TANK, a co-inducer with TRAF2 of TNF- and CD40L-mediated NF- κ B activation

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We describe a new signal mediator of NF- κ B activation, TANK, that acts in a pathway common to two surface receptors CD40 and TNFR II. TRAF family members interact directly with these receptors. Using the yeast two-hybrid system, TANK was identified as an intracellular protein without previous homologs that interacts with all three known TRAF family members. In cotransfection experiments, TANK and TRAF2 activate NF- κ B synergistically, requiring both the amino-terminal portion of TANK and the ring finger domain of TRAF2. TANK has a negatively acting carboxyl terminus and is constitutively inactive, but TRAF2 binding overcomes the internal inhibitory influence. We propose that ligand binding to CD40 or TNFR II leads to the formation of a TRAF2/TANK complex, mediating NF- κ B activation.

[**Key Words:** TANK; NF- κ B activation; TRAF family; TNF; CD40; TNFR II]

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Members of the tumor necrosis factor receptor (TNFR) superfamily play important roles in a wide range of biological effects including acute phase responses, cell growth and apoptosis, and lymphocyte activation (for review, see Smith et al. 1994). To date, 12 such family members have been identified. Most are type I transmembrane proteins with a characteristic cysteine-rich pseudorepeat in the extracellular region. The eight corresponding ligands isolated so far also share significant sequence homology and belong to the TNF family. The structure of a soluble human 55-kd TNFR (TNFR I) complex with human TNF- β suggests that the ligand-dependent activation of the TNFR superfamily involves receptor trimerization (Banner et al. 1993). Each trimeric complex contains three receptors bound to one ligand trimer. The cytoplasmic domains of the TNFR superfamily members are relatively short and contain no known catalytic motif. They also lack significant sequence homology among themselves except for the death domains in TNFR I and Fas (Itoh and Nagata 1993; Tartaglia et al. 1993).

The mechanisms of signal transduction from the TNFR superfamily members are uncertain. There is evidence for involvement of a sphingomyelinase and a ceramide-activated kinase (Schutze et al. 1992). The recently identified TNFR-associated factors (TRAF) family members (Hu et al. 1994; Rothe et al. 1994; Cheng et al. 1995; Mosialos et al. 1995; Sato et al. 1995) and the death domain-containing proteins such as TRADD, FADD, and RIP (Chinnaiyan et al. 1995; Hsu et al. 1995; Stanger et al. 1995) are strong candidates as initial mediators of signals. The TRAF family includes TRAF1, TRAF2, and CRAF1 (also called CD40bp, TRAF3, CAP1, LAP1) mol-

ecules, which share an extensive sequence homology through their carboxy-terminal ends called the TRAF-C domain (Cheng et al. 1995). The TRAF-C domain is involved in both ligand binding and homo- or heterodimerization (Cheng et al. 1995). TRAF2 binds to the cytoplasmic tails of TNFR II and CD40 (Rothe et al. 1994, 1995), whereas CRAF1 binds to the tails of CD40 and the latent membrane protein 1 (LMP-1) of the Epstein-Barr virus (Hu et al. 1994; Cheng et al. 1995; Mosialos et al. 1995; Sato et al. 1995). Overexpression of a truncated CRAF1 containing the TRAF-C domain in human B cells produced a dominant inhibition of CD40-mediated up-regulation of CD23 thus indicating that TRAF family members are in the CD40 signaling pathway (Cheng et al. 1995). Dominant negative CRAF1 also inhibits CD40-induced up-regulation of B7-1 and, perhaps more interestingly, abolishes the ability of CD40 to rescue Fas-induced B-cell apoptosis (A. Cleary, G. Cheng, S. Lederman, and D. Baltimore, unpubl.). However, CD40-mediated up-regulation of the adhesion molecule ICAM-1 and B-cell homotypic aggregation are not affected by the same dominant-negative CRAF1. Thus, CD40 could have at least two signaling pathways, one TRAF family-dependent and the other independent.

Occupancy of either member of the TNFR superfamily CD40 or TNFR II activates NF- κ B, typically a heterodimeric transcription factor composed of p50 and p65 subunits. NF- κ B is thought to function in the regulation of the acute-phase response, inflammation, lymphocyte activation, and cell growth and differentiation (for review, see Grilli et al. 1993). In most cell types, NF- κ B is located in the cytoplasm as an inactive NF- κ B/I κ B complex (Baeuerle and Baltimore 1988). A wide variety of stimuli including viruses, bacterial lipopolysaccharide

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(LPS), antigen receptor engagement, stress factors, cytokines, phorbol esters, oxidants, and UV irradiation activate NF- κ B (Grilli et al. 1993). Activation by such agents appears to require phosphorylation and subsequent degradation of I κ B, allowing translocation of NF- κ B to nucleus (for review, see Finco and Baldwin 1995). It is still not clear, however, which kinase or kinases phosphorylate I κ B and what molecules link signaling from various stimuli to the activation of these kinases. All the proteins known to interact with TRAF family members, including CD40, TNFR II, and LMP-1, have been shown to activate NF- κ B (Laherty et al. 1992; Berberich et al. 1994; Rothe et al. 1994), suggesting that TRAF family members may be involved in NF- κ B activation.

Here we provide evidence that CD40 and TNFR II share a common signal transduction pathway for NF- κ B activation. This pathway involves synergistic activation of NF- κ B by TRAF2 and a novel TRAF2-associated protein TANK. The domains in TRAF2 and TANK responsible for this cooperation have been identified. TANK contains a carboxy-terminal inhibitory region whose activity is overcome by TRAF2 binding, suggesting a model for the early stages of the signaling pathway.

Results

Identification of the minimum CRAF1/TRAF2-binding site in the CD40 cytoplasmic tail

To identify regions of the CD40 cytoplasmic tail responsible for signaling through TRAF proteins, we used *in vitro* binding assays to map the minimum CRAF1-binding site. Glutathione *S*-transferase (GST) fusion proteins containing the entire cytoplasmic tail of mouse

CD40 (90 amino acids) or various deletion mutants were tested for their ability to interact with CRAF1 overexpressed in mammalian cells (Fig. 1A,B). A 17-amino-acid peptide bound to the full-length CRAF1 as strongly as the entire CD40 tail or any of the peptides of intermediate size (Fig. 1B, lanes 2–7). Two overlapping 10-amino acid segments from either end of the 17-amino-acid peptide, however, showed no binding (lanes 8,9), suggesting that the 17-amino-acid peptide is close to the minimum binding site for CRAF1 (its sequence is in the legend to Fig. 1A). Human CD40 cytoplasmic tail contains 62 amino acids and shares 45% sequence identity to the mouse CD40 tail in its amino-terminal portion. However, the sequence for the 17-amino acid CRAF1 minimum-binding site is identical between human and mouse, demonstrating its evolutionary conservation. To examine whether this site also binds to the other TRAF family members, cell extracts from TRAF1- or TRAF2-producing transiently transfected 293 cells were used for the interaction assays. The full-length CD40 tail and the 17-amino-acid CRAF1-binding motif bound to TRAF2 with a similar affinity as to CRAF1, but did not bind to TRAF1 (data not shown). Several mutations that altered the sequence of this 17-amino acid peptide abolished the association with CRAF1 and also failed to bind TRAF2, suggesting that CRAF1 and TRAF2 interact with the same site on the CD40 cytoplasmic tail (G. Cheng and D. Baltimore, unpubl.). Because CRAF1 and TRAF2 are both widely expressed, the CRAF1-dependent pathway we characterized previously using a dominant-negative CRAF1 in human B cells could involve either CRAF1 or TRAF2, or both. This pathway appears to involve binding of CRAF1 or TRAF2 to the 17-amino-acid

Figure 1. Defining TIMct, the minimum CRAF1 and TRAF2 binding site in the mouse CD40 cytoplasmic tail. (A) Diagram of deletion clones in the mouse CD40 cytoplasmic tail. CD40CT represents the entire cytoplasmic tail of mouse CD40, and other clones are named according to the number of amino acid residues in each construct. The sequence of the 17-amino acid TIMct is PVQETLHGCQPVTQEDG. (B) *In vitro* binding of CD40 tail to CRAF1. GST fusion proteins with CD40 tail or various deletion mutants were incubated with cell extracts from either 3T3 cells or 3T3 cells overexpressing Flu-tagged full-length mouse CRAF1. The bound Flu-CRAF1 was detected by Western analysis using anti-Flu antibody.

sequence in the cytoplasmic tail of CD40, which we will refer to as TIMct for TRAF family member interacting motif in CD40 tail.

TIMct is able to mediate NF- κ B activation

Cross-linking of CD40 leads to the activation of NF- κ B in B cells (Berberich et al. 1994). To further understand the mechanism of this activation, we developed a luciferase reporter system to monitor reconstitution of CD40 ligand-dependent NF- κ B activation in transiently transfected 293 cells. The reporter was driven by an interferon- β minimal promoter plus three repeats of the NF- κ B-binding κ B site from the immunoglobulin κ gene (Ig κ) (Fujita et al. 1993). Coexpression of the reporter with full-length CD40 in 293 cells increased NF- κ B activity three- to fourfold over the empty vector control (Fig. 2A, lanes 1,5). When a vector encoding the CD40 ligand was included, NF- κ B activity was increased >150-fold (lane 6). A similar level of NF- κ B stimulation was also obtained by cross-linking with anti-CD40 antibody

(data not shown). A luciferase construct lacking the κ B-binding sites showed minimal induction by expression of CD40 with or without CD40 ligand (Fig. 2A, lanes 7,8). Importantly, CD40 lacking its cytoplasmic tail failed to activate NF- κ B (lanes 3,4). In contrast, a CD4/CD40CT chimeric molecule containing the CD4 extracellular and transmembrane regions with the CD40 cytoplasmic tail did activate NF- κ B and was strongly stimulated by anti-CD4 antibody (Fig. 2B, lanes 5,6). The CD4 lacking a tail did not activate and activation required the κ B sites (Fig. 2B, lanes 3,4,7,8). Therefore, the cytoplasmic tail of CD40 is sufficient to deliver signals for NF- κ B activation. The likely function of the CD40 extracellular portion is to interact with the specific ligand and trimerize the tail leading to the subsequent intracellular events. However, because cross-linking by anti-CD4 monoclonal antibody alone without any secondary antibodies should cause only dimerization, these results also suggest that aggregation but not necessarily trimerization is essential for CD40-mediated NF- κ B activation.

Figure 2. NF- κ B activation by the TIMct. (A) The CD40 cytoplasmic tail is required for the ligand dependent NF- κ B activation by CD40. Luciferase constructs driven by the interferon- β minimum promoter either with or without NF- κ B-binding sites [(κ B)₃-IFN-LUC or IFN-LUC, respectively] were cotransfected into 293 cells with 1 μ g of either empty pBABE vector or vectors expressing CD40 or the CD40 mutant lacking the cytoplasmic tail (CD40ΔC) in the presence (+) or absence (-) of 1 μ g of the CD40 ligand construct (CD40L). Constructs are specified in Table 1. The y-axis represents the normalized luminescence numbers recorded from the luminometer (see Materials and methods). (B) The CD40 cytoplasmic tail is sufficient to deliver signals for NF- κ B activation. Twenty nanograms of (κ B)₃-IFN-LUC or IFN-LUC was cotransfected with 1 μ g of either the pBABE vector, a vector expressing a mouse CD4 mutant lacking the cytoplasmic tail (CD4ΔC), or the CD4/CD40CT chimeric molecule that contains the mouse CD4 extracellular and transmembrane regions with the human CD40 cytoplasmic tail. Twenty-four hours after transfection, cells were either untreated (-) or treated (+) with anti-mouse CD4 monoclonal antibody. Luciferase assays were carried at ~48 hr after transfection. (C) NF- κ B activation by the TIMct. Similar experiments were carried out as described in A and the (κ B)₃-IFN-LUC was used as the reporter. CD40C17 and CD40N22 represent constructs expressing extracellular and transmembrane domains of CD40 plus either the TIMct (or C17) described in Fig. 1 or the 22-amino-acid amino-terminal portion of the CD40 cytoplasmic tail, respectively. CD40C17M is a mutant form of CD40C17 carrying a double point mutation [Glu²⁵³Thr²⁵⁴ to AlaAla], which fails to bind to CRAF1 or TRAF2.

To further dissect and identify the motifs in CD40 tail responsible for NF- κ B activation, we made CD40 mutant constructs with deletions or mutations in the cytoplasmic tail of CD40. A CD40 construct containing only the 17-amino-acid TIMct (CD40C17) was capable of mediating CD40 ligand-dependent NF- κ B activation. A mutant construct, CD40C17M, containing alanines in place of Glu²⁵³Thr²⁵⁴ that abolished binding to CRAF1 and TRAF2 also failed to activate NF- κ B, indicating that CRAF1 or TRAF2 is involved in CD40-mediated NF- κ B activation (Fig. 2C, lanes 5,6). It may be significant that the CD40 construct containing the 22-amino-acid peptide from the amino-terminal portion of the CD40 cytoplasmic tail can also weakly activate NF- κ B (lanes 7,8), perhaps indicating that it mediates a TRAF family-independent pathway to NF- κ B activation.

Isolation of a TRAF family-associated protein, TANK, by yeast two-hybrid screening

The fact that both TIMct and TRAF2 can activate NF- κ B suggest that TRAF family members mediate this response to CD40 activation (Fig. 2C; Rothe et al. 1995). Because the TRAF family contains no domains known to be on other signaling pathways, we chose to search for downstream elements in this pathway using yeast two-hybrid screening to find interacting proteins. Using the full-length mouse CRAF1 as bait, 76 and 40 positive clones were obtained from 4×10^6 transformants from mouse T-cell and human B-cell libraries, respectively. Forty T-cell clones and 10 B-cell clones showing high β -galactosidase activities in liquid assays were sequenced partially. Most of them were overlapping clones that fell into five different groups. One of the three groups of clones tested so far was able to activate NF- κ B weakly when overexpressed alone in 293 cells using the luciferase assays described above. Including 12 mouse cDNA clones and 2 human cDNA clones, this group represents a single novel gene that we named TANK (for TRAF family member associated NF- κ B activator). Northern hybridization using a TANK probe revealed an mRNA band of ~ 2.3 kb in all tissues tested including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis (data not shown). Thus, like CRAF1, TANK appears to be widely expressed. Of the 12 mouse cDNA clones, 3 contained full-length coding sequences for a 413-amino-acid protein (Fig. 3). Data base searches failed to detect significant homology to known proteins.

The yeast two-hybrid system was used with fragments of CRAF1 to show that only the TRAF-C domain would interact with TANK (data not shown). To determine whether in mammalian cells TANK can interact with CRAF1 or the TRAF proteins, we carried out coimmunoprecipitation assays. Cell extracts were prepared from 293 cells cotransfected with Flu-tagged CRAF1, TRAF1, or TRAF2 and GST-tagged TANK constructs. GST-TANK was evident in the immunoprecipitates of CRAF1, TRAF1, and TRAF2, indicating that all three TRAF domains are found in complex with TANK in cell extracts from overproducing cells (Fig. 4A, lanes 2–4).

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MDKNIGEQLNRAYEAFRQACMDRDSAVRELQKQKTENYEQRIREQQEQLSF 50
QQNLIDRLKSQLLLVDSRDNSYGYVPLEDSRRKNNLTLDPEHDKVKL 100
GTLRDQKQSKVRRQEVSSGKESAKGLNIPLHHERDNIKTFWDLKEEFHRI 150
CLLAKAQKDHLSKLNIPDIATDTQCSVPIQCTDKTEKQEALFKPQAKDDI 200
NRGMSCVTAVTPRGLGRDEEDTSFESLSKFNVKFPMDNDSIFLHSTPEA 250
PSILAPATPETVCQDRFNMEVRDNPNGNFVKTEETLFEIQGIDPITSAIQN 300
LKTTDKTNPSNLRATCLPAGDHNVFYVNTFFLQDPPDAPFSLDSPGKAV 350
RGPPQPFWKPFLLNQDTDLVVPSDSSELLKPLVCEFCQELFPSPITSRGD 400
FLRHLNTHFNGET 413
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Figure 3. Predicted amino acid sequences of mouse TANK. The full-length mouse sequence is shown and numbered. The underlined sequence indicates the minimum TRAF interacting motif, TIMtk. The cDNA nucleotide sequences are deposited in GenBank with accession number U51907.

On the basis of the overlapping of partial TANK clones from both mouse and human cDNA clones as well as two deletion constructs we were able to narrow down the CRAF1 binding site to a small region in the middle of TANK. Association could be detected even when a GST fusion protein containing a 21-amino-acid peptide in the middle of TANK was incubated with cell extracts expressing CRAF1 or TRAF2 (Fig. 4B, lanes 3,6). In addition, a mutant TANK lacking the 21-amino-acid peptide failed to bind to CRAF1 or TRAF2 in a coimmunoprecipitation assay (Fig. 4A, lane 5). Thus, TRAF family members can associate with TANK through this 21-amino-acid peptide located in the middle of the TANK, which we called TIMtk for TRAF family member interacting motif in TANK.

Because both TIMct and TIMtk bind to the TRAF-C domain of either CRAF1 or TRAF2, we asked whether these two binding sites would compete with each other for binding to CRAF1 or TRAF2. Cell extracts from 293 cells cotransfected with a constant amount of Flu-tagged TRAF2 and increasing amounts of TANK were immunoprecipitated with the GST fusion protein containing TIMct (C17). Flu-TRAF2 that bound to GST-TIMct was then detected by Western blots with anti-Flu antibody. As the amount of transfected TANK was increased, the association between GST-TIMct and TRAF2 decreased (Fig. 4C) suggesting that TIMct and TIMtk compete for the same binding pocket in the TRAF-C domain.

TRAF2 and TANK activate NF- κ B synergistically

As described, TIMct is sufficient to provide CD40L-mediated NF- κ B activation (see Fig. 2C). In addition, overexpressed TRAF2 activates NF- κ B (Rothe et al. 1995). We then tested whether overexpression of TANK can also activate NF- κ B. Although we used a transcriptionally strong vector for its expression, TANK activated NF- κ B only weakly (two- to fivefold over the empty vector control) (Fig. 5, lane 2). Using a weaker transcriptional system, TRAF2 also activated NF- κ B marginally (lane 3), although a stronger vector could lead to higher activation (Rothe et al. 1995; G. Cheng and D. Baltimore, unpubl.). When the TANK vector was titrated into the

Figure 4. TIMct and TIMtk compete with each other for interaction with TRAF family members. (A) Coimmunoprecipitation of TANK and TRAF family members. Cell extracts from 293 cells, or 293 cells cotransfected with GST-tagged TANK or TANK Δ 21 and Flu-tagged TRAF family members, were immunoprecipitated with anti-Flu antibody. The Western blot was probed with anti-GST antibody to detect coimmunoprecipitated GST-TANK or GST-TANK Δ 21. The protein expression levels of GST-tagged TANK and TANK Δ 21 were similar by Western blot analysis using cytoplasmic cell extracts. (B) In vitro binding of TIMct and TIMtk to CRAF1 and TRAF2. Cell extracts from 3T3 cells (lane 1), 3T3 cells overexpressing Flu-tagged CRAF1 (lanes 2,3), 293 cells (lane 4), and 293 cells overexpressing Flu-tagged TRAF2 (lanes 5–7) were incubated with glutathione-agarose beads containing GST-TIMct (C17) (lanes 1,2,5), GST-TIMtk (T21) (lanes 3,4,6), and GST (lane 7). The Western blot from the precipitated products was probed with anti-Flu antibody to detect Flu-CRAF1 and Flu-TRAF2. (C) TIMct and TIMtk compete for binding to TRAF2. Cell extracts from 293 cells transfected with 1 μ g of vector for Flu-tagged TRAF2 and increasing amount of vector-expressing Flag-tagged TANK were precipitated with glutathione-agarose beads containing GST-TIMct (C17) and a Western blot was performed as in B.

same cells as received the TRAF2 vector, NF- κ B activity was greatly enhanced suggesting that TRAF2 and TANK could activate NF- κ B synergistically. The synergistic activation was biphasic (Fig. 5, lanes 4–8); a peak of activation was observed when 100–300 ng of TANK-encoding plasmid was cotransfected with 1 μ g of pGD-TRAF2 but an increase of TANK DNA to 1 μ g caused a decline of NF- κ B activity and a further increase could abolish activation (data not shown). This decrease in NF- κ B activity was not attributable to cell toxicity because the activity of the cotransfected *lacZ* reporter used for normalization was unaffected (data not shown). The levels of GST-TANK protein increased linearly with the amount of TANK-encoding plasmid DNA as assayed in a Western blot using anti-GST antibody (data not shown). Similar synergistic NF- κ B activation with TRAF2 was observed when a vector expressing FLAG-tagged TANK was used instead of GST-TANK (data not shown). To examine whether the association of TRAF2 and TANK is required for their synergistic activation of NF- κ B, parallel experiments were also carried out using a mutant TANK in which TIMtk was deleted. Although the levels of protein expressed were comparable to wild-type TANK (data not shown), this mutant TANK failed to cooperate with TRAF2 (Fig. 5, lanes 9–13), suggesting the importance of the binding between these two proteins for NF- κ B activation. Consistent with this suggestion, a TRAF2 mutant with a deletion of the TRAF-C domain (TRAF Δ C), which was unable to bind to TANK, also failed to activate NF- κ B synergistically with TANK (data not shown).

When CRAF1 or TRAF1 was cotransfected with

Figure 5. Synergistic activation of NF- κ B by TRAF2 and TANK. One microgram of pGD vector expressing TRAF2 was cotransfected with 10 ng (lanes 4,9), 30 ng (lanes 5,10), 100 ng (lanes 6,11), 300 ng (lanes 7,12), and 1 μ g (lanes 8,13) of pEBG-expressing TANK (lanes 4–8), or TANK Δ 21 (lanes 9–13) that lacks the TIMtk. Vector alone, 100 ng of pEBG-TANK, and 1 μ g of pGD-TRAF2 were used for transfections in lane 1, 2, and 3, respectively. The luciferase assays were carried out as described in Materials and methods.

TANK, no significant NF- κ B coactivation was observed (G. Cheng and D. Baltimore, unpubl.). Because the three TRAF family members bind to TANK through its TIMtk, it appears that this binding per se is not sufficient for NF- κ B activation. It is possible that TRAF2 is the only member of the family that actually activates NF- κ B through the TANK pathway. Therefore, we used it for subsequent NF- κ B activation experiments.

TANK contains both positive and negative-regulatory domains

Because TANK titration gave a biphasic NF- κ B activation response, we dissected the TANK protein to learn which region of TANK might carry positive or negative elements. TANK-expressing constructs encoding the amino-terminal (TANK-N) and carboxy-terminal (TANK-C) portions of TANK were made, both of which lack the TIMtk. Strikingly, when TANK-N was cotransfected with TRAF2 into 293 cells, NF- κ B activity was two- to threefold higher than the maximal response with full-length TANK plus TRAF2 (Fig. 6A, lane 6, cf. Fig. 5, lane 6, experiments performed in parallel). A larger input of the TANK-N vector than the one encoding full-length TANK was required to show the synergistic effect, probably because TRAF2 and TANK-N do not interact directly. In addition, TANK-N did not show the biphasic pattern seen for the full-length TANK (data not shown), suggesting that the carboxy-terminal half of TANK might exert a negative influence on NF- κ B activation.

Deletion of the ring finger domain of TRAF2 completely abolished the ability of TRAF2 to cooperate with TANK-N (Fig. 6A, lanes 7–9). However, synergistic NF- κ B activation still occurred with TRAF2 Δ C, which only carries the ring finger and zinc finger domains but

lacks the TRAF-C domain (data not shown). Thus, there appear to be two positive elements involved in the NF- κ B activation: One is within the amino-terminal region of TANK and the other is the ring finger (alone or together with the zinc finger) of TRAF2. These two positive factors need not bind directly to each other but apparently function cooperatively in NF- κ B activation.

To further understand the role of the carboxy-terminal portion of TANK in NF- κ B activation, various amounts of the TANK-C vector were cotransfected with either TRAF2 alone or TRAF2 plus TANK-N. Increasing expression of TANK-C strongly inhibited the synergistic activation of NF- κ B by TRAF2 and TANK-N (Fig. 6B, lanes 4,5). When equal amounts of the TANK-N and TANK-C vectors were cotransfected with TRAF2, the cooperation between TANK-N and TRAF2 was abolished. Similar levels of TRAF2 and TANK-N proteins were obtained at all TANK-C concentrations by Western blot assays (data not shown), indicating that the inhibition of NF- κ B activation by TANK-C was not attributable to the reduction in the level of TRAF2 or TANK. In addition, the TANK-C inhibition was not attributable to TANK-N and TANK-C dimerization through the GST tag as similar results were also observed with Flu-tagged TANK-C instead of GST-tagged TANK-C (data not shown). To test the specificity of TANK-C inhibition, we cotransfected into 293 cells various amounts of TANK-C DNA with vectors expressing either CD40 and CD40 ligand or the NF- κ B subunits p50 and p65 themselves. TANK-C strongly inhibited the CD40 and CD40 ligand-mediated NF- κ B activation but it did not affect NF- κ B activity induced by p50 and p65 (Fig. 7), indicating that TANK-C does not block NF- κ B directly but does act in the signal transduction pathway from CD40. Consistent with this, we found that TANK-C also inhibits

Figure 6. Dissection of TANK. (A) The amino-terminal portion of TANK cooperates with TRAF2 in NF- κ B activation. One microgram of pBABE vector, the vector expressing TRAF2, or TRAF2 Δ N was cotransfected with either an empty pEBG vector, 0.1 μ g, or 1 μ g of the vector expressing TANK-N, which contains the amino-terminal portion of TANK but lacks the TIMtk. (B) The carboxy-terminal portion of TANK contains a negative regulator that interferes with cooperation between TRAF2 and TANK-N. One microgram of pGD-TRAF2 was cotransfected in combination with various amount of pEBG-TANK-N and pEBG-TANK-C, a vector that expresses the carboxy-terminal portion of TANK but lacks the TIMtk.

Figure 7. TANK-C does not block NF- κ B directly but acts through a signal transduction pathway. (A) TANK-C inhibits CD40- and CD40L-induced NF- κ B activation. One microgram of pBABE-CD40 and pBABE-CD40L were cotransfected with 1 μ g of pEBG vector (lane 1), 0.3 μ g of pEBG-TANK-C (lane 2), and 1 μ g of pEBG-TANK-C (lane 3). (B) TANK-C does not inhibit p50- and p65-induced NF- κ B activation. 0.5 μ g of vectors expressing p50 and p65 were cotransfected with 1 μ g of pEBG vector (lane 1), 0.3 μ g of pEBG-TANK-C (lane 2), and 1 μ g of pEBG-TANK-C (lane 3).

NF- κ B activation induced by TRAF2, and TRAF2 plus full-length TANK (data not shown).

Discussion

Stimulation of members of the TNF family of cell surface receptors leads to the activation of NF- κ B (Berberich et al. 1994; Rothe et al. 1994). To study the pathway of CD40L-stimulated activation, initially we used the yeast two-hybrid system to identify CRAF1 (Cheng et al. 1995), a protein that we show here binds CD40 through a 17-amino-acid TIMct motif on the receptor tail. We implicated CRAF1 in signaling by use of a dominant-negative fragment (Cheng et al. 1995) but we now know that such a fragment could interfere with either CRAF1 or TRAF2 binding and the properties of the two proteins suggest that it may be TRAF2 that is the primary transducer of CD40-mediated signaling to NF- κ B. While still unaware of this perspective, we again used the two-hybrid system to find CRAF1-binding proteins and discovered TANK, a protein that binds to CRAF1, TRAF2, and TRAF1 through a TIMtk motif of ≤ 21 amino acids. TANK as well as CRAF1 and the TRAFs are so widely expressed that we have no cell line lacking them. Therefore, we have used overexpression in 293 cells by transient transfection to analyze their functions. We used a reporter construct with κ B sites as a measure of NF- κ B activation, as done by others (Rothe et al. 1995), but we have not yet investigated the actual proteins that are

binding to the sites. Activation of NF- κ B by transfected CD40 in this system is strictly dependent on CD40 ligand stimulation or cross-linking with anti-CD40 antibody; it clearly uses the TRAF2/TANK pathway because signaling is blocked by the TANK carboxyl terminus. Because TRAF2 also binds to the cytoplasmic tail of TNFR II, this TRAF2/TANK pathway likely represents a common NF- κ B activation pathway shared by members of the TNF receptor superfamily.

Activation of NF- κ B in 293 cells can be accomplished by overexpression of TRAF2 but not CRAF1 (Rothe et al. 1995). At a limiting concentration of TRAF2, TANK coexpression can produce an ~ 20 -fold stimulation but a further increase in TANK leads to inhibition. We traced this biphasic response to a bipartite organization of TANK. Its amino-terminal one-third can coactivate NF- κ B ~ 60 -fold, whereas its carboxy-terminal two-thirds can counter completely this stimulation. Because the carboxyl terminus inhibits signaling from intact CD40 or TRAF2 but does not affect NF- κ B directly, it may be a *cis*-acting regulator of the activity of the amino-terminal segment of TANK.

These data suggest a unique model of signaling from the TNF family of receptors (Fig. 8). CRAF1 and TRAF2 bind to the receptors without activation, suggesting that they are bound in the ground state. Aggregation of receptors by ligand could lead to local release of these TRAF family proteins, with free TRAF2 stimulating NF- κ B and free CRAF1 activating along an undetermined pathway. The released TRAF2 might be modified, perhaps by phosphorylation. Other potential TRAF family members may also participate. Therefore, overexpressed TRAF2 may mimic the action of ligand by providing such an excess of TRAF2 that the receptors cannot hold it all. Overexpressed TRAF2 appears to activate NF- κ B by combining with TANK; the ability of the TANK carboxy-terminal region to inhibit TRAF2-mediated activation indicates this. TANK in its ground state is probably autoinhibited by interaction of its carboxyl terminus and amino terminus, possibly by direct association. The binding of TRAF2 to TANK relieves this inhibition and the coordinate activity of the amino-terminal region of TANK and the ring finger of TRAF2 then causes NF- κ B activation, presumably by activating an I κ B kinase. Although many details remain unclear and the final step is still a black box, the outlines of the model are strongly suggested by the accumulated data.

The model of NF- κ B activation is applicable only to TRAF2; the three known TRAF family members (CRAF1, TRAF1, and TRAF2) are likely to play different roles in the TNF receptor superfamily-mediated signal transduction pathways. Although all of them share the obviously homologous TRAF-C domain, their binding specificities to receptor tails are different. TRAF2 binds to both CD40 and TNFR II, CRAF1 binds to CD40, and TRAF1 does not bind to either of them directly (Rothe et al. 1995). In contrast, TANK interacts with the TRAF-C domains of all three TRAF family members. Therefore, TANK may participate in events other than NF- κ B activation because CRAF1 plus TANK does not activate NF-

Figure 8. A model for the CD40 and TNFR II-mediated NF- κ B activation pathway. The model postulates that in unactivated cells CD40 and TNFR II are bound to CRAF1 and TRAF2 because the interaction of these proteins does not require any known modification. The interaction involves TIMct on CD40 with the TRAF-C domain on CRAF1 and TRAF2 and an equivalent interaction of the tail of TNFR II. The model then suggests that activation of the receptors by ligand binding will cause release of CRAF1 and TRAF2. This postulate derives from the known activity of free TRAF2 to activate NF- κ B [Rothe et al. 1995]. Having found that TANK can bind to TRAF2 and signal activation of NF- κ B, we postulate that the free TRAF2 binds to inactive TANK and releases the activation potential of the TANK-N domain. The TRAF-C domain of TRAF2 binds to TANK through the TIMtk motif of TANK and that interaction would only be possible if TRAF2 is freed from the receptor because TIMct and TIMtk compete (Fig. 4). We also postulate that TANK is autoinhibited in the absence of TRAF2 by an internal interaction of the TANK-N positive activator (+) with the TANK-C inhibitory region (-). The model indicates that it is the synergistic activity of TANK-N and TRAF2 that activates NF- κ B.

κ B. In spite of the competition between them for TRAF-C binding, the minimum CRAF/TRAF-binding sites in the CD40 tail and TANK do not share an obvious consensus sequence (see Fig. 1A legend and Fig. 3). In addition, both CRAF1 and TRAF2 form homodimers through their TRAF-C domains but only TRAF2 can form a heterodimer with TRAF1 [Cheng et al. 1995; Rothe et al. 1995]. How the TRAF-C domains determine their specificities of both ligand binding and dimerization is not clear. Furthermore, other domains of this family might also function differently. Both CRAF1 and TRAF2 contain ring finger and zinc finger domains but TRAF1 does not. CRAF1 has an additional isoleucine zipper domain [Cheng et al. 1995]. The ring finger of TRAF2 but not CRAF1 cooperates with TANK-N, suggesting there is functional difference between these two ring finger domains. Thus, the specificities of each TRAF family member in receptor binding, dimer formation, and functional domains could modulate the very divergent functions that the TNF receptor superfamily mediates.

The role of the receptor in signaling NF- κ B activation appears surprisingly simple. The ability of TIMct alone as an intracellular region to mediate activation and the ability of the CD4 extracellular region to substitute for its CD40 counterpart indicates that all of the action is in the 17 amino acids of TIMct. It binds TRAF-C and it is hard to imagine that it could bind much else at the same time. Release of TRAF2 may be its only function. Previously, we showed that overexpression of the TRAF-C domain, which should displace TRAF2, is inhibitory, not activating [Cheng et al. 1995]. This might seem to negate the model until one realizes that overexpressed TRAF-C will bind to TANK and block its action. Therefore, it could well be that signaling involves no other event than release of TRAF2, although it is not immediately apparent why aggregation of receptors should lead to such a release.

An aspect of this data that is most unusual is the activation of TANK by TRAF2. TANK is inactive without its 21-amino-acid TIMtk motif and therefore, it needs to interact with a TRAF-C domain for it to be active. The

amino-terminal portion of TANK (TANK-N), in the absence of carboxy-terminal inhibitory region, can activate NF- κ B synergistically with both full-length TRAF2 and the amino-terminal portion of TRAF2 (TRAF2-N) containing only the ring finger and zinc finger domains. This cooperative activation does not appear to require direct association between TANK-N and TRAF2-N. Thus, the role of TIMtk/TRAF-C binding is apparently to neutralize the inhibitory effect of the carboxyl terminus of TANK. The inhibitory effect of the carboxy-terminal portion of TANK (TANK-C) is very potent. When equal amounts of TANK-N and TANK-C vectors are cotransfected with TRAF2, the synergistic NF- κ B activation by TRAF2 and TANK-N is abolished completely. TANK has no homology to any other protein, so we have no clue to its mechanism of activation or inhibition. Whether it has homologs remains unknown but given the extensive data bases available today, it may not.

Materials and methods

Cell culture

293T, a variant of the human embryonic kidney cell line 293, was used throughout. This variant and NIH-3T3 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% FCS, 1% (wt/vol) penicillin/streptomycin,

and 1% glutamine (GIBCO-BRL) in an atmosphere of 5% CO₂ in air humidified at 37°C. 3T3 cells overexpressing Flu-tagged CRAF1 were selected in the above medium in the presence of 2.5 μ g/ml of puromycin (Sigma).

Constructs

DNA fragments for most of the deletion or mutation constructs were obtained by PCR amplification using Pfu polymerase (Stratagene). All constructs were confirmed by restriction enzyme analysis and partial DNA sequence. Their particulars are indicated in Table 1. The luciferase reporter constructs p(κ B)₃-INF-LUC and pINF-LUC have been described previously (Fujita et al. 1993). They contain luciferase cDNA driven by the interferon- β minimum promoter either with or without three repeats of the NF- κ B-binding site from the Ig κ gene.

Transient transfection and luciferase reporter assays

293 cells were seeded 18–24 hr before transfection (5×10^5 cells/well in six-well dishes). Various constructs were cotransfected with 20 ng of p(κ B)₃-INF-LUC and 50 ng of pCSK-LacZ or 250 ng of pmf β -LacZ by standard calcium phosphate methods. Total amounts of plasmid DNA were kept constant by adding empty vectors. Antibody cross-linking of CD4 or CD40, if required, was carried out 24 hr after transfection. At 36–48 hr after transfection, the culture medium was removed and the cells were washed with PBS and lysed in 250 μ l of reporter lysis buffer (Promega). Twenty microliters of the cell extract from

Table 1. Constructs used in this paper

Constructs	Description	Position ^a	Vector no. ^b
CD40CT	mouse CD40 cytoplasmic tail	216–305	pGex2T
C45	deletion clone of CD40CT	234–278	pGex2T
C39	deletion clone of CD40CT	234–272	pGex2T
C38	deletion clone of CD40CT	241–278	pGex2T
C32	deletion clone of CD40CT	241–272	pGex2T
C17	deletion clone of CD40CT	251–267	pGex2T
C10N	deletion clone of CD40CT	251–260	pGex2T
C10C	deletion clone of CD40CT	258–267	pGex2T
T21	CRAF/TRAF binding site in TANK	169–189	pGex2T
CD40	full-length human CD40	1–277	pBABE
CD40 Δ C	hCD40 lacking cytoplasmic tail	1–222	pBABE
CD40C17	CD40 Δ C plus CRAF1 binding site	1–222 + 250–266	pBABE
CD40C17M	mutant form of CD40C17	E253A; T254A	pBABE
CD40N22	CD40 Δ C plus amino-terminal of CD40 tail	1–244	pBABE
CD4 Δ C	mouse CD4 lacking cytoplasmic tail	1–431	pBABE
CD4/CD40CT	CD4 Δ C plus hCD40CT	(1–431) + (223–277)	pBABE
CD40L	mouse CD40 ligand	1–260	pBABE
CRAF1	mCRAF1 with 5' Flu tag	1–576	pBABE
TRAF1	mTRAF1 with 5' Flu tag	1–409	pBABE
TRAF2	mTRAF2 with 3' Flu tag	1–501	pGD
TRAF2 Δ N	TRAF2 with deletion of Zn ring	94–501	pGD
TRAF2 Δ C	TRAF2 with deletion of TRAF-C	1–271	pGD
TANK	mTANK with 5' GST tag	1–413	pEBG
FLAG-TANK	FLAG-tagged TANK	1–413	pCMV5
TANK Δ 21	TANK with deletion of 21 amino acids	1–168 + 190–413	pEBG
TANK-N	amino-terminal portion of TANK	1–168	pEBG
TANK-C	carboxy-terminal portion of TANK	190–413	pEBG

^aAmino acid positions start with the translation initiation methionine as position 1.

^bpGex2T was obtained from Pharmacia. Other vectors are described in the following papers: pBABE (Morgenstern and Land 1990); pGD (Daley et al. 1990); pEBG (Sanchez et al. 1994); pCMV5 (Anderson et al. 1989).

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each sample was used to measure the luciferase activity using the luciferase assay system according to the manufacture's protocol (Promega). We used β -galactosidase activity as the internal control to monitor each transfection. For the β -galactosidase assays, 50 μ l of the cell extract was incubated with 0.5 ml of Z buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , 50 mM β -mecaptoethanol) and 0.1 ml of 4 mg/ml ONPG (*O*-nitrophenyl- β -D-galactopyranoside) at 30°C for 30 min. The reaction was stopped by adding 0.25 ml of 1 M Na_2CO_3 , and the β -galactosidase activity was measured by spectrophotometer at 420 nm wavelength of visible light. Each construct has been tested for its expression. The luminescence values shown in Figures 2, 5, 6, and 7 were normalized by the individual β -galactosidase activity from each transfection and are averages of two to four independent experiments.

In vitro-binding assays and immunoprecipitation

The GST fusion proteins containing various fragments of the CD40 cytoplasmic tail and the 21-amino-acid peptide in TANK were purified from *Escherichia coli* as described (Cheng et al. 1994). Approximately 2×10^7 3T3 cells overexpressing Flu-tagged CRAF1 or 293 cells transfected transiently with Flu-tagged CRAF1, TRAF1, or TRAF2 were lysed in 1 ml of lysis buffer [1% Triton X-100, 150 mM NaCl, 20 mM HEPES (pH 7.2), 10 mM NaF, 0.4 mM EDTA, 1 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1% aprotinin] on ice for 30 min. The supernatants were incubated with the glutathione-agarose beads carrying the GST fusion proteins for 2 hr at 4°C. The beads were washed three times with the lysis buffer before Western assays with anti-Flu antibody (12CA5; Berkeley Antibody Company).

For immunoprecipitation assays, 293 cells were cotransfected with Flu-tagged TRAF family members and GST-tagged TANK or TANK Δ 21. Cytoplasmic extracts from 2×10^7 transfected 293 cells were incubated with anti-Flu antibody for 2 hr at 4°C. The TRAF protein complexes were then precipitated by protein G-Sepharose (Pharmacia). Western blots were probed with anti-GST antibody.

Yeast two-hybrid screening

The full-length coding sequence of the mouse CRAF1 cDNA was amplified by PCR using Pfu polymerase and cloned in-frame into the bait vector pAS1-CYH2, which contains the GAL4 DNA-binding domain and TRP1 selection marker. The CRAF1 bait (pAS-CYH2-CRAF1) was then transformed into the yeast strain Y190. Two lambda phage libraries made from either human B cells or mouse T cells were obtained from Stephen Elledge's laboratory (Elledge et al. 1991). After infection of the bacterial strain BNN132 with these two libraries, $\sim 8 \times 10^7$ individual colonies that contain plasmids carrying cDNA inserts were grown up from each library. The purified library plasmids were then transformed into Y190, which carries the pAS-CYH2-CRAF1. Approximately 4×10^6 transformants from both the B and T cell libraries were selected on 150-mm yeast synthetic plates that lack histidine, leucine, and tryptophan but contain 50 mM 3-AT (3-aminotriazole; Sigma, A8056). Four to 5 days later, ~ 100 and 50 yeast colonies had grown up from the T and B cell library transformants, respectively. They were then patched onto fresh plates 1 day before the filter lift assays for β -galactosidase activity. Of them, 76 T cell clones and 40 B cell clones showing blue color were picked up and grown in liquid culture to measure the β -galactosidase units. Among them, 40 T-cell clones and 10 B-cell clones gave >25 β -galactosidase units. Yeast DNA was prepared from those clones and trans-

formed into *E. coli* strain TOPP 10. Colonies carrying the library plasmid were identified by restriction enzyme analysis, and cDNAs were sequenced with an automatic DNA sequencer.

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