Anatomy of TRAF2

DISTINCT DOMAINS FOR NUCLEAR FACTOR-κB ACTIVATION AND ASSOCIATION WITH TUMOR NECROSIS FACTOR SIGNALING PROTEINS

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The tumor necrosis factor (TNF) receptor-associated factor (TRAF) family of proteins interact with and transduce signals for members of the TNF receptor superfamily. TRAF1, TRAF2, and TRAF3 share a conserved C-terminal TRAF domain. TRAF2 plays a key role in transducing signals for activation of the transcription factor nuclear factor-κB (NF-κB). We have performed extensive mutational analysis on TRAF2, examining the requirements for NF-κB activation, self-association, and interaction with other molecules involved in TNF signaling. Examination of point mutants and TRAF2-TRAF3 chimeric proteins indicates that the N-terminal RING finger and two adjacent zinc fingers of TRAF2 are required for NF-κB activation. The two distinct TRAF-N and TRAF-C subdomains of the TRAF domain appear to independently mediate self-association and interaction with TRAF1. Interaction of TRAF2 with TNF-R2 and TRADD requires sequences at the C terminus of the TRAF-C domain, whereas interaction with the protein kinase receptor-interacting protein V(RIP) occurs via sequences at the N terminus of the TRAF-C domain. Thus, distinct domains of TRAF2 are involved in recruitment and signaling functions.

TNF is a pleiotropic cytokine that functions as a mediator of immune regulation and inflammatory responses. Inappropriate TNF expression has been linked to the development of diseases such as septic shock and certain autoimmune disorders (reviewed by Beutler, 1992). The cellular responses triggered by TNF are initiated through its interaction with two distinct cell surface receptors, TNF-R1 (55 kDa) and TNF-R2 (75 kDa) (reviewed by Tartaglia and Goeddel, 1992; Rothe et al., 1992). Both TNF receptors are part of the larger TNF receptor superfamily whose members, including the Fas antigen, CD27, CD30, and CD40, share characteristic cysteine-rich pseudorepeats in their extracellular domains (reviewed by Smith et al., 1995). TNF-related cytokines generate partially overlapping cellular responses, including differentiation, proliferation, NF-κB activation, and cell death, by triggering the aggregation of receptor monomers (reviewed by Tartaglia and Goeddel, 1992; Smith et al., 1994).

Until recently, the mechanisms of signal transduction by TNF and TNF-related cytokines remained unclear. In general, the cytoplasmic domains of the TNF superfamily receptor members lack recognizable common motifs, which suggested that they may use distinct signaling mechanisms. The exception occurs in TNF-R1 and Fas antigen, both of which contain ~80 amino acid “death domains” sharing ~30% identity near their C termini (Tartaglia et al., 1993; Itoh and Nagata, 1993). The recent identification of two families of proteins that directly associate with the cytoplasmic domains of TNF receptor superfamily members has provided a first step toward the understanding of signaling mechanisms used by these receptors.

TRADD and Fas-associated death domain protein (also called MORT) are novel death domain-containing proteins that were isolated on the basis of their interaction with the death domains of TNF-R1 and Fas antigen, respectively (Hsu et al., 1995; Boldin et al., 1995; Chinnaiyan et al., 1995). RIP is another Fas-interacting protein that was identified by yeast two-hybrid cloning (Stanger et al., 1995). In addition to its C-terminal death domain, RIP also contains a kinase domain at its N terminus (Stanger et al., 1995). Overexpression of TRADD, Fas-associated death domain protein, or RIP mimics ligand-binding-induced cellular responses such as apoptosis and NF-κB activation, suggesting that they may be important elements in the TNF-R1 and Fas signal transduction machinery (Hsu et al., 1995, 1996a, 1996b; Boldin et al., 1995; Chinnaiyan et al., 1995; Stanger et al., 1995).

The TRAF proteins comprise the second family of signal transducers that interact with members of the TNF receptor superfamily. TRAF1, TRAF2, and TRAF3 (also known as CD40bp, CRAF1, LAP1, and CAP1) were isolated based on their interaction with the cytoplasmic tails of TNF-R2 and CD40 (Rothe et al., 1994; Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995; Song and Donner, 1995). The TRAF proteins are defined by several distinct structural features. A C-terminal TRAF domain of approximately 230 amino acids (Rothe et al., 1994) can be further divided into the TRAF-N and TRAF-C subdomains (Cheng et al., 1995). The highly conserved C-terminal TRAF-C domains of the three TRAF proteins share approximately 60% identity over 150 amino acids (Cheng et al., 1995). Immediately adjacent is the TRAF-N domain, which has the potential to form an α-helical coiled-coil structure. TRAF1 and TRAF2 share 34% identity over 77 amino acids in this domain (Rothe et al., 1994) but bear very little primary amino acid sequence similarity to the TRAF-N domain of TRAF3. TRAF2 and TRAF3, unlike TRAF1, contain an N-terminal RING finger structure, followed by five zinc fingers.

TRAF1 and TRAF2 can form both homo- and heterodimers.
(Rothé et al., 1994). However, whereas TRAF2 can directly interact with cytoplasmic domains of both TNF-R2 and CD40, the association of TRAF1 with these receptors appears to occur via the TRAF2/TRAF1 heterodimer (Rothé et al., 1994, 1995a). TRAF3, in contrast, forms a homodimer that interacts with both CD40 (Hu et al., 1994; Cheng et al., 1995) and the Epstein-Barr virus protein LMP-1 (Mosialos et al., 1995) but not with TNF-R2 (Rothé et al., 1995a; Cheng et al., 1995). The TRAF domain is sufficient for both self-association and receptor interaction (Rothé et al., 1994, 1995a; Cheng et al., 1995).

One biological property shared by CD40, TNF-R1, and TNF-R2 is the ability to signal activation of the transcription factor NF-κB (Krupp et al., 1992; Rothé et al., 1994; Berberich et al., 1994), which in turn leads to the transcriptional activation of numerous genes encoding proteins involved in inflammatory, immune, and acute phase responses (reviewed by Baueerle and Henkel, 1994). We have shown that overexpression of TRAF2, but not TRAF1 or TRAF3, leads to activation of NF-κB and that a truncated TRAF2 lacking its N-terminalRING finger behaves as a dominant-negative inhibitor of NF-κB activation mediated by TNF-R2 and CD40 (Rothé et al., 1995a). More recently, we found that TRAF2 may also be involved in TNF-R1-mediated NF-κB activation because the TRAF2 dominant-negative mutant blocks this activity (Hsu et al., 1996a). TRAF2 is recruited to the TNF-R1 signaling complex by binding to the death domain protein TRADD (Hsu et al., 1996a). Despite significant differences in their cytoplasmic sequences, TNF-R1, TNF-R2, and CD40 each appear to use TRAF2 to transduce the signal for NF-κB activation.

With the aim of gaining further insight into the functional and structural properties of TRAF proteins, we performed extensive mutational analysis on hTRAF2. We examined the requirements for NF-κB activation, homo- and hetero-oligomerization, receptor association, and interaction with other signal transducers such as TRADD and RIP that are involved in signaling by the TNF receptor superfamily. This analysis defined the different subdomains of TRAF2 required for each of these properties.

MATERIALS AND METHODS
Reagents—Recombinant hTNF and interleukin 1β were provided by Genentech, Inc. Rabbit anti-hTNF-R2 polyclonal antibody was described previously (Rothé et al., 1994). Monoclonal anti-FLAG and anti-HA were purchased from Kodak International Biotechnologies, Inc. and Berkeley Antibody Company, respectively. The anti-myc monoclonal antibody has been described (Hsu et al., 1994). Monoclonal anti-FLAG and anti-p65 antibodies were purchased from Santa Cruz Biotechnology, Inc. and Berkeley Antibody Company, respectively. The anti-myc monoclonal antibodies were provided by Dr. V. M. Dixit (University of Michigan Medical School). The glutathione S-transferase-hTRADD fusion protein has been described (Hsu et al., 1994). Recombinant hTNF and interleukin 1β were provided by Genentech, Inc. (5-CGCGGATCCTGAGCTAAGCTGGAGGTTCCCAACAGTGAAG-3' and 5'-TGATCCCCGACCCATCTCTGGAAGAGGCGCC-3'). DNA sequencing using the oligonucleotide combinations shown: for T2(1–355), MO148, MO159 (5'-TGCATTACCTCTCTGACGACTGTC-3'); for T2(1–271), MO148, MO155 (5'-TTAGGCTGAGGCTGAGGTCAACCT-3'); and for T2(321–371), MO148 and MO159 (5'-CCCCTCTGAGGCTGAGGTCAACCT-3').

Expression plasmids encoding hTRAF2/TRAF3 and TRAF3/TRAF2 chimeric proteins were constructed by two-step PCR amplification of hTRAF2 cDNA using the oligonucleotide combinations as shown, digestion with BamHI and Xhol cleaved pcDNA3HA for T3, and for T3(1–355), 5'-GGCGGGATCCCTGAGCTAAGCTGGAGGTTCCCAACAGTGAAG-3' and 5'-TGATCCCCGACCCATCTCTGGAAGAGGCGCC-3'. DNA sequencing using the oligonucleotide combinations shown: for T2(1–355), MO148, MO159 (5'-TGCATTACCTCTCTGACGACTGTC-3'); for T2(1–271), MO148, MO155 (5'-TTAGGCTGAGGCTGAGGTCAACCT-3'); and for T2(321–371), MO148 and MO159 (5'-CCCCTCTGAGGCTGAGGTCAACCT-3').
RESULTS

NF-κB Activation by Overexpression of hTraf2—We demonstrated previously that overexpression of mTraf2 is a potent activator of NF-κB (Rothe et al., 1995a). Here we confirmed these observations with human Traf2 (hTraf2) by both electrophoretic mobility shift and reporter gene assays (Fig. 1). A single complex composed of the NF-κB p65/p50 heterodimer was observed in nuclei from both TNF-treated and hTraf2-overexpressing human 293 cells (Fig. 1A). In addition, hTraf2 increased NF-κB-dependent luciferase expression approximately 10-fold over the level seen in vector-transfected cells (Fig. 1B). HA epitope-tagged hTraf2 showed the same enhancement of NF-κB-dependent luciferase expression as untagged hTraf2. Therefore, all subsequent experiments were performed using HA-tagged hTraf2 constructs.

Three Distinct Domains Contribute to NF-κB Activation by Traf2—To map the structural features of hTraf2 required for NF-κB activation, we generated a series of expression vectors that direct the expression of hTraf2 deletion mutants. These expression plasmids and an NF-κB reporter gene with a wild-type (wt) or a mutated (m) NF-κB site, a β-galactosidase expression plasmid, and an empty vector (C) or expression plasmids encoding Traf2 (T2) or HA-tagged hTraf2 (HA-T2). Thirty hours posttransfection, cells were either left untreated (C) or treated with 10 ng/ml hTNF (B) for 4 h before harvest. β-Galactosidase activities were used to normalize luciferase values for transfection efficiency. Values shown are averages (means; bars, S.D.) of experiments performed in triplicate.

Overexpression of hTraf2 activates NF-κB. A, induction of NF-κB DNA binding activity by Traf2. Approximately 2 × 10^6 293 cells/100-mm plate were transiently transfected with 3 μg of control plasmid or Traf2 expression vector. After 24 h, cells were treated with 10 ng/ml hTNF for 4 h. Three-μl aliquots of nuclear extracts were incubated with a 32P-labeled E-selectin NF-κB site and an irrelevant antibody (mIgG), anti-p50, or anti-p65 for 30 min at 4°C. The free probe runs at the bottom of the gel (●). B, induction of NF-κB reporter activity by Traf2. 293 cells were transiently cotransfected with an E-selectin-luciferase reporter gene with a wild-type (wt) or a mutated (m) NF-κB site, a β-galactosidase expression plasmid, and an empty vector (C), or expression plasmids encoding Traf2 (T2) or HA-tagged hTraf2 (HA-T2). Thirty hours posttransfection, cells were either left untreated (●) or treated with 10 ng/ml hTNF (■) for 4 h before harvest. β-Galactosidase activities were used to normalize luciferase values for transfection efficiency. Values shown are averages (means; bars, S.D.) of experiments performed in triplicate.
Activation by TRAF2—TRAF2 and TRAF3 share many structural similarities, yet overexpression of TRAF3 fails to activate NF-κB (Rothe et al., 1995a). Our finding that the TRAF-N domain of TRAF2 is not required for NF-κB activation, coupled with the fact that TRAF2 and TRAF3 have highly homologous TRAF-C domains, suggested that sequences in the N-terminal zinc binding domains might determine why TRAF2 can activate NF-κB, whereas TRAF3 cannot. To examine this hypothesis, we constructed a series of plasmids that direct the expression of chimeric TRAF2/TRAF3 proteins and evaluated their ability to activate NF-κB (Fig. 3A). Neither the N-terminal 287 amino acids nor the C-terminal 281 amino acids of TRAF3 were able to activate NF-κB when expressed separately. However, when the N-terminal 271 amino acids of TRAF2 comprising the entire zinc binding region were fused to amino acids 288–568 of TRAF3, NF-κB was activated to a greater extent than that achieved by full-length TRAF2. Conversely, the chimera consisting of the N-terminal half of TRAF3 and the C-terminal half of TRAF2 failed to activate NF-κB. Taken together, these data demonstrate that sequences specifying the NF-κB activation potential of TRAF2 reside in the RING and zinc finger domains, whereas the TRAF domains appear to be interchangeable.

To assign in greater detail the sequence requirements in the RING and zinc fingers for NF-κB activation, we performed site-directed mutagenesis on these structures and generated additional TRAF2/TRAF3 chimeras (Fig. 3). Mutations that would be expected to disrupt zinc binding or either of the two halves of the RING finger inactivated TRAF2 (Fig. 3B). This result is consistent with the observation that the RING deletion mutant cannot activate NF-κB (Fig. 2A). This suggests that a correctly folded RING domain is essential to NF-κB activation by hTRAF2 because mutations of cysteines at either zinc binding site should interfere with the folding of the entire domain (Borden et al., 1995). However, a chimeric TRAF protein (T2/T3-2) in which the RING domain (residues 1–99) of hTRAF2 is linked to the C-terminal 458 amino acids of TRAF3 (residues 111–568) did not mediate NF-κB activation (Fig. 3A), indicating that TRAF2 sequences in addition to the RING finger are required for this activity. Further mutational analysis showed that the two N-terminal Zn fingers (F1 and F2) of TRAF2 were also critical for NF-κB activation, whereas zinc fingers 3, 4, and 5 were not (Fig. 3B). Nonetheless, a TRAF2 deletion mutant (Δ163-249) lacking only zinc fingers 3, 4, and 5 was unable to activate NF-κB (Fig. 2A). However, a chimeric protein in which the RING and zinc fingers 1 and 2 from TRAF2 (amino acids 1–162) were linked to the remainder (amino acids 177–568) of TRAF3, resulted in strong NF-κB activation (Fig. 3A). Thus, it appears that the RING domain and two zinc fingers of TRAF2 are what distinguish TRAF2 from TRAF3 with respect to NF-κB activating potential.

Dominant-negative Effect of TRAF2 Mutants on TNF-induced NF-κB Activation—In 293 cells, TNF-mediated NF-κB activation occurs exclusively through TNF-R1 (Rothe et al., 1995a). However, overexpression of TNF-R2 in these cells also activates NF-κB via a TRAF2-dependent mechanism (Rothe et al., 1995a). We reported previously that overexpression of a mTRAF2 deletion mutant lacking amino acids 1–86 blocked NF-κB activation mediated by both TNF-R1 (Hsu et al., 1996a) and TNF-R2 (Rothe et al., 1995a). Here, we examined the ability of additional inactive TRAF2 mutants to act as dominant-negative inhibitors of TNF-induced NF-κB activation (Fig. 4). The activation of NF-κB by TNF-R2 overexpression was completely blocked by coexpression of hTRAF2 containing mutations in, or deletions of, the RING domain or zinc finger 1 or 2. TNF-R2-induced NF-κB activation was partially inhibited by overexpression of the TRAF domain alone [T2(272–501)], whereas the Zn binding region alone (T2(1–271)) had no effect (Fig. 4A). These mutants behaved similarly when evaluated for their ability to inhibit TNF-induced NF-κB activation (Fig. 4B). However, they did not display dominant-negative effects against interleukin 1β-induced NF-κB activation (Fig. 4B), con-

![Anatomy of TRAF2](image)
fingering that this latter pathway is TRAF2-independent (Hsu et al., 1996a).

Both the TRAF-C and TRAF-N Domains Are Involved in Self-association—The C-terminal 230 amino acid "TRAF domain" of TRAF2 can be subdivided into an N-terminal putative coiled-coil "TRAF-N domain" and a more highly conserved C-terminal "TRAF-C domain" (Rothe et al., 1994; Cheng et al., 1995). We have shown by yeast two-hybrid analysis that the TRAF domain of mTRAF2 can associate with itself and with the TRAF domain of TRAF1 (Rothe et al., 1994). To map in more detail the region of hTRAF2 that mediates self-association in mammalian cells, we performed communoprecipitation assays. Various TRAF2 mutants (Fig. 5A) harboring N-terminal HA epitopes were coexpressed in 293 cells with FLAG-tagged full-length TRAF2. Following communoprecipitation of intact TRAF2 with a FLAG monoclonal antibody, coprecipitating TRAF2 mutants were detected by Western blotting with an anti-HA antibody (Fig. 5B). No detectable interaction occurred between the N-terminal zinc binding region (amino acids 1–271) and intact TRAF2. In contrast, TRAF2 interacted with

FIG. 3. TRAF2 RING finger and two zinc fingers are required for NF-κB activation. A, schematic structure of TRAF2-TRAF3 chimeras and their ability to activate NF-κB when overexpressed. The numbers below the TRAF2 and TRAF3 diagrams refer to amino acid number. The five individual zinc fingers are labeled 1-5 and I–V for TRAF2 and TRAF3, respectively. The portions of the various TRAF2-TRAF3 chimeric proteins derived from TRAF3 are indicated by shading. +, NF-κB-dependent luciferase expression ∼10-fold over the level seen in the vector control; ++, a greater than 20-fold induction. B, alanine scan mutagenesis of RING and zinc fingers of TRAF2. The amino acid sequences of the Zinc binding regions of TRAF2 and TRAF3 are aligned with the amino acids that contact zinc are boxed. The pairs of cysteine residues converted to alanines in the seven TRAF2 mutants (R1m, R2m, F1m, F2m, F3m, F4m, and F5m) are indicated by asterisks. +, NF-κB-dependent luciferase expression equivalent to levels induced by wild-type TRAF2. --, no activation of NF-κB.
its TRAF domain (amino acids 272–501) as well as it does with full-length TRAF2. TRAF2 deletion mutants lacking either the TRAF-N or TRAF-C domain retained their ability to associate with intact TRAF2. However, neither of these domains alone was capable of associating with TRAF2 when analyzed by this immunoprecipitation assay.

In general, the same TRAF2 deletion mutants that interact with TRAF2 also interact with TRAF1. However, there are two significant differences because both the TRAF-C domain alone and TRAF2(272–358) interact with TRAF1 but not TRAF2 (Fig. 5A). One interpretation of these results is that TRAF2/TRAFC heterodimers are favored over TRAF2 homodimers, a possibility that had been suggested based on earlier studies (Rothe et al., 1994, 1995b).

The TRAF Domain of TRAF2 Interacts with TNF-R2, TRADD, and RIP—We have shown by yeast two-hybrid assays that the TRAF domain of TRAF2 associates with TNF-R2 (Rothe et al., 1994). To more carefully define this interaction, we performed immunoprecipitation assays. Expression vectors encoding TNF-R2 and various HA-TRAF2 mutants were cotransfected into 293 cells. TNF-R2 was immunoprecipitated using a polyclonal antibody against the extracellular domain of TNF-R2, and the resulting TNF-R2 immunocomplex was subjected to Western blotting with an anti-HA antibody (Fig. 5C). The interaction between TRAF2 and TNF-R2 required the entire TRAF domain because the C-terminal deletion mutants T2(1–484) and T2(1–358) as well as the TRAF-N deletion mutant T2(Δ272-355) failed to associate with hTNF-R2. However, these mutants are capable of NF-κB activation (Fig. 2A), demonstrating that this TRAF2 activity does not require receptor interaction.

We also tested the interaction of the TRAF2 mutants with TRADD and RIP, two proteins that interact with TRAF2 and are used for TNF-R1 signal transduction (Hsu et al., 1995, 1996b). Overexpression of either TRADD or RIP mimics TNF stimulation, leading to both apoptosis and NF-κB activation (Hsu et al., 1995, 1996b). To examine the TRAF2-TRADD interaction, we coexpressed Myc epitope-tagged TRADD and HA epitope-tagged TRAF2 proteins. Following anti-HA immunoprecipitation, coprecipitating Myc-TRADD was detected by immunoblotting (Fig. 5C). Full-length TRAF2 and the complete TRAF domain bound specifically to TRADD. There was no interaction of TRADD with TRAF2(1–271) alone or deletion mutants lacking either the TRAF-N or TRAF-C domains. These data indicate that TRAF2 interacts similarly with both TRADD and TNF-R2.

Next, we examined the interaction of TRAF2 with the protein kinase RIP. HA epitope-tagged TRAF2 mutants and FLAG epitope-tagged RIP were expressed in 293 cells. RIP was immunoprecipitated, and coprecipitating TRAF2 was detected by Western analysis. RIP was found to interact with the TRAF domain of TRAF2 and the deletion mutant lacking the TRAF-N domain but not with the TRAF-C domain deletion mutant. We could not detect expression of T2(1–358), the minimal TRAF2 construct for NF-κB activation, when it was coexpressed with RIP, but T2(97–358) was able to interact with RIP. These data indicate a strong correlation between TRAF domain sequences needed for NF-κB activation and those required for association with RIP.

DISCUSSION

The recent identification of distinct classes of receptor-associated signal transducers provided insights into how members of the TNF receptor superfamily initiate downstream signaling events (reviewed by Vandenabeele et al., 1995). Signaling proteins containing “TRAF domains” were originally found to associate with TNF-R2 and CD40 (Rothe et al., 1994; Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995; Song and Donner, 1995). TRAF2 mediates NF-κB activation triggered by TNF-R2 and CD40 stimulation (Rothe et al., 1995a), and TRAF3 has been implicated in CD40-mediated CD23 induction (Cheng et al., 1995). More recently, it became apparent that TRAF domain proteins function as adaptors that can recruit additional signaling proteins into the cognate receptor complexes as well as being recruited themselves to noncognate receptors via other signaling molecules. For example, TRAFs mediate the indirect association of TNF-R2 with members of the “inhibitor of apoptosis” protein family, c-IAP1 and c-IAP2 (Rothe et al., 1995b). Also, TRAF’s associate with “death domain” containing proteins such as RIP and TRADD, which enables them to bind to the TNF-R1 signaling complex (Hsu et al., 1996a, 1996b).

To understand better the mechanisms of action of members of the TRAF family, we have begun to define sequences within TRAF2 that are required for function by mutational analysis. These studies suggest a TRAF2 anatomy composed of modular domains mediating distinct activities.

Previous evidence had indicated that the regions of TRAFs
containing zinc-binding motifs are required for signaling distinct downstream responses (Cheng et al., 1995; Rothe et al., 1995b). Consistent with this, the NF-κB activating ability of TRAF2 was found to reside within its N-terminal half, which comprises a RING finger domain and five zinc finger-like structures. In particular, site-directed mutagenesis and the analysis of chimeric proteins between TRAF2 and TRAF3 revealed that both zinc binding motifs within the RING finger domain and the immediately adjacent two zinc finger structures of TRAF2 are indispensable for NF-κB activation. However, the zinc binding regions of TRAF2 are not sufficient for NF-κB activation. Rather, this TRAF2 function also requires at least a partial TRAF domain. Interestingly, the TRAF domains of TRAF2 and TRAF3 are interchangeable in this respect, indicating a common functionality.

A comparison of the sequences of TRAF2 and TRAF3 in the region responsible for conferring NF-κB activation potential (RING finger and the first two zinc fingers) reveals a highly related RING finger (49% sequence identity) and less conserved zinc finger structures (Fig. 3B). The differences in primary amino acid sequence between TRAF2 and TRAF3 may result in important overall structural differences because RING and zinc fingers appear capable of adopting significantly varied three-dimensional structures (Freemont, 1993; Klug and Schwabe, 1995; Borden et al., 1995). How such structural differences might affect NF-κB activation, however, is not

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**Fig. 5.** Mapping of TRAF2 domains required for self-association and interaction with TRAF1, TNF-R2, TRADD, and RIP. A, expression vectors (3 µg) for the indicated HA-tagged TRAF2 mutants were transiently cotransfected with expression vectors (3 µg) for FLAG-TRAF2, FLAG-TRAF1, TNF-R2, Myc-TRADD, or FLAG-RIP for 24 h. Extracts were prepared and immunoprecipitated as described under "Materials and Methods" with either mouse IgG (data not shown), anti-FLAG, anti-HA, or anti-TNF-R2. Coprecipitating HA-tagged TRAF2 proteins or Myc-tagged TRADD were detected by immunoblot analysis using anti-HA or anti-myc epitope monoclonals, respectively. +, an interaction was seen; −, no binding was seen; ND, not done; *, no T2(1–358) expression could be detected when it was coexpressed with RIP. B, C, and E, Western blot analyses of TRAF2 mutants coprecipitating with TRAF2, TNF-R2, TRADD, and RIP, respectively. D, Western blot analysis of TRADD coprecipitating with TRAF2 mutants. Full-length TRAF2 was expressed poorly (lane 1) in this experiment (data not shown), resulting in a weak TRADD band in this lane.
clear. It is still unknown whether the zinc binding region is involved in direct protein-protein interactions or whether it regulates the TRAF domain or proteins that associate with the TRAF domain.

Earlier analysis had shown that TRAF domain association mediates the formation of homo- and heteromeric complexes between TRAF1 and TRAF2 (Rothe et al., 1994). Deletion mutants of TRAF2 lacking either the TRAF-N or the TRAF-C subdomains were still capable to bind to full-length TRAF1 or TRAF2, suggesting multiple regions of association within the TRAF domain. However, neither the TRAF2(272–358) nor the TRAF-C domain alone of TRAF3, which shares 62% identity with that of TRAF2, can self-interact in yeast two-hybrid assays. It may be that the individual TRAF-N or TRAF-C domains of TRAF2 can self-associate but cannot interact with full-length TRAF2 and, therefore, cannot be detected in our immunoprecipitation assay, which measures only those proteins that coprecipitate with full-length TRAF2. Our data could also be interpreted to suggest that the zinc binding region may stabilize oligomerization of TRAF2 that occurs through the TRAF-N or TRAF-C domains. In any event, the above data suggest that TRAF2/TRA1 heterodimers are preferred over TRAF2 homodimers, whereas TRAF3 may favor homo-oligomerization.

In addition to oligomerization, the ability of TRAF2 to bind to TNF-R2 localizes within its TRAF domain (Rothe et al., 1994). Deletion of the N-terminal half of TRAF2 showed that the TRAF domain of TRAF2 alone (amino acids 264–501) is sufficient for this association (Rothe et al., 1994). Our present analysis extends these observations and indicates that both the TRAF-N and the TRAF-C domains are involved in the interaction with TNF-R2 since deletion of either of these domains abolished the ability of TRAF2 to bind to TNF-R2. Interestingly, several of these TRAF2 mutants retained the NF-κB activating function. This is consistent with an activation mechanism in which TRAF2 clustering induced by its overexpression is similar to that induced by ligand-triggered receptor aggregation, thereby activating the NF-κB signaling pathway independently of TNF-R2 association with TNF-R2 (Rothe et al., 1995a). However, the TRAF-C deletion mutant, T2(1–35), can self-associate but cannot activate NF-κB suggests that oligomerization itself is not sufficient for the NF-κB activation function of TRAF2.

Besides receptor association and oligomerization, the TRAF domain mediates the interaction of TRAF2 with other proteins involved in TNF signal transduction. These include members of the cellular inhibitor of apoptosis protein family, c-IAP1 and c-IAP2 (Rothe et al., 1995b), and the death domain-containing proteins TRADD (Hsu et al., 1996a) and RIP (Hsu et al., 1996b). The interaction with TRADD involves both the TRAF-N and the TRAF-C domains. In contrast, the interaction with RIP was delineated to a small region at the junction of the TRAF-N and TRAF-C domains. Since TRADD can recruit both TRAF2 and RIP to TNF-R1 and since RIP also binds to TRAF2, differences in the association of these two death domain proteins within the TRAF domain of TRAF2 may contribute to the formation and/or stabilization of an active TNF-R1 signaling complex. Interestingly, association of TRAF2 with RIP correlated with NF-κB activation, raising the possibility that RIP and in particular its protein kinase activity (Hsu et al., 1996b) may be involved in signaling this TRAF2 activity. Presently, the functional contribution of RIP and also of the RING and zinc finger regions of TRAF2 to NF-κB activation await further characterization.

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