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A Novel Mechanism of TRAF Signaling Revealed by Structural and Functional Analyses of the TRADD–TRAF2 Interaction

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Summary

TRAF proteins are major mediators for the cell activation, cell survival, and antiapoptotic functions of the TNF receptor superfamily. They can be recruited to activated TNF receptors either by direct interactions with the receptors or indirectly via the adaptor protein TRADD. We now report the structure of the TRADD-TRAF2 complex, which is highly distinct from receptor–TRAF2 interactions. This interaction is significantly stronger and we show by an in vivo signaling assay that TRAF2 signaling is more readily initiated by TRADD than by direct receptor–TRAF2 interactions. TRADD is specific for TRAF1 and TRAF2, which ensures the recruitment of cIAPs for the direct inhibition of caspase activation in the signaling complex. The stronger affinity and unique specificity of the TRADD–TRAF2 interaction are crucial for the suppression of apoptosis and provide a mechanistic basis for the perturbation of TRAF recruitment in sensitizing cell death induction.

Introduction

The TNF receptor superfamily consists of more than 20 structurally related type I transmembrane proteins that are specifically activated by the corresponding superfamily of TNFα-like cytokines, eliciting a wide spectrum of cellular responses including transcriptional gene activation and induction of apoptosis (Smith et al., 1994; Gravestein and Borst, 1998). Members of this receptor superfamily are widely distributed and play key roles in many crucial biological activities including lymphoid and neuronal development, innate and adaptive immunity, and maintenance of homeostasis. Agents that manipulate the signaling of these receptors are being used or showing promise toward the treatment and prevention of many human diseases such as rheumatoid arthritis, coronary heart disease, transplantation rejection, insulin resistance, multiple organ failure, and neoplasm (Ashkenazi and Dixit, 1998; Leonen, 1998; Newton and Decicco, 1999).

The TNF receptor superfamily can be divided into two subgroups, depending on whether the intracellular region contains a death domain. Receptors that contain death domains are known as death receptors (Nagata, 1997; Ashkenazi and Dixit, 1998). The best studied death receptors are TNFR1 and Fas; while TNFR1 only induces cell death under certain circumstances and more often induces transcriptional gene activation, Fas is efficient in cell death induction. TNF receptors that do not contain death domains are represented by TNFR2, CD40, CD30, and many others. These receptors are involved primarily in gene transcription for cell survival, growth, and differentiation.

The cell activation, cell survival, and antiapoptotic functions of the TNF receptor superfamily are mostly mediated by the family of TNF receptor–associated factors (TRAF1–6) (Arch et al., 1998). The TRAF proteins are also genetically conserved across other multicellular organisms including *Drosophila* (Liu et al., 1999), *C. elegans* (Wajant et al., 1998), and *Dictyostelium discoideum* (Regnier et al., 1995). The downstream effectors of TRAF signaling are transcription factors in the NF-κB and AP-1 family (Malinin et al., 1997; Nishitoh et al., 1998; Baud et al., 1999), which can turn on numerous genes involved in various aspects of cellular and immune functions. In addition, the activation of NF-κB and AP-1 have been shown to render cells protection from apoptosis via the transcription of antiapoptotic genes (Beg and Baltimore, 1996; Minden and Karin, 1997).

Both subgroups of the TNF receptor superfamily can recruit TRAF proteins. Those without death domains recruit many TRAF family members directly for signal transduction (Rothe et al., 1994; Arch et al., 1998). Death receptors related to TNFR1, but not Fas, recruit TRAF2 indirectly via the N-terminal domain of the adaptor protein TRADD (Hsu et al., 1996b). TRADD also contains a death domain and interacts with the intracellular death domain of TNFR1 via homotypic associations (Hsu et al., 1995). The death domain of TRADD is multifunctional and capable of recruiting two additional signaling proteins, FADD and RIP (Hsu et al., 1996a, 1996b). FADD can directly recruit and activate caspase-8, giving rise to the initiation of apoptosis (Boldin et al., 1996; Muzio et al., 1996). RIP, on the other hand, reinforces the stimulation of gene transcription by activating the NF-κB signaling pathways (Kelliher et al., 1998).

Even though the establishment of TRAF proteins as common signaling molecules explains the partially overlapping cellular effects by members of the TNF receptor superfamily, it is not clear whether and how TRAF signaling differs between the two subgroups of receptors and whether these differences may effect different biological functions. The indirect TRAF recruitment by TRADD exists in the context of the opposing proapoptotic and antiapoptotic pathways. Targeted gene deletion has implicated TRAF2 as essential for the suppression of the intrinsic apoptotic tendency of the TNFR1 signaling

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complex (Yeh et al., 1997), but the precise mechanism of this function has not been well established. In addition, there appear to be differences in the abilities of different TNF receptors in responding to soluble versus cell-bound forms of ligands.

Here, we use structural studies as leads, in conjunction with further biological experiments, to expand our understanding on this complex network of TRAF signaling. By determining the crystal structure of the complex between the N-terminal domain of TRADD (TRADD-N) and the TRAF domain of TRAF2, we reveal a novel mode of interaction mediated by a relatively extensive protein–protein interface. This is distinct from the linear motif–mediated receptor–TRAF2 interactions shown earlier by us and others (McWhirter et al., 1999; Park et al., 1999; Ye et al., 1999). We further show by characterizing the native and mutant TRADD–TRAF2 interactions using biosensor measurements that this novel form of TRADD–TRAF2 interaction confers significantly higher affinity and distinct specificity in comparison with receptor–TRAF2 interactions. The higher affinity of the TRADD–TRAF2 interaction suggests that TRADD is a stronger inducer of TRAF2 signaling, which we further confirm experimentally in vivo. This affinity difference also provides a structural basis for the different avidity requirements of direct TRAF signaling versus TRADD-mediated TRAF signaling. In contrast with the rather promiscuous interactions of receptors with many members of the TRAF family, we show that TRADD is specific for TRAF1 and TRAF2. This specificity of TRADD suggests a more prominent role of the cellular caspase inhibitors cIAPs for the immediate inhibition of caspase activation in the multifunctional signaling complex of TNFR1, as cIAPs are constitutively associated with these two TRAF proteins only. Therefore, any perturbation on TRAF-mediated recruitment of cIAPs to the TNFR1 signaling complex may sensitize TNFR1-induced apoptosis. We show that the high affinity, and perhaps the specificity, of the TRADD-TRAF2 interaction, is crucial for the suppression of apoptosis.

Results and Discussion

Crystal Structure of the TRADD-TRAF2 Complex and Its Placement in the Postreceptor Signaling Complex of TNFR1

Crystal structure of the complex between TRADD-N and the TRAF domain of TRAF2 was determined at 2.0 Å resolution using multiwavelength anomalous diffraction of the selenomethionyl crystal. TRADD-N folds into an α-β sandwich with a four-stranded β sheet and six α helices, each forming one layer of the structure (Figure 1A). The basic polypeptide topology of TRADD-N has been observed in other protein structures (Orengo and Thornton, 1993). However, TRADD-N shares no recognizable sequence homology to any known structures and is much more elaborate with only approximately half of its residues superimposable with its structural neighbors.

The trimeric TRAF domain of TRAF2 imposes a 3-fold symmetry to the stoichiometrically bound TRADD-N (Figures 1B and 1C). The TRAF domain of TRAF2 has the shape of a mushroom when viewed along a vertically orientated 3-fold axis, with its β sandwich domain as the cap and its coiled-coil domain as the stalk (Park et al., 1999). Each TRADD-N contacts one protomer of the TRAF domain only. The binding is at the β sandwich domain, away from the coiled-coil domain. This interaction of TRADD-N with the TRAF domain at the upper rim of the mushroom cap adds a wing-like structure to the mushroom (Figure 1B). The 3-fold axis of the complex coincides with the crystallographic 3-fold axis in the crystal, suggesting that the complex obeys near perfect symmetry in solution.

The TRADD-TRAF2 complex can be placed in the large postreceptor signaling complex of TNFR1 and used to suggest the relative locations and proximities of the signaling molecules (Figure 1D). The carboxyl terminus of TRADD-N projects up and away from the TRADD-TRAF2 complex when viewed in the orientation shown in Figure 1B. The direction of the projection is likely to indicate the possible location of the carboxy-terminal death domain of TRADD, which directly binds the intracellular death domain of TNFR1. This fixes the orientation of the complex relative to the cellular membrane (Figures 1B and 1D). The death domain of TRADD is the central platform for recruiting other intracellular signaling molecules such as FADD for caspase-8 binding and activation and RIP for NF-κB activation. The amino-terminal effector domain of TRAF2 would also locate more intracellularly for engaging those molecules required for JNK and NF-κB activation. More importantly, the coiled-coil domain of TRAF2, which is responsible for its interaction with cIAPs, would likely situate close to the FADD-recruited caspase-8 in this signaling complex.

The Bipartite TRADD-TRAF2 Interaction Occupies a Similar Surface of TRAF2 but Bears No Resemblance to Receptor-TRAF2 Interactions

The interface between TRADD-N and the TRAF domain of TRAF2 possesses dual "ridge into groove" contacts, which serve to divide the interface into two partially connected regions (Figure 2). Region I is smaller in surface area but largely hydrophobic. It is mediated by the exposed shallow face of the β sheet of TRADD and a surface protrusion of TRAF2 formed by β7, the following loop and the connection between β3 and β4. Region II of the interface is mediated by a highly charged prominent ridge formed by TRADD residues 143–149 in the EF loop and a surface depression of TRAF2 presented by strand β6 and the following loop. This hydrophilic interface is rich in hydrogen bonds and salt bridges. In addition, there are many water-mediated interactions in this region and the boundary between regions I and II.

The protein–protein interface of the TRADD–TRAF2 interaction occupies a similar surface at the edge of the β sandwich of TRAF2 in comparison with receptor–TRAF2 interactions determined previously (McWhirter et al., 1999; Park et al., 1999; Ye et al., 1999). This indicates the competitive nature of direct and indirect TRAF2 recruitment by the TNF receptor superfamily. However, the actual molecular contacts of TRAF2 with TRADD and with receptors are entirely different. In all six different receptor–TRAF2 complexes, the receptor peptides exhibit an extended main chain conformation to form an additional strand adjacent to the edge of the β sandwich of TRAF2 through main chain hydrogen

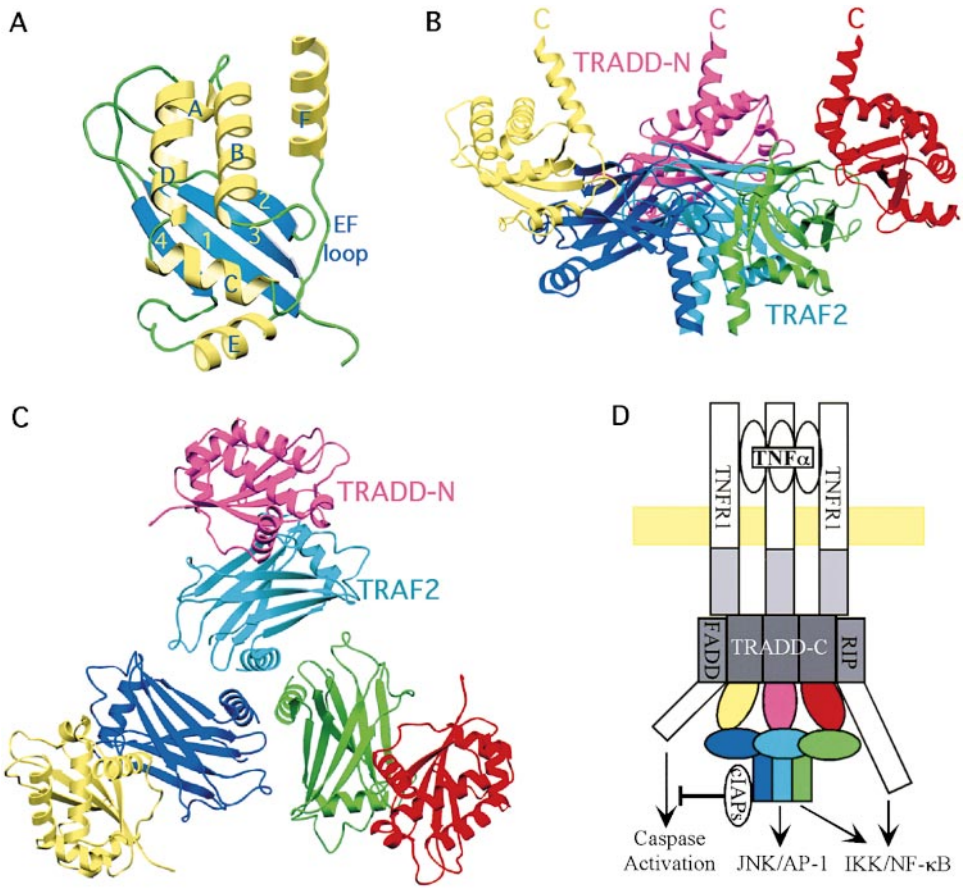


Figure 1. Structural Overview of TRADD-N and the TRADD-N/TRAF2 Complex

(A) Ribbon representation of TRADD-N, showing the two-layer arrangement of the α-β sandwich. Helices are colored yellow (A-F), β strands blue (1–4), and loops green. The β sheet is entirely antiparallel and slightly twisted with a strand order of β2, β3, β1, and β4. There are two helices each in the β1-β2 and β3-β4 cross-over connections while the β2-β3 connection is hairpin-like. The remaining two helices (E and F) are near the carboxyl terminus of the domain; the loop in between (EF loop) partly covers one end of the exposed face of the β sheet. A single hydrophobic core is present in TRADD-N between the buried face of the β sheet and the opposing α helices. The closed nature of this hydrophobic core supports that this domain folds independently of the carboxy-terminal death domain.

(B and C) Ribbon representations of the TRADD-N/TRAF2 complex, showing with the 3-fold axis vertical in (B) and into the page in (C). Three molecules of TRADD-N are shown respectively in magenta, red, and yellow. The protomers of the trimeric TRAF domain of TRAF2 are shown respectively in cyan, green, and dark blue. The death domain of TRADD (TRADD-C) is proposed to be locate above the C-terminal helix of TRADD-N in (B).

(D) A hypothetical molecular arrangement in the signaling complex of TNFR1 and related death receptors. The cell membrane is represented in yellow. The trimeric TNFα, shown by ovals, mediates TNFR1 trimerization. TNFR1 is shown by straight rectangles, while FADD and RIP are shown by bent rectangles. Death domains in TNFR1, TRADD (labeled as TRADD-C), FADD, and RIP are shaded in gray. TRADD-N and the TRAF domain of TRAF2 are highlighted using the same color-coding in (B) and (C). cIAPs (oval shape) are recruited by TRAF2 and shown to inhibit caspase activation by this signaling complex. For clarity, only single molecules of FADD and RIP are shown, even though they are expected to multimerize in the signaling complex.

bonding interactions. Specific side chain interactions define linear TRAF2 binding motifs, which are absent in the TRADD sequence.

The interaction between TRADD and TRAF2 buries a total of 1500 Å^2 surface area, in contrast with the smaller peptide–protein contacts in receptor–TRAF2 interactions. There are small local conformational adjustments in the Cα positions of TRAF2 (0.5–1.0 Å) within or immediately adjacent to the TRADD binding site, while no significant local conformational changes have been observed in the receptor-bound TRAF2 structures. In both cases, overall structural superpositions between the bound and several free TRAF domain structures of TRAF2 gave rise to main chain deviations similar with observed variations among different crystal forms of TRAF2. Receptor peptides appear to undergo significant conformational changes upon binding to TRAF2 (Ye and Wu, 2000). We suspect that TRADD-N (especially the EF loop) may also undergo a certain degree of conformational rearrangement upon TRAF2 binding based on the relative instability of TRADD-N in its isolated state.

Biosensor Measurements Reveal the Significantly Higher Affinity of the TRADD–TRAF2 Interaction, Contributed Mostly by Hydrophobic Contacts

To characterize the affinity and the energetic determinants of the TRADD–TRAF2 interaction, surface plasmon resonance biosensor analyses were used to assess

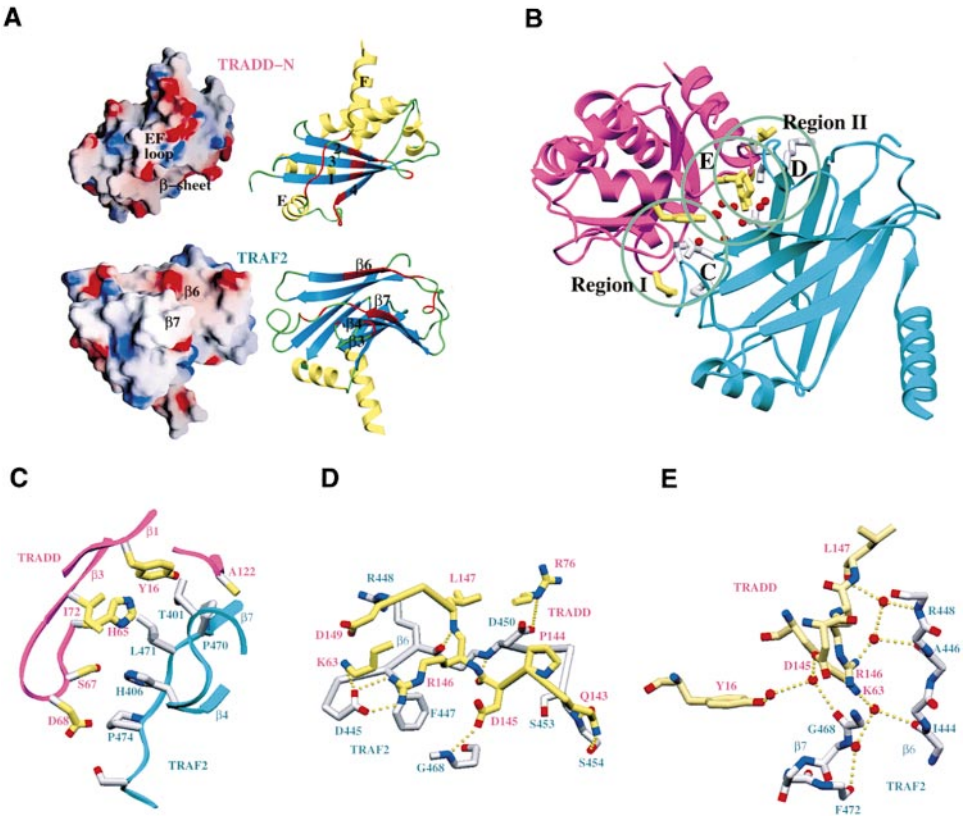
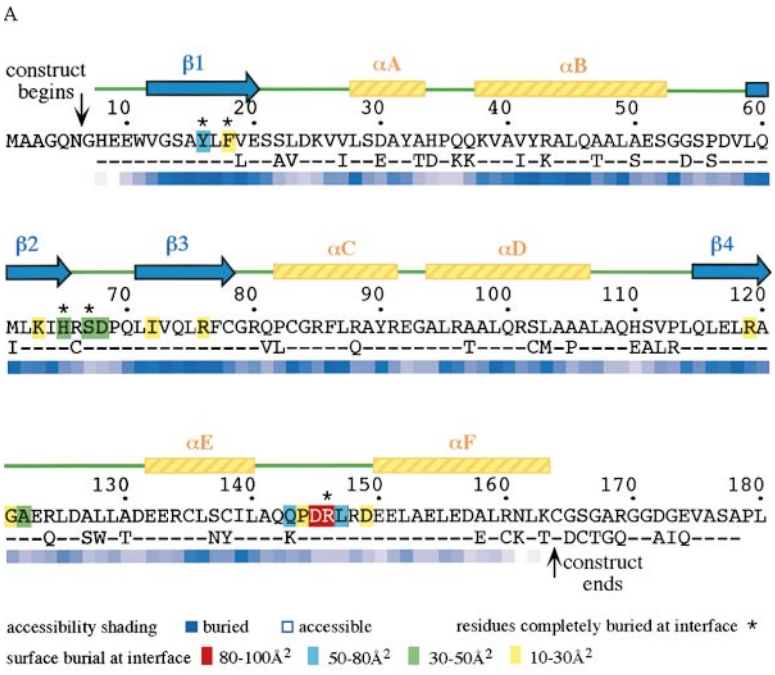


Figure 2. Structural Details of the TRADD-N/TRAF2 Complex

(A) Bipartite interacting surfaces formed by neighboring regions of the proteins. The TRADD-N structure is shown in the same orientation as the magenta-colored TRADD-N, while the TRAF domain of TRAF2 is rotated 180° along the vertical axis relative to the cyan protomer in Figure 1B. The surface representations are colored based on their surface electrostatic potentials. The ribbon drawings have the color coding of red for residues involved in the interaction. (B), (C), and (E) are rotated approximately 90° along the vertical axis relative to (A), and (D) has an orientation similar to (A). Ribbons of TRADD-N are shown in magenta and the TRAF domain of TRAF2 in cyan. For stick models, either the entire side chains or the carbon atoms are shown in yellow for TRADD-N and gray for the TRAF domain of TRAF2. Standard color coding applies to other atoms. Water molecules are represented by red balls. Labels for TRADD-N are in magenta and those for the TRAF domain of TRAF2 in cyan. (B) A ribbon drawing of the complex. Selected side chains and water molecules at the interface are shown. Approximate locations of the enlargements in (C), (D) and (E) are circled and labeled. (C) Details of the lower part of the interface (region I), showing the hydrophobic interactions. The interface is represented by a ribbon drawing, showing side chains involved in the interaction. (D) Details of the upper part of the interface (region II), showing the polar interactions. The interface is represented by a stick model. Part of the main chain that is not involved in the polar interaction is simply shown by the Cα trace. Potential hydrogen bonds and salt bridges are represented by yellow dotted lines. Residues 145–147 of TRADD form antiparallel main chain hydrogen bonds with residues 448–450 of TRAF2 in the connection between β6 and β7. Many side chain hydrogen bonds and salt bridges exit at this interface including the bidentate hydrogen bonds between the guanidinium group of R146 in TRADD and the carboxylate of D445 in TRAF2. (E) Details of water-mediated interactions near the center of the interface. Most of these interactions are between the polar and charged side chain atoms of TRADD and the polar main chain atoms of TRAF2 at the edge of the β sandwich. In addition to bridging the TRADD-TRAF2 interface, the water molecules may also provide hydration to the polar and charged atoms in the vicinity.

the native interaction and the effects of TRADD-N mutations (Figure 3). To avoid potential avidity effects, the trimeric TRAF domain of TRAF2 was coupled to the biosensor chip to measure the binding of monomeric TRADD-N. The native interaction gave rise to a dissociation constant of 7.8 ± 3.6 μM with an on-rate of 1.65 ± 0.7 × 10^5 $M^{-1}s^{-1}$ and an off-rate of 1.3 ± 0.2 s^{-1}. This interaction is significantly stronger than direct receptor–TRAF2 interactions (K_D = 40 μM–1.0 mM) (Ye and Wu, 2000). However, it is still rather weak compared to other protein–protein interactions (Kuriyan and Cowburn, 1997), which is indicative of the importance of multimeric interaction in this TRAF2 recruitment and in the assembly of the TNFR1 signaling complex. The importance of avidity is further supported by the significant affinity enhancement in the binding of trimeric TRAF2 to Biosensor chips coupled with increasing densities of TRADD-N (data not shown).

Residues in regions I and II showed differential effects on the binding affinity, in a manner unrelated to their surface area burial at the interface (Figure 3). In general, alanine substitutions of residues in region I (Y16, F18, H65, and S67) had much more drastic effects, despite their smaller surface area contribution. This may be explained by their complete solvent inaccessibility in the complex and the largely hydrophobic nature of the contact. Residues in region II were selected for their large surface area burial and their abilities to form direct and



B

	K_D (μM)	K_D (relative)	ΔΔG (kcal/mol)
wTRADD	**7.8±3.6**	**1**	
Y16A	**250±30**	**32**	**2.0**
Y16A,F18A	**>5000**	**>641**	**>3.8**
H65A	**71±1**	**9.1**	**1.3**
S67A	**720±130**	**92**	**2.6**
Q143A	10±1	1.3	0.2
D145K	17±1	2.2	0.5
R146A	**130±5**	**17**	**1.6**
L147A	25±1	3.2	0.7

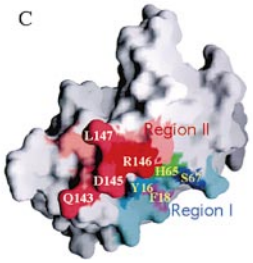


Figure 3. Energetics of the Interaction

(A) Mapping of structural features onto the TRADD-N sequence. The first line is human TRADD and the second line is mouse TRADD with identical residues shown as dashes. Solvent accessibility of each TRADD-N residue when the TRAF2 structure is pulled away is shaded below the sequences. Residues involved in TRAF2 interaction are colored based on their total surface area burials and those completely buried at the interface are labeled by asterisks. TRADD residues involved in TRAF2 interaction are almost entirely identical between the human and mouse sequences, suggesting a functional conservation among the mammalian species.

(B) Characterization of the interactions of wild-type and mutant TRADD-N with wild-type TRAF domain of TRAF2 using biosensor analysis. The dissociation constants (K_D) in μM and relative to the wild-type interaction are shown, as well as the calculated ΔΔG of the mutational effects (25°C).

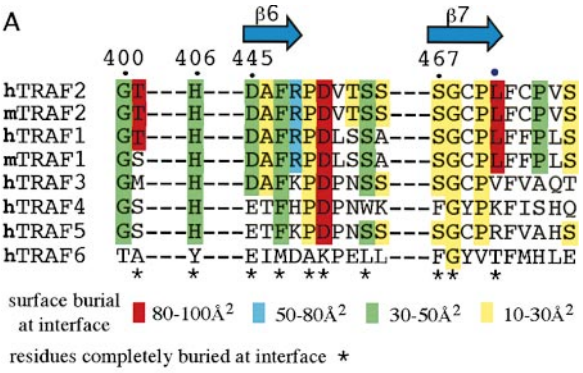
(C) TRAF2-interacting surface of TRADD-N. Region I is shown in cold colors (light blue, blue, dark blue, green, and purple) and region II in different shades of red. Colors for each mutated residue follow the same text color in (B).

water-mediated hydrogen bonds or salt bridges at the interface (Q143, D145, R146, and L147). Rather unexpectedly, mutations on Q143, D145, and L147 had minimal effects on the binding energy (≤3-fold). The only residue that exhibits a large effect is R146, which forms buried bidentate hydrogen bonds and a salt bridge to residue D445 of TRAF2. For most of the mutations in both regions I and II, the direct removal of a particular interaction rather than local conformational perturbation accounts for a majority of the effects. This is supported by the additive nature of the ΔΔG of TRADD-N mutants and the ΔΔG of TRAF2 mutants, as compared with the measured ΔΔG between mutant TRADD-N and mutant TRAF2 proteins (data not shown). Therefore, it appears that region I is the primary energetic determinant of the interaction, while region II contributes less, perhaps due to the plasticity afforded by water-mediated interactions. The electrostatic nature of region II, on the other hand, may provide long-range attraction forces to facilitate the interaction.

Swapping Mutagenesis Shows the Specificity of TRADD for TRAF1 and TRAF2: Implication for the Recruitment of cIAPs to the Signaling Complex

To fully elucidate the functional consequence of the TRADD–TRAF2 interaction, it is important to establish whether other TRAF family members may also interact with TRADD. Existing experimental data have produced conflicting results regarding this specificity (Hsu et al., 1996b; Shu et al., 1996). The availability of the crystal structure allowed us to resolve this issue by careful examination of the sequence conservation among different TRAF proteins at the TRADD-N binding site followed by structure-based mutagenesis. These "switching" mutations from residues in TRAF2 to corresponding residues in other TRAF family members (Figure 4) were then assessed by biosensor measurements.

The severe effects of TRAF2 mutations T401M, L471K, and L471R (Figures 4B and 4C) established that TRAF3, TRAF4, and TRAF5 have much decreased ability to interact with TRADD. Both T401 and L471 constitute part of the region I of the interaction and are completely buried at this mostly hydrophobic interface (Figure 4A). Therefore, the bulkier size of M401 and the charge of K/R471 could create unfavorable contacts in the interaction. In TRAF6, almost all TRADD-interacting residues are substituted, including residues T401 and L471. In addition, mutations D450K and S467F also had unfavorable effects on the interaction. The predicted additive effects of this double mutation alone could lead to a ΔΔG of 2 kcal/mol, corresponding to a 30-fold decrease in affinity. The only TRAF family member that appears to retain the



B

	K_D (μM)	K_D (relative)	ΔΔG (kcal/mol)
wTRAF2	7.8±3.6		
S454A TRAF1	4.6±0.1	0.6	-0.3
T401M TRAF3	140±20	18	1.7
L471K TRAF4	U. D.	U. D.	U. D.
L471R TRAF5	U. D.	U. D.	U. D.
D450K TRAF6	45±1	5.8	1.0
S467F TRAF4/6	42±1	5.4	1.0

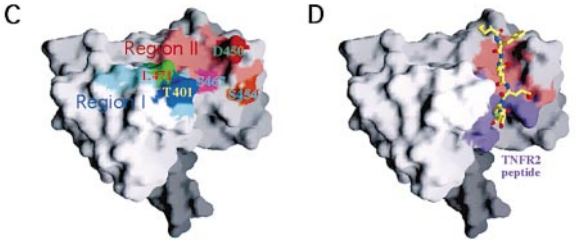


Figure 4. Specificity of the Interaction

(A) Sequence alignment of different human and mouse TRAF family members at the TRADD binding site. TRAF2 residues involved in TRADD interaction are shaded as in Figure 3A. Identical residues in other TRAFs are also colored with the same scheme. Residues completely buried at the interface are labeled by asterisks.

(B) Characterization of the interactions of wild-type and mutant TRAF2 with wild-type TRADD-N using biosensor analysis. The dissociation constants (K_D) in μM and relative to the wild-type interaction are shown, as well as the calculated ΔΔG of the mutational effects (25°C). U. D., undetectable.

(C) TRADD-interacting surface of TRAF2. Region I is shown in cold colors (light blue, dark blue and green) and region II in warm colors (light red, dark red, magenta, and dark orange). Colors for each mutated residue follow the same text color in (B).

(D) TRAF2 in complex with a TNFR2 peptide bearing the sequence of QVPFSKEEC (Park et al., 1999). The TNFR2 peptide is shown in a stick model with carbon atoms in yellow, nitrogen atoms in blue, oxygen atoms in red, and sulfur atoms in green. TNFR2-interacting surface of TRAF2 is colored in light red for the region in overlap with the TRADD-interacting surface and purple for the remaining region.

ability to interact with TRADD is TRAF1, which contains almost entirely identical residues at the TRADD binding site. The only nonconserved substitution S454A produced little change in the affinity (Figure 4B), even though the side chain of S454 appears to mediate a hydrogen-bonding interaction with Q143 of TRADD. Consistently, Q143A mutation on TRADD also had minimal effect on the binding (Figure 3B), perhaps due to the plasticity of region II of the interface. The avidity contribution during assembly of the signaling complex should further enhance the specificity of TRADD for TRAF1 and TRAF2, as compared with the affinity differences shown in the monovalent interactions.

The specificity of TRADD for TRAF1 and TRAF2 ensures the recruitment of cIAPs to the signaling complex, which may be important for direct caspase-8 inhibition (Wang et al., 1998) and the immediate suppression of apoptosis at the apical point of the cascade (Figure 1D). The cellular caspase inhibitors cIAP1 and cIAP2 were originally isolated from the TNFR2 signaling complex and shown to interact constitutively with TRAF1 and TRAF2 (Rothe et al., 1995). This interaction is rather unique as other inhibitors of apoptosis proteins (IAPs) do not interact with any TRAF proteins and other TRAF proteins do not interact with cIAPs (Roy et al., 1997). In addition, this interaction with cIAPs requires both TRAF1 and TRAF2 (Rothe et al., 1995). Therefore, like TRADD, the cIAPs have the same specificity for TRAF1 and TRAF2. This is in contrast with the direct TRAF recruitment by the subgroup of TNF receptors that do not contain a death domain, which exhibits a more promiscuous specificity for TRAF1, 2, 3, and 5 (Park et al., 1999; Ye et al., 1999). Interestingly, TRAF1 does not contain an amino-terminal effector domain for survival signaling via transcriptional gene activation, while other TRAF proteins such as TRAF5 and TRAF6 do. Therefore, the inclusion of TRAF1 in the TNFR1 signaling complex further implicates the importance of cIAPs for the intrinsic antiapoptotic function of TNFR1.

The Higher Affinity of the TRADD-TRAF2 Interaction versus the Receptor-TRAF2 Interactions Leads to More Effective TRADD-Mediated TRAF2 Signaling

The higher affinity of the TRADD-TRAF2 interaction (K_D = 7.8 μM), as compared with the interactions between TRAF2 and receptor peptides (K_D = 40 μM–1.0 mM) (Ye and Wu, 2000), suggests that TRADD might be a stronger inducer of TRAF2 signaling. To test this hypothesis, we compared TRAF2 signaling from TNFR1, a TRADD-mediated signaling, and from TNFR2 (K_D = 0.5 mM for the TNFR2-TRAF2 interaction), a direct receptor-mediated signaling, since both TNFR1 and TNFR2 bind with high affinity to soluble TNFα.

As many cell types coexpress TNFR1 and TNFR2, we isolated embryonic fibroblasts from mice deficient in TNFR1 (Pfeffer et al., 1993) or in TNFR2 (Erickson et al., 1994), in order to obtain cells that contain only one of the TNF receptors. The number of TNF receptors on each type of fibroblast was first determined for the normalization of TRAF2 signaling. The binding of each type of fibroblasts to ^{125}I-labeled TNFα in the absence and presence of excess cold TNFα was measured. Scatchard analyses of the binding data showed that the TNFR1-deficient fibroblasts contain approximately 3800 TNFR2 per cell with a dissociation constant of 0.09 nM and that the TNFR2-deficient fibroblasts contain ~9600 TNFR1 per cell with a dissociation constant of 0.07 nM.

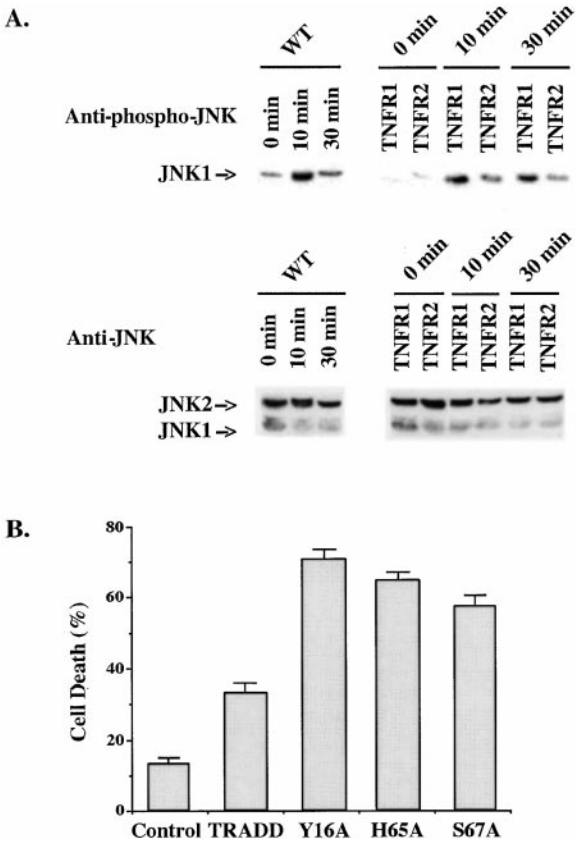


Figure 5. Functional Analysis of the Interaction between TRADD and TRAF2

(A) Comparison of JNK signaling in fibroblasts containing either TNFR1 or TNFR2 only. Embryonic fibroblasts unstimulated (0 min) or stimulated (10 and 30 min) with TNFα were lysed, loaded based on normalized receptor numbers, and probed with antibodies against the doubly phosphorylated JNK (upper panel) and total JNK (lower panel). JNK was activated upon TNFα treatment and down-regulation of JNK was observed.

(B) TRADD-mediated apoptosis assay. Percentages of apoptotic cells and the standard deviations for the control vector (CDM8), the wild-type TRADD, and TRADD mutants H65A, Y16A, and S67A are shown.

As TRAF2-deficient mice and transgenic mice expressing a dominant-negative form of TRAF2 have shown that TRAF2 is the major activator of JNK by TNFα (Lee et al., 1997; Yeh et al., 1997), we used JNK activation as the readout for TRAF2-mediated signal transduction. The primary embryonic fibroblasts were treated with mouse TNFα and the levels of doubly phosphorylated JNK were detected at 10 and 30 min poststimulation using specific anti-phospho-JNK antibodies. The TNFR1-containing fibroblasts had significantly higher levels of JNK activation than the TNFR2-containing fibroblasts (Figure 5A), substantiating TRADD as the more effective initiator of TRAF2 signaling. The higher level of TRAF2 signaling by TNFR1 also indicates an effective interaction between the death domain of TRADD and TNFR1. Interestingly, it has been shown that in the absence of receptor activation, the death domain of TNFR1 is bound to a silencer protein to suppress its tendency to signal (Jiang et al., 1999).

The High Affinity of the TRADD–TRAF2 Interaction Is Required for Efficient Suppression of Apoptosis

TNFR1 and related death receptors constitute one of the most intriguing intracellular signaling pathways that are capable of both cell survival promotion and cell death induction. Under most circumstances, however, TNFα treatment rarely induces cell death and the survival pathway through TRAF2 and RIP appears to dominate the signaling process. TRAF2 signaling plays an important role in protection of apoptosis as TRAF2-deficient mice are overly sensitive to TNFα-induced cell death (Yeh et al., 1997). The much higher affinity of TRAF2 recruitment by TRADD versus the direct TRAF2 recruitment by TNF receptors without a death domain, suggests the importance of preserving this high affinity in the signal transduction by TNFR1 and related receptors. Using TRADD mutants with various degrees of affinity decrease to TRAF2, we analyzed the effects of these mutations in providing apoptosis protection.

We selected three single-site mutations of TRADD, H65A, Y16A, and S67A with decreasing affinities to TRAF2 (Figure 3B), and tested their apoptosis-inducing effects in a transfection assay. It has been shown that TRADD transfection or overexpression could induce significant cell death in TNFα-sensitive cell lines such as L929 (Liou and Liou, 1999). If the interaction between TRADD and TRAF2 is sensitive to affinity modulation, TRADD mutants with decreased TRAF2 recruiting capability would be expected to exert more potent cell death effects. The experimental results showed that indeed the TRADD mutants were highly potent in apoptosis induction, as compared with the wild-type TRADD (Figure 5B). Only a small percentage of transfected cells were viable two days posttransfection. The H65A mutant exhibits a mere 9-fold decrease in TRAF2 binding and falls into the same range of affinities to the interactions of TRAF2 with linear receptor motifs. This mutant was surprisingly as potent in cell killing as the most severe S67A mutant. These data suggest that the in vivo interaction between TRADD and TRAF2 is extremely sensitive to affinity changes. A modest decrease in the monomeric affinity, which may translate into a larger difference in the multimeric interaction, could lead to an imbalance in the regulation between cell survival and cell death.

There may be two ways that TRAF2 can protect the cells from apoptosis and the cooperation of the two mechanisms may be necessary for efficient apoptosis suppression. The first mechanism has to do with recruitment of the cellular caspase inhibitors, cIAP1 and cIAP2, to the TNFR1 signaling complex by TRAF1 and TRAF2 to inhibit caspase-8 activation (Rothe et al., 1995; Wang et al., 1998). This mechanism acts at the entry point of TNFα-mediated apoptosis, is independent of gene transcription, and explains the specificity of TRADD for TRAF1 and TRAF2 but not other members of the TRAF family. The second mechanism may be related to the ability of TRAF2 to activate JNK and NF-κB, both of which may induce the expression of antiapoptotic genes to suppress cell death (Beg and Baltimore, 1996; Minden and Karin, 1997). Since apoptosis induction can be fast and does not require gene transcription and protein

Table 1. Structure Determination

MAD Phasing (30–2.1 Å)

Cell dimensions: a = b = 131.5 Å, c = 62.8 Å (R3)

					Diffraction Ratio			
	Wavelength	Energy	R_{merge}	Completeness	λ_1	λ_2	λ_3	λ_4
λ_1	0.992 Å	12500ev	5.6%	80.0%	4.0%	4.4%	4.5%	6.3%
λ_2	0.979 Å	12660ev	5.6%	78.4%		5.0%	4.1%	7.1%
λ_3	0.979 Å	12664ev	5.5%	81.7%			6.5%	5.2%
λ_4	0.968 Å	12820ev	6.0%	85.4%				5.0%
	MADSYS statistics		$\Delta(\Delta\phi) = 46.8°$		$R_{FA} = 41.8\%$			

Refinement (20–2.0 Å)

Cell dimensions: a = b = 132.4 Å, c = 62.8 Å (R3)

Wavelength	R_{merge}	Completeness	Atoms	H_2O	R (R_{free})	Rms Bond	Rms Angle
0.992 Å	3.6%	96.9%	2542	209	22.9 (26.1%)	0.008 Å	1.7°

synthesis, the first mechanism is likely to be crucial in placing apoptosis under check while the second mechanism may further strengthen the antiapoptotic function of TRAF2.

The Affinity Differences of TRAF2 Recruitment Reveal the Different Avidity Requirements for Efficient Signaling

It has been indicated that the entire TNF receptor superfamily assembles their intracellular signaling complex in response to ligand-induced receptor trimerization (Banner et al., 1993) or higher order oligomerization. In particular, the ligand dependence of TRADD recruitment to TNFR1 and the sequential binding of TRAF2 following TRADD recruitment have been demonstrated experimentally (Hsu et al., 1996b; Shu et al., 1996). Therefore, the avidity provided by different degrees of multimeric interactions, as well as the intrinsic affinity between the signaling proteins, drive the signal transduction cascade of this family of receptors.

The consideration on the combined effects of affinity and avidity explains why TNFR2 is rather nonresponsive to soluble TNFα but mostly responds to cell-bound TNFα (Grell et al., 1995), while TNFR1 can be stimulated efficiently with soluble TNFα. TNFα is synthesized as a transmembrane ligand and efficiently converted to soluble TNFα under most but not all circumstances. The affinity of the TNFR2–TRAF2 interaction (K_D = 0.5 mM) is remarkably lower than the TRADD–TRAF2 interaction. The cell-bound TNFα may be capable of creating higher order of receptor clustering than soluble TNFα and may therefore provide enhanced avidity to TRAF2 recruitment by TNFR2 to potentiate this signal transduction. TNFR2 signaling can also be induced by the upregulation of expression, which again increases the avidity contribution of TRAF2 recruitment.

The generally weaker affinity between TRAF2 and many members of the TNF receptor superfamily, such as CD40, CD30, Ox40, and 4-1BB (K_D = 40 μM–1.0 mM), implies that a higher order of aggregation may be required for the optimal signal transduction of these receptors. In keeping with this observation, the corresponding ligands for these receptors are membrane bound, which could induce more aggregation and provide higher avidity for the receptor–TRAF2 interactions. In these cases, soluble ligands are often inefficient in eliciting signal transduction and may even act as decoys to downregulate the signal (Kehry and Castle, 1994; Hodgkin et al., 1997).

The Competitive Recruitment of TRAF1, TRAF2, and the Associated cIAPs in Sensitizing TNFR1-Induced Cell Death

Although TNFR2 does not directly couple to apoptosis, mice deficient in TNFR2 showed decreased cell death following TNFα treatment (Erickson et al., 1994), while overexpression of TNFR2 could lead to increased sensitivity to TNFα-induced apoptosis (Heller et al., 1992; Vandenabeele et al., 1995; Weiss et al., 1997; Haridas et al., 1998; Chan and Lenardo, 2000). Moreover, TNFα-induced apoptosis of activated primary T lymphocytes has also been shown to require TNFR2 (Sarin et al., 1995; Zheng et al., 1995). Functionally, this may be important for Fas-independent peripheral deletion of T lymphocytes and the regulation of mature T cell homeostasis.

We propose that the observed cooperation of TNFR2 in TNFR1-mediated apoptosis could be explained by a decreased recruitment of TRAF1, TRAF2, and cIAPs in the TNFR1 signaling complex. Overexpression or upregulation of expression of TNFR2 upon T cell activation could lead to an increased association of TRAF1 and TRAF2 with TNFR2, sequestering intracellular TRAF1, TRAF2, and the constitutively associated cIAPs away from the signaling complex of TNFR1 and related receptors. In keeping with this proposal, the TRAF2 binding region of TNFR2 is required for this potentiation of TNFR1-mediated apoptosis (Weiss et al., 1998).

This interplay between TNFR2 and TNFR1 may be extended to other members of the TNF receptor superfamily such as CD40, CD30, LT-βR and CD27. These receptors have been shown to induce cell death under certain circumstances (Grell et al., 1999). Similar to TNFR2, activation of any of these receptors could lead to a sequestration, and possibly degradation (Duckett and Thompson, 1997), of TRAF1, TRAF2, and cIAPs. As these receptors have been shown to induce the expression of TNFα (Grell et al., 1999), the thus activated TNFR1 would be tipped to apoptosis induction under the lack of recruitment of TRAF1, TRAF2, and cIAPs.

Experimental Procedures

Complex Formation and Crystallization

Protein preparation for the TRAF domain of human TRAF2 (residues 327–501) was performed as described earlier (Park et al., 1999). Bacterial expression of the TRAF2 binding domain of TRADD (residues 1–169), previously identified from deletion analysis (Hsu et al., 1996b), yielded protein that was severely prone to aggregation and did not associate appreciably with purified TRAF domain of TRAF2 in vitro. In contrast, an alternative construct (TRADD-N, residues 7–163), designed based on amino acid sequence analysis (Rost and Sander, 1994), gave rise to a monomeric protein under overnight induction at 20°C. The protein contained a carboxy-terminal polyhistidine tag and was purified by nickel affinity chromatography and gel filtration. For complex formation, slightly excess TRADD-N was incubated with the TRAF domain of TRAF2 at 37°C for 30 min. The resulting mixture was further purified by gel filtration. The complex exhibited much improved stability over isolated TRADD-N.

The complex between TRADD-N and the TRAF domain of TRAF2 was crystallized by vapor diffusion using 11% PEG4K and 4% isopropanol at pH 5.6. Although crystals may be obtained with PEG4K alone, the addition of isopropanol dramatically improved the diffraction limit from 3.0 Å to 2.4 Å using a rotating anode X-ray source. There are seven methionine residues in the TRAF domain of TRAF2 and only one in TRADD-N. Therefore, the TRAF domain of TRAF2 was produced in the selenomethionyl form (Hendrickson et al., 1990) for MAD phasing using selenium-containing crystals. These crystals diffracted to 2.0 Å spacing under synchrotron radiation and belong to space group R3 with one complex per crystallographic asymmetric unit. The molecular 3-fold axis of the complex coincides with the crystallographic 3-fold axis.

Structure Determination

Crystal structure of the complex was phased by MAD analysis and partial model phase combination (Table 1). The MAD data set was collected at the HHMI X4A beamline of NSLS using a Quantum 4 CCD detector. Four wavelengths were chosen for the experiment: 12500ev (0.992 Å, λ1, low energy remote), 12660ev (0.979 Å, λ2, edge), 12664ev (0.979 Å, λ3, peak), and 12820ev (0.968 Å, λ4, high energy remote). The data set was somewhat incomplete due to crystal decay. A complete data set at 2.0 Å resolution from another selenomethionyl crystal was collected at 12500ev, which was used for phase and model refinement. Data processing was carried out in the HKL package (Otwinowski, 1990). The scaled reflections were left unmerged for subsequent MAD phasing.

Phase calculations were performed at a resolution of 2.1 Å using the program MADSYS (Hendrickson, 1991). The complete data set at 2.0 Å resolution is sufficiently isomorphous with the MAD data set to allow the direct transfer of the ABCD phase coefficients from the MAD analysis. The TRAF domain of TRAF2 structure in the crystal was located by molecular replacement using the REPLACE package (Tong, 1993). Phases calculated from this partial model were combined with the experimental phases from the MAD analysis. The combined phases were then refined and extended by solvent flattening and histogram matching using the DM program in the CCP4 suite.

The initial map showed recognizable secondary structures but with poor connectivity and side chain densities. Iterative model building (Jones et al., 1991) and refinement (Brunger et al., 1998) were necessary for sequence assignment and complete tracing of the TRADD polypeptide chain (residues 8–163). The main chain atoms of two additional residues from the His-tag (residues LEHHHHHH) were also visible and built. Ribbon diagrams and stick models were generated using the program Setor (Evans, 1993) and molecular surface representations were produced with the program GRASP (Nicholls et al., 1991).

Surface Plasmon Resonance Biosensor Analysis

The interaction of TRADD-N with the TRAF domain of TRAF2 was analyzed by surface plasmon resonance using a BIACORE 2000 optical biosensor (Biacore AB, Uppsala, Sweden). The TRAF domain of TRAF2 was immobilized onto a B1 biosensor chip using amine coupling chemistry described previously (Johnsson et al., 1991). For the wild-type interaction, the entire biosensor experiment was repeated four times using immobilized TRAF2 levels of 300, 500, 800, and 1000 RU. For mutant interactions, each TRAF2 variant was immobilized onto a separate biosensor surface at high response levels (1200–1300 RU). A separate reference flow cell was activated and blocked to correct for refractive index changes. The experiments were performed at 25°C using a flow rate of 100 μl/min. TRADD-N was injected over the sample and reference flow cells at concentrations of 60, 20, 6.6, 2.2, 0.74, and 0 μM. The running buffer contained 10 mM HEPES at pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20. Each concentration of TRADD-N was injected three times in random order. No regeneration condition was required as the bound TRADD-N dissociated back to baseline in a few seconds.

Binding data were prepared for kinetic analysis by subtracting responses from the reference surface and average blank injections (Myszka, 1999). The association and dissociation phase data were fitted simultaneously to a single bimolecular interaction model ($A + B = AB$) using program CLAMP (Morton and Myszka, 1998; Myszka and Morton, 1998). The standard deviations of the measurements were determined from averaging four independently fitted parameters for the wild-type interaction and from statistical errors of a single global fitting for the mutant proteins.

Scatchard Analysis for the Binding of TNFα to Embryonic Fibroblasts

Recombinant mouse TNFα (Pepro Tech) was labeled by ^{125}I to a specific activity of 1.7×10^8 cpm/nmol. Embryonic fibroblasts were isolated from mice deficient in TNFR1 or TNFR2 (Jackson Laboratories) and maintained in DMEM supplemented with 15% fetal bovine serum. Binding of ^{125}I-TNFα to TNFR1- or TNFR2-containing fibroblasts (0.25×10^6 and 0.36×10^6 cells, respectively) was carried out at 4°C in 6-well culture dishes at concentrations of 0.075 nM, 0.15 nM, 0.3 nM, 0.6 nM, and 1.2 nM in PBSA, both in the absence and presence of 20-fold excess unlabeled TNFα. The binding mixtures were incubated for 2 hr with gentle rocking. The unbound TNFα was then washed off and the cells were dissolved in 1 M NaOH for counting. The binding data were then subjected to standard Scatchard analyses to determine receptor numbers and binding affinities.

JNK Signaling Assay for Mice Deficient in TNFR1 or TNFR2

For JNK signaling assay, cells were plated at $3–5 \times 10^5$/well of a six-well dish the day before the treatment with saturating concentrations of recombinant mouse TNF. Cells in each well were lysed at times 0, 10, and 30 min in lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM PMSF. Whole cell lysates corresponding to the same total receptor numbers were loaded onto 10% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore). Blots were probed with anti-JNK and anti-phospho-JNK polyclonal IgG (New England Biolabs) and HRP-conjugated secondary antibody. Detection was carried out using ECL plus chemiluminescence substrate (Amersham Pharmacia).

TRADD-Mediated Apoptosis Assay

Full-length wild-type TRADD was subcloned into the pCDNA3 vector and single-site mutagenesis was performed and confirmed by double-stranded DNA sequencing. The L929 cell line was maintained in 10% bovine serum in DMEM and 1.5×10^5 cells/well were seeded on 6-well dishes the day before transfection. Cells were cotransfected with 4 μg of CMV-LacZ plasmid and 6 μg of either CDM8 (DNA control), wild-type TRADD, or mutant TRADD for 48 hr. Methods for transfection, fixing, and X-Gal staining were performed as described previously (Liou and Liou, 1999). The number of blue cells (both shrunken dense-nucleated apoptotic cells and spindle-shaped healthy cells) were determined microscopically from eight zones on each dish, from which the average and the standard deviation of the percentage cell death were obtained.

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Protein Data Bank ID Code

The atomic coordinates reported in this paper have been deposited in the Protein Data Bank under ID code 1F3V.