further the expression of *TRIP* in lympho-

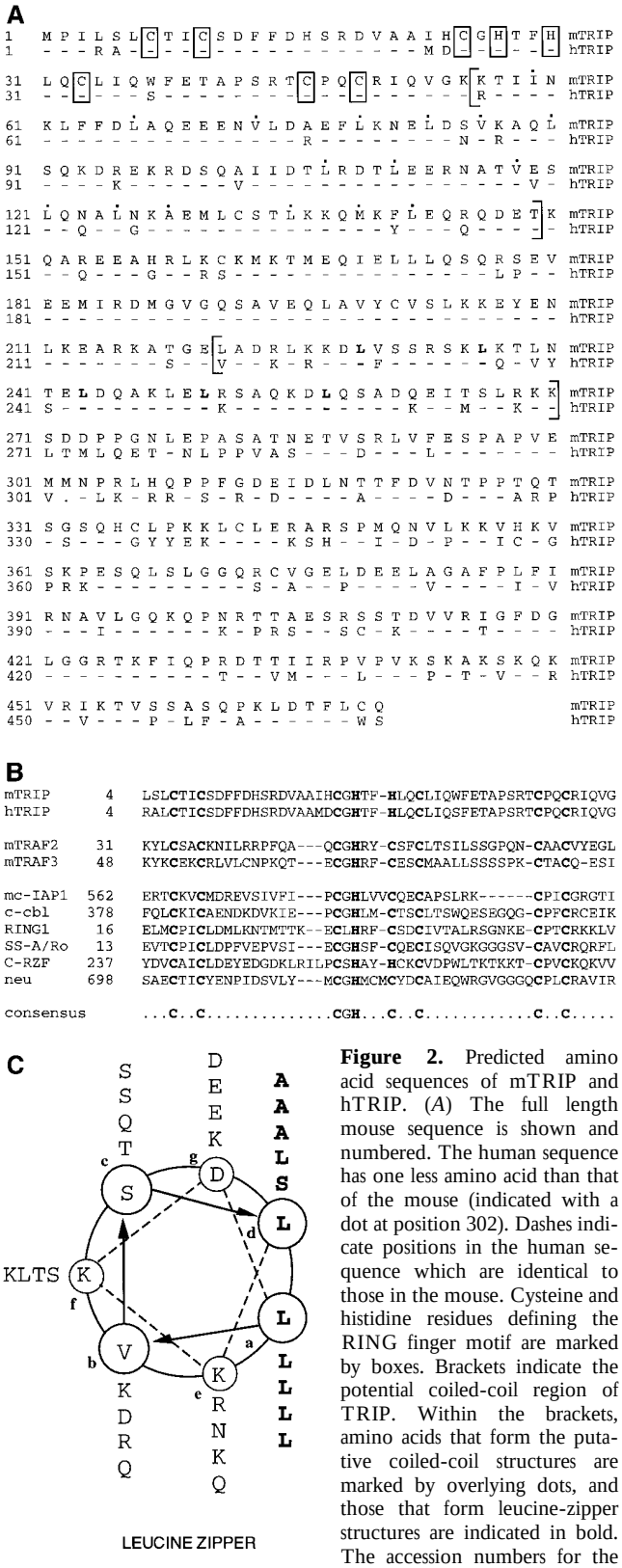

Figure 2. Predicted amino acid sequences of mTRIP and hTRIP. (*A*) The full length mouse sequence is shown and numbered. The human sequence has one less amino acid than that of the mouse (indicated with a dot at position 302). Dashes indicate positions in the human sequence which are identical to those in the mouse. Cysteine and histidine residues defining the RING finger motif are marked by boxes. Brackets indicate the potential coiled-coil region of TRIP. Within the brackets, amino acids that form the putative coiled-coil structures are marked by overlying dots, and those that form leucine-zipper structures are indicated in bold. The accession numbers for the mouse and human TRIP sequences reported in this paper are U77844 and U77845, respectively. (*B*) Comparison of amino acid sequences from various proteins that contain RING finger motifs. The RING finger domains of mTRIP and hTRIP are aligned with those of TRAFs, c-IAP1, human protooncogene c-cbl, human RING1, human ribonucleoprotein SS-A/Ro, chicken C-RZF, and *Drosophila* neuralized gene (38). Residues corresponding to the consensus sequence are indicated in bold. (*C*) Helical wheel representation of residues 225 to 260 of TRIP. The wheel starts with the inner residue Leu[225] at position *d* and finishes with the outer residue Ala[260] at position *d*.

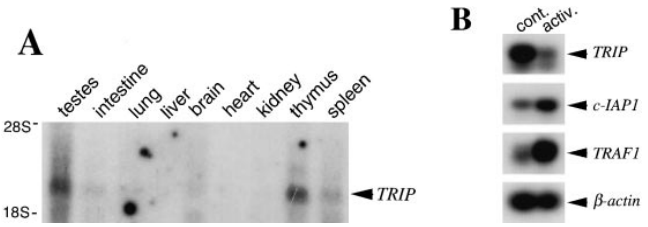

Figure 3. *TRIP* mRNA expression in mouse tissues and its regulation during cell activation. (*A*) Northern blot analysis of *TRIP* mRNA in mouse tissues. Total RNA isolated from various tissues was hybridized with a *TRIP*-specific probe. The *TRIP* probe hybridized to an ~2.1-kb mRNA, indicated by the arrow. Positions of 18S and 28S ribosomal RNA are indicated. The amount of total RNA loaded in each lane was similar based on the intensity of EtBr-stained rRNAs (data not shown). (*B*) Expression of *TRAF*, *c-IAP1*, and *TRIP* during lymphocyte stimulation. cDNAs were prepared from lymph node cells stimulated with anti-TCR Ab plus anti-CD28 Abs for 0 (*cont.*) and 48 (*activ.*) h. The cDNAs were then subjected to semiquantitative PCR using primers specific for *mTRIP*, *mTRAF1*, and *m-c-IAP1* as described in Materials and Methods.

cytes, we analyzed its expression during lymphocyte proliferation by semiquantitative PCR. The expression of *TRIP* was significantly reduced when lymphocytes were stimulated to proliferate via antigen receptors (Fig. 3 *B*). This is in contrast to that of other components of the TNFR–TRAF signaling complex. For example, the expression of *TRAF1* and *c-IAP1* was upregulated upon lymphocyte proliferation (Fig. 3 *B*). These results suggest that the repertoire of signal transducers available in a given cell type can change depending on the state of the cell.

The Putative Coiled-coil Domain of TRIP Interacts with the TRAF Domains of TRAF1 and TRAF2. The yeast two-hybrid assay was used to determine the structural requirements for the interaction of TRIP with TRAF1 or TRAF2. In the yeast two-hybrid assay, a mutant TRIP comprising the NH_2-terminal half of the protein (TRIP[1–275]) interacted with TRAFs, whereas a mutant TRIP lacking the NH_2-terminal RING finger and the coiled-coil domain (TRIP[275–470]) failed to interact with TRAFs (Fig. 4 *A*). Further deletion analysis suggested that the putative coiled-coil region of TRIP mediates the interaction with TRAFs, since a mutant TRIP lacking the NH_2-terminal RING finger motif still interacted with TRAFs (TRIP[56–275]) (Fig. 4 *A*). In addition, both TRIP(56–185) and TRIP (186–275) interacted with TRAFs, suggesting that TRIP contains two independent TRAF binding sites within the long coiled-coil domain of the protein (Fig. 4 *A*).

TRIP Interacts with TRAF Domains of TRAF1 and TRAF2. To delineate a region in TRAF that is required for TRIP binding, the interaction of TRIP with various truncation mutants of TRAF1 or TRAF2 was determined by the yeast two-hybrid assay or by a transfection-based coprecipitation assay in 293 cells. TRIP interacted with an

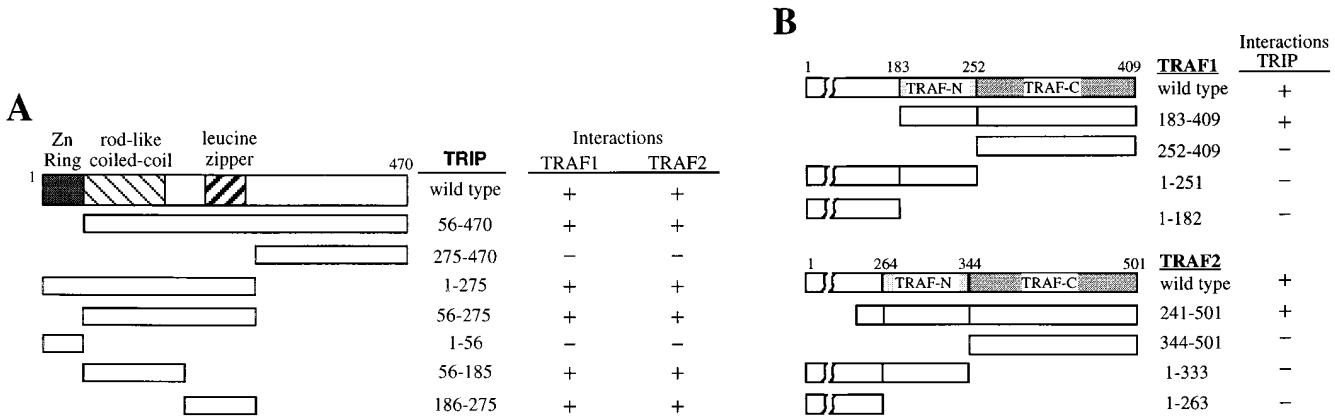

Figure 4. Mapping of TRIP–TRAF interaction domains. (*A*) Interaction of TRAF1 or TRAF2 with the NH_2- and COOH-terminal domains of TRIP. Expression vectors encoding wild-type TRIP or the indicated deletion mutants of TRIP fused to the transcription activation domain were co-transformed into yeast with plasmids expressing LexA DNA binding domain–TRAF1 or –TRAF2 fusion proteins. Interactions between fusion proteins were scored by measuring β-gal activity of yeast transformants. (+) Average β-gal activity of three independent yeast transformants was higher than 1,000 Miller units; (−) average β-gal activity of three independent yeast transformants was about 100 Miller units, which was similar to that of negative controls (bait plasmid alone). (*B*) Interaction of TRIP with TRAFs. Expression vectors encoding the NH_2-terminal deletion mutants of TRAF fused to the LexA DNA binding domain were co-transformed into yeast with plasmids expressing TRIP fused to the transcription activation domain. Interactions between fusion proteins were scored by measuring β-gal activity of yeast transformants as described in Fig. 4 *A*. For the analysis of the COOH-terminal deletion mutants of TRAFs, a transient transfection-based coprecipitation experiment was used. The indicated COOH-terminal deletion mutants of TRAFs were coexpressed with GST–TRIP fusion proteins in 293 cells. Cell lysates were subjected for purification with glutathione–Sepharose beads, followed by Western blot analysis with anti-TRAF1 or anti-TRAF2 polyclonal antibodies as described in Fig. 1.

NH_2-terminal deletion mutant of TRAF1 expressing the entire TRAF domain (TRAF1[183–409]), but failed to interact with an NH_2-terminal deletion mutant of TRAF1 expressing only the TRAF-C domain (TRAF1[252–409]) (Fig. 4 *B*). TRIP did not interact with a COOH-terminal deletion mutant of TRAF1 lacking the TRAF-C domain (TRAF1[1–251]), suggesting that the interaction of TRIP with TRAF1 requires the entire TRAF domain (Fig. 4 *B*). Mutational analysis of TRAF2 also showed that TRIP interacts with TRAF2 through the TRAF domain (Fig. 4 *B*).

TRIP Associates with the Receptor–TRAF Signaling Complex. TRIP did not directly interact with the cytoplasmic domains of TNFR2 or CD30 in the yeast two-hybrid assay (data not shown). However, since the interaction of TRAFs with the cognate members of the TNFR superfamily is mediated through the TRAF-C domain rather than the entire TRAF domain (4, 5, 10, 11, 15, 24, 26, 27), it was important to determine whether TRIP can indirectly interact with the receptors through TRAFs. To test this, HA-epitope–tagged TRIP and GST fusion proteins with the cytoplasmic domains of TNFR2 (GST-TNFR2) or CD30 (GST-CD30) (30) were coexpressed in 293 cells in the presence or absence of TRAFs. Cell lysates were precipitated with glutathione–Sepharose beads, and analyzed on Western blots with anti-HA, anti-TRAF1, and anti-TRAF2 antibodies. Consistent with the yeast two-hybrid assay, TRIP was not coprecipitated by the GST-TNFR2 or GST-CD30 (Fig. 5). When TRAF2 was coexpressed, however, TRIP was readily coprecipitated by the GST-TNFR2 (Fig. 5). GST-TNFR2, which does not strongly interact with TRAF1 oligomer (4), did not readily coprecipitate the TRAF1–TRIP complex (Fig. 5). Coexpression of both TRAF2 and TRAF1 did not increase the amount of TRIP coprecipitated with GST-TNFR2 (Fig. 5). Similar to GST-TNFR2, GST-CD30 also coprecipitated TRIP efficiently in the presence of TRAF2 (Fig. 5). Although TRAF1 homooligomer can interact with CD30 or TRIP efficiently in 293 cells, only low level of TRIP was coprecipitated by GST-CD30 in the presence of TRAF1 alone (Fig. 5). Taken together, these results suggest that TRIP can be recruited to the TNFR2 or CD30 through the TRAF2 homooligomer. However, whether TRIP is also recruited to the receptor via TRAF2–TRAF1 heterooligomer cannot be excluded.

TRIP Overexpression Inhibits TRAF2-mediated NF-κB Activation. The ability of TRIP to bind the receptor–TRAF signaling complexes raised the possibility that TRIP may regulate the receptor-mediated signal transduction. In particular, the association of TRIP with TRAF2 suggests that TRIP expression may regulate TRAF2-mediated effector function such as NF-κB activation (9, 28–30). Therefore, we tested the effect of TRIP expression on TRAF2-mediated NF-κB–dependent reporter gene activation using a transient transfection assay in 293 cells. When overexpressed, TRIP significantly inhibited TRAF2-mediated NF-κB activation (Fig. 6 *A*). This inhibition was similar to that exerted by overexpression of a dominant negative form of TRAF2 (TRAF2[241–501]; Fig. 6 *A*; references 28–30). The inhibition of NF-κB activation by TRIP required the same domains of TRIP which mediate the interaction. An NH_2-terminal deletion mutant of TRIP which lacks the TRIP–TRAF interaction domain (TRIP[275–470]) failed to inhibit TRAF2-mediated NF-κB activation (Fig. 6 *A*). Moreover, a COOH-terminal deletion mutant of TRIP containing the NH_2-terminal RING finger motif and the putative coiled-coil domain (TRIP[1–185]) was

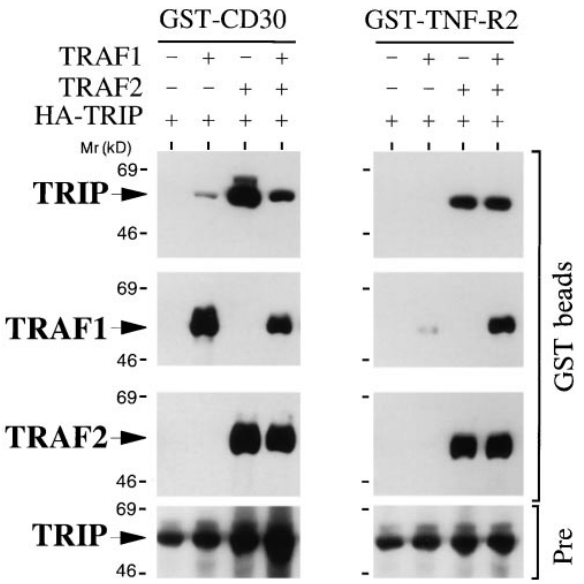

Figure 5. TRAF2-mediated interaction of TRIP with CD30 or TNFR2. 293 cells were transiently transfected with the indicated combinations of equal amounts of HA-TRIP, TRAF1, TRAF2, GST-CD30, or GST-TNFR2 expression vectors for 36 h. Aliquots of cell lysates were subjected for purification with glutathione–Sepharose beads as described in Materials and Methods. Proteins coprecipitated with GST fusion proteins were analyzed by Western analysis with an anti-HA mAb (12CA5), and anti-TRAF1 or anti-TRAF2 polyclonal antibodies. In control experiments, GST proteins did not coprecipitate any of the proteins tested (data not shown). *Pre*, cell lysates before purification with glutathione–Sepharose beads were analyzed by Western analysis with anti-TRIP polyclonal antibodies to show that equal amounts of TRIP were expressed in each case. The positions of molecular mass markers are shown on the left. Arrows indicating the positions of TRAF1, TRAF2, or TRIP are also shown on the left.

sufficient to inhibit TRAF2-mediated NF-κB activation (Fig. 6 *A*). However, further deletion analysis showed that the RING finger motif of TRIP was not required for inhibition of TRAF2-mediated NF-κB activation because a mutant TRIP containing only the putative coiled-coil domain (TRIP[56–275]) was sufficient to inhibit TRAF2-mediated NF-κB activation (Fig. 6 *A*). Overexpression of a mutant TRIP expressing only the NH_2-terminal RING finger motif failed to inhibit NF-κB activation (data not shown). These results suggested that the coiled-coil domain of TRIP (amino acids 56–275) is required for TRIP–TRAF interaction and also for inhibition of TRAF2-mediated NF-κB activation.

Since TRIP associates with the receptor complex, we also tested the effect of TRIP on NF-κB activation induced via TNFR2 or CD30. As previously shown, overexpression of chimeric receptors with the extracellular domain of CD8 fused to the cytoplasmic domain of TNFR2 (CD8-TNFR2) or CD30 (CD8-CD30) induced NF-κB activation in 293 cells without further cross-linking (Fig. 6 *B*; reference 30). This is similar to the activation of NF-κB induced by overexpression of wild-type TNFR2, CD40, or other chimeric receptors in 293 cells, which will trigger the clustering of signal transducers without additional cross-linking by cognate ligands or antibodies (19, 28, 29, 34, 41). When TRIP was coexpressed, the receptor-mediated NF-κB activation was significantly inhibited (Fig. 6 *B*). Because NF-κB activation by TNFR2 and CD30 is mediated by TRAF2 (29, 30), the results are consistent with the fact that TRIP works as a proximal negative regulator of TRAF2-mediated NF-κB activation by members of the TNFR superfamily.

TRIP Inhibits NF-κB Activation Induced by TNF, but Not by IL-1. Since TRAF2 also mediates NF-κB activation triggered by the TNFR1–TRADD complex (9), we tested the effect of TRIP overexpression on TNF-induced NF-κB activation in 293 cells, which is mediated by TNFR1 (29). Overexpression of TRIP in 293 cells inhibited TNF-induced NF-κB activation (Fig. 6 *C*). Consistent with this, TRIP overexpression also inhibited NF-κB activation mediated by TRADD overexpression in 293 cells (Fig. 6 *D*). While TRAF2 is required for TNF- or TRADD-induced NF-κB activation, it is not required for NF-κB activation induced by IL-1 in 293 cells (9, 29). To test whether TRIP affects TRAF2-mediated NF-κB activation specifically, the effect of TRIP overexpression on NF-κB activation by IL-1 was also tested. In contrast to TNF-induced NF-κB activation, IL-1–induced NF-κB activation was not inhibited by TRIP overexpression (Fig. 6 *C*). Recent experiments have shown that IL-1–induced NF-κB activation is mediated by another member of the TRAF family, TRAF6 (31). These results suggest that TRIP is a specific inhibitor of TRAF2-mediated NF-κB activation, rather than a general inhibitor of NF-κB activation.

Discussion

Stimulation of members of the TNFR superfamily activates signaling cascades leading to the regulation of cell activation/growth or death (1). The recent identification of distinct families of receptor-associated signal transducers have provided insight into how members of the TNFR superfamily may induce pleiotropic effects on cells (4–19). Many of these signal transducers contain either TRAF or death domains, which mediate protein–protein interactions. The TRAF family proteins interact directly with some members of the TNFR family (TNFR2, CD40, LT-βR, or CD30) and play pivotal roles in the activation of signaling pathways induced by these receptors (4, 5, 10, 11, 15, 23–27). For example, the activation of NF-κB triggered by these receptors is mediated by TRAF2 or TRAF5 (9, 10, 28–30). Proteins containing death domains like FADD/MORT1, RIP, or TRADD interact with Fas(CD95) or TNFR1 (6, 12, 17, 20). Once associated with the receptors, these proteins recruit downstream signal molecules that act to initiate cascades leading to cell death or activation (2, 3, 6, 9, 12–14, 17, 20, 21). For example, TRADD recruits FADD/MORT1 or TRAF2 to the TNFR1 complex to initiate the signal transduction required for cell death or NF-κB activation, respectively (9).

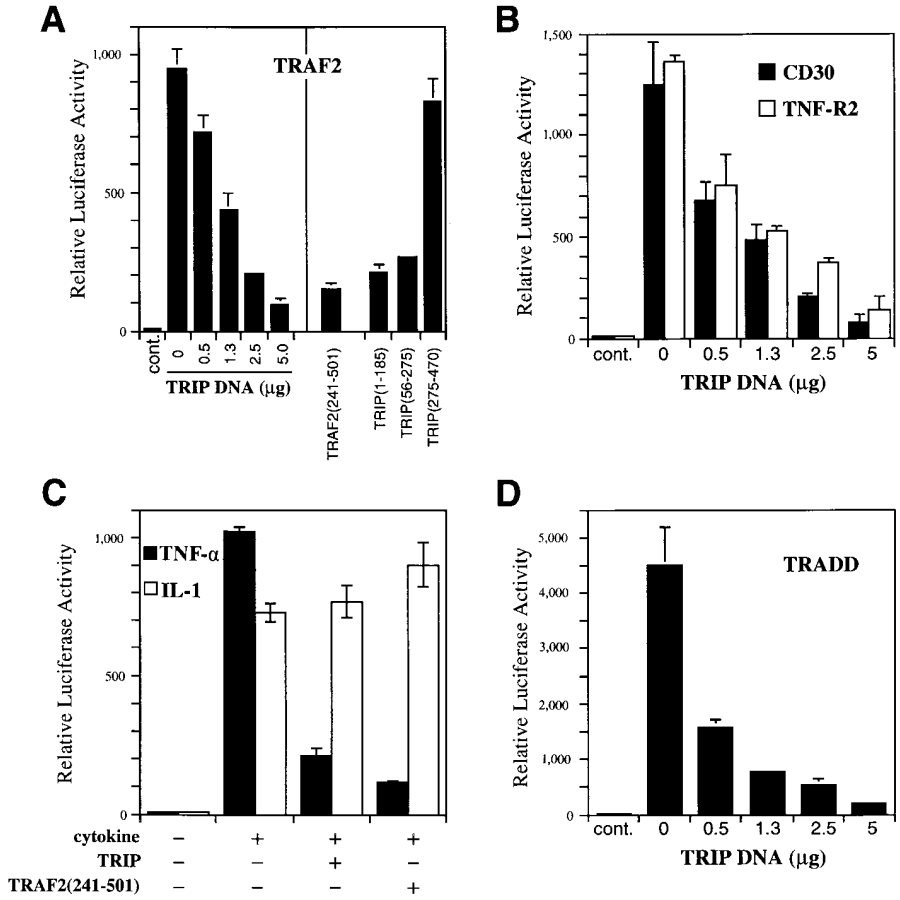

Figure 6. Inhibition of TRAF2-mediated NF-κB activation by TRIP overexpression. (*A*, *left*) A dose-dependent effect of TRIP expression on TRAF2-mediated NF-κB activation. 293 cells were transfected with 0.5 μg of TRAF2 expression vector together with 0.5 μg of p(κB)$_3$-IFN-LUC (28) in the presence of the indicated amount of TRIP expression vectors. Controls were transfected with 0.5 μg of pcDNA3.1 control vector and 0.5 μg of p(κB)$_3$-IFN-LUC. All the transfections included 0.25 μg of pCMV–β-gal plasmids. 48 h after transfection, cell lysates were prepared and used for luciferase assay. All values represent luciferase activities normalized to β-gal activities and are shown as means with their respective SEMs for representative experiments performed in duplicate. (*A*, *right*) The putative coiled-coil domain of TRIP is required to inhibit TRAF2-mediated NF-κB activation. 293 cells were transfected with 0.5 μg of TRAF2 expression vector together with 0.5 μg of p(κB)$_3$-IFN-LUC in the presence of 5 μg of plasmids expressing a dominant negative form of TRAF2 (TRAF2[241–501]), or expressing the indicated TRIP mutants. For the control experiment, cells were transfected with 0.5 μg of pcDNA3.1 control vector and 0.5 μg of p(κB)$_3$-IFN-LUC. All the transfections included 0.25 μg of pCMV–β-gal plasmids. 48 h after transfection, cell lysates were prepared and used luciferase assay. All values represent luciferase activities normalized to β-gal activities and are shown as means with their respective SEMs for representative experiments performed in duplicate. Luciferase activity of the control experiments is shown *A*, *left*. (*B*) Dose-dependent inhibition of TNFR2- or CD30-mediated NF-κB activation by TRIP. 293 cells were transfected with 1 μg of plasmids expressing the chimeric receptors, CD8-TNFR2 or CD8-CD30 (30), together with 0.5 μg of p(κB)$_3$-IFN-LUC in the presence of the indicated amount of TRIP expression vectors. For the control experiment, cells were transfected with 0.5 μg of pcDNA3.1 control vector and 0.5 μg of p(κB)$_3$-IFN-LUC. All the transfections included 0.25 μg of pCMV–β-gal plasmids. All values represent luciferase activities normalized to β-gal activities and are shown as means with their respective SEMs for representative experiments performed in duplicate. (*C*) TRIP overexpression inhibits TNF-induced NF-κB activation. 293 cells were transfected with 0.5 μg of p(κB)$_3$-IFN-LUC in the presence or absence of 5 μg of plasmids expressing a dominant negative form of TRAF2 (TRAF2[241–501]), or TRIP. For the control experiment, cells were transfected with 0.5 μg of pcDNA3.1 control vector and 0.5 μg of p(κB)$_3$-IFN-LUC. All the transfections included 0.25 μg of pCMV-β-gal plasmids. 36 h after transfection, cells were treated for 6 h with 100 pg/ml of either TNF or IL-1. All values represent luciferase activities normalized to β-gal activities and are shown as means with their respective SEMs for representative experiments performed in duplicate. (*D*) TRIP overexpression inhibits TRADD-mediated NF-κB activation. 293 cells were transfected with 0.5 μg of plasmids expressing TRADD together with 0.5 μg of p(κB)$_3$-IFN-LUC in the presence of the indicated amounts of TRIP expression vectors. For the control experiment, cells were transfected with 0.5 μg of pcDNA3.1 control vector and 0.5 μg of p(κB)$_3$-IFN-LUC. All the transfections included 0.25 μg of pCMV-β-gal plasmids. All values represent luciferase activities normalized to β-gal activities and are shown as means with their respective SEMs for representative experiments performed in duplicate.

In this study we have carried out experiments to study how TRAF proteins contribute to the signal transduction pathway triggered by the TNFR superfamily because TRAFs do not contain any domains of known signaling function despite their importance as signal transducers. It has been previously suggested that TRAFs might work as adapters to recruit different types of effector proteins to the receptor complex, which will induce specific signals (28, 29). For example, TRAF2 may interact with downstream signal transducers which phosphorylate IkB to activate NF-κB. Other classes of signal transducers are also likely to be recruited to the receptor complex to regulate various biological effects exerted by the TNFR superfamily. For example, members of the c-IAP family (c-IAP1 and c-IAP2) are recruited to the TNFR2 signaling complex by their interaction with TRAFs (7). The role of c-IAPs in the signal transduction pathway of the TNFR superfamily is not clear, but some members of the c-IAP family are involved in the protection of cells from apoptosis (42–44). Since members of the TNFR superfamily can interact with different sets of TRAF proteins, a diverse collection of downstream signal transducing molecules are likely to be recruited to the receptor complex.

We now report the identification and characterization of a novel signal transducer of the TNFR superfamily. TRIP interacts with the receptor–TRAF signaling complex and inhibits the TRAF2-mediated NF-κB activation. The structural features of TRIP include an NH_2-terminal RING

finger motif followed by a long putative coiled-coil structure. The putative coiled-coil domain of TRIP is divided into two subdomains. Amino acid sequences of the NH_2-terminal half of the coiled-coil domain of TRIP shows ~50% similarity to the rod-like coiled-coil structure of myosin heavy chain (39), while those of the COOH-terminal half of the coiled-coil domain of TRIP are characteristic of a leucine zipper (40). The putative coiled-coil domain of TRIP was shown to be required not only for TRIP–TRAF interactions, but also for the inhibition of TRAF2-mediated NF-κB activation by TRIP. Although the RING finger domain of TRIP has not been implicated in the regulation of NF-κB activation in this study, it may play some other regulatory role based on analogy to other RING-finger proteins (38). The COOH-terminal half of TRIP distal to the coiled-coil domain does not show any significant homology to other proteins but contains several potential phosphorylation sites, suggesting that TRIP may be regulated by kinases.

TRIP is recruited to the receptors TNFR2 or CD30 via its interaction with TRAF proteins. The recruitment of TRIP to these receptors was efficient in the presence of TRAF2 oligomer. Although only TNFR2 and CD30 have been tested in this study, TRIP may affect the signaling pathway mediated by many other members of the TNFR superfamily because TRAF2 is expressed ubiquitously and interacts with most of the TRAF-binding members of the TNFR superfamily. This suggestion is supported by the fact that TRIP also inhibits the induction of NF-κB activation mediated by TNFR1, which indirectly interacts with TRAF2 via TRADD (9). When the role of TRIP was examined by a transient transfection assay in 293 cells, TRIP inhibited NF-κB activation induced by TNFR2, CD30, and TNFR1, and also by TRADD, all of which activates NF-κB via TRAF2 (9, 29, 30). However, TRIP did not inhibit the activation of NF-κB by IL-1R which is mediated by TRAF6 (31), suggesting that a negative effect of TRIP on NF-κB activation was specific to a TRAF2-mediated pathway.

This specificity of TRIP makes it unique among other signal transducers (I-TRAF and A20) which inhibit TRAF2-mediated NF-κB activation (8, 19, 45). In contrast to TRIP, both I-TRAF and A20 inhibit the activation of NF-κB induced by IL-1R as well as by TNFRs (8, 19). In addition to its specificity, TRIP differs from I-TRAF or A20 in several additional aspects. First, TRIP is recruited to the cognate receptor–TRAF signaling complex, but I-TRAF is not (8). Whereas TRIP can be recruited to the cognate receptors via its interaction with TRAF2 homooligomer, A20 interacts only with TRAF2–TRAF1 heterooligomer (19). Second, the inhibitory mechanism acting on NF-κB activation by I-TRAF, A20, and TRIP appears to be different. I-TRAF inhibits TRAF2-mediated NF-κB activation by blocking the recruitment of TRAF2 to the receptor complex, which would normally initiate the clustering of TRAF proteins (8). In contrast, TRIP is recruited to the receptor complex by its association with TRAF2. Although A20 interacts with TRAFs, its inhibitory effect on TRAF2-mediated NF-κB activation does not require direct protein–protein interaction in a transfection assay in 293 cells (19). TRIP, however, inhibits TRAF2-mediated NF-κB activation only when its coiled-coil domain required for TRIP–TRAF interaction is intact. TRAF3 also inhibits TRAF2-mediated NF-κB activation when overexpressed in 293 cells. However, TRAF3 does not interact with TRAF1 or TRAF2 in the yeast two-hybrid assay (29), suggesting that the inhibitory mechanism regulating TRAF2 effector function by TRAF3 is different from that by TRIP. Therefore, a unique property of TRIP that distinguishes it from other inhibitors of TRAF2 function is that TRIP is a component of the receptor–TRAF complex and inhibits proximal events necessary for TRAF2-mediated NF-κB activation. However, the mechanism of TRIP's inhibitory effect is difficult to predict. Future identification of signal transducers required for TRAF2-mediated NF-κB activation will be required to understand how TRIP might negatively regulate the function of TRAF2.

Apart from TRIP, c-IAPs are the only other proteins which have been shown to be recruited to the receptor–TRAF complex (7). In contrast to TRIP, however, c-IAPs do not exert a negative effect on the activation of NF-κB induced by these receptors (7), rather, they have been implicated in the inhibition of cell death (42–44). In addition to their functional differences, TRIP and c-IAPs are recruited differently to their cognate receptors. c-IAPs are recruited to TNFR2 only through the TRAF2–TRAF1 heterooligomer (7), but TRIP can be recruited to the cognate receptors (TNFR2 or CD30) in the presence of the TRAF2 homooligomer. Therefore, the level of TRAF1 expression which is controlled differently among various tissues (4) may influence whether the cognate receptor will recruit c-IAPs or TRIP.

A Balance between Cell Activation/Proliferation and Death. The studies in this paper have identified TRIP as a novel signaling component of the TNFR superfamily and also shown that TRIP works as a receptor-proximal negative regulator of NF-κB activation. In addition, the studies of the regulation of TRIP suggested a model of how the signals mediated by the TNFR2– or CD30–TRAF signaling complex can initiate such seemingly opposing effects on cells, namely cell activation/growth or cell death (Fig. 7).

In this model, the balance of proactivation/growth or procell death signals mediated by the receptor–TRAF complex may be controlled by the particular set of signal transducers (e.g., c-IAPs or TRIP) which are recruited to the receptor complex. When c-IAPs are recruited to the receptor complex, TRAF2-mediated NF-κB activation proceeds unaffected. The activation of NF-κB induces the expression of various genes and also suppresses cell death (22, 46–48) which drives the cells towards the proactivation/growth state. In addition, c-IAPs themselves may contribute to antiapoptotic signals (42–44). For example, c-IAP1 (also known as MIHB and HIAP-1) was shown to inhibit the apoptosis triggered by IL-1β converting enzyme overexpression or serum deprivation (42, 44). Manganese superoxide dismutase or A20 induced by NF-κB activation will also contrib-

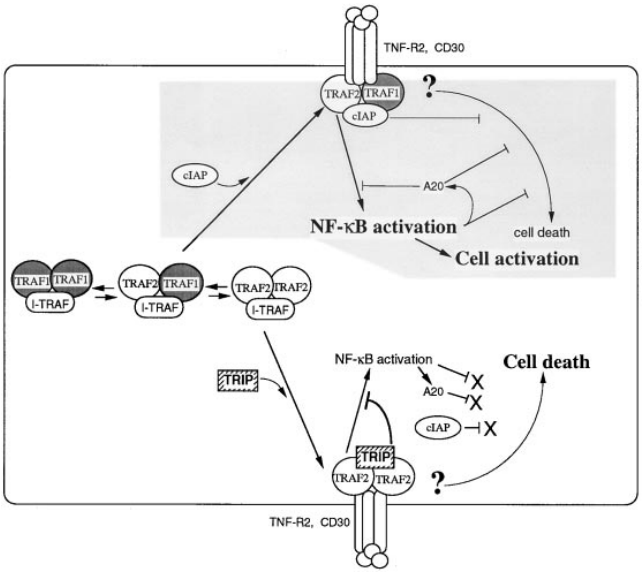

Figure 7. A model of interrelationship of TRAFs, c-IAP, and TRIP, and the switch of the TRAF-mediated signals between cell activation and cell death. The upper part of the diagram (*shaded*) describes how the receptor–TRAF signaling complex will inhibit cell death and promote cell activation/growth (7, 8, 19, 28, 29), in which A20 can work as a negative feedback regulator for TRAF2 (19). In this model, the members of the TNFR family which do not contain the death domains (e.g., TNFR2 or CD30) are postulated to trigger the induction of cell death by yet to be identified mechanism which is indicated by question mark. The lower part of the diagram explains how TRIP inhibits the TRAF-mediated cell activation/growth and contributes to the promotion of signals for cell death. For simplicity, the model does not include the receptor–TRAF2–TRAF1–TRIP complex, the presence of which cannot be excluded. However, the signals from this complex may be similar to those from the receptor–TRAF2–TRIP complex. All indicated protein association may represent dimers or higher oligomers. Three types of proximal signal transducers (TRAFs, c-IAP, or TRIP) are described. TRAFs are kept inactive in the cytoplasm due to their association with I-TRAF (also known as TANK) (8). For simplicity, a costimulatory role of I-TRAF/TANK is not included (28).

ute to the survival of cells during cell proliferation/growth (45, 49). When TRIP is recruited to the receptor complex, however, TRIP inhibits NF-κB activation which is required for antiapoptotic signals. In addition, the contribution of antiapoptotic proteins like c-IAPs, manganese superoxide dismutase, or A20 will be diminished. Therefore, the signals by the receptor–TRAF–TRIP complex will drive cells toward the antiactivation/procell death state. Consistent with this idea and also with recent findings solidifying the anti-apoptotic role of NF-κB during TNF-mediated apoptosis (22, 46–48), TRIP overexpression enhanced TNF-mediated cell death in HeLa cells (data not shown).

The choice of which type of signal transducers (c-IAPs or TRIP) is to be recruited to the cognate receptors may be determined by their availability and also by the presence of different TRAF proteins (e.g., TRAF1). This idea is consistent with several observations. First, the expression of TRAF1 is tissue-specific, whereas that of TRAF2 is not (4). Second, when lymphocytes are stimulated to proliferate via their antigen receptors, the expression of c-IAP1 or TRAF1 is upregulated, whereas TRIP expression is decreased (Fig. 3 *B*). In contrast, TRAF2 expression is not significantly affected during lymphocyte proliferation (27). During antigen-stimulation of lymphocytes, therefore, the formation of TRAF2–TRAF1–c-IAP complex will be favored and recruited to the cognate TNFR family members, which may exert costimulatory signals for lymphocyte proliferation (1). Third, TRAF1 overexpression which may antagonize the formation of TRAF2 homooligomer in cells, impairs the activation-induced cell death of mature $CD8^+$ T cells (data not shown) which is partly mediated by the TNFR2 signaling complex (32). Lastly, TRIP expression is most abundant in thymocytes, most of which are destined to die during clonal deletion, which is in part mediated by CD30 (36). In addition to TRAF1, the repertoire of other TRAF proteins present in a particular cell and the repertoire of downstream signal transducing molecules expressed in a given cell type may control the switch between cell activation/growth and cell death triggered by the receptor–TRAF signaling complex. Future studies will be directed towards identifying downstream signal transducers which are responsible for cell activation/growth (e.g., a protein which directly activates NF-κB) or those which are responsible for cell death in this signaling pathway.

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