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Structural basis for lack of E2 interaction in the RING of TRAF2

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Abstract

TRAF proteins are intracellular signal transducers for a number of immune receptor superfamilies. Specifically, TRAF2 interacts with members of the TNF receptor superfamily and connects the receptors to downstream signaling proteins. It has been assumed that TRAF2 is a ubiquitin ligase like TRAF6 and mediates K63-linked polyubiquitination of RIP1, a kinase pivotal in TNF α -induced NF- κ B activation. Here we report the crystal structure of the RING and the first zinc finger domains of TRAF2. We show that the TRAF2 RING structure is very different from the known TRAF6 RING structure. The differences are multifaceted, including amino acid differences at the critical Ubc13-interacting site, local conformational differences, and a unique 9-residue insertion between the RING and the first zinc finger in TRAF2. These structural differences prevent TRAF2 from interacting with Ubc13 and other related E2s via steric clash and unfavorable interfaces. Our structural observation should prompt a reevaluation of the role of TRAF2 in TNF α signaling and may indicate that TRAF2-associated proteins such as cIAPs may be the ubiquitin ligases for NF- κ B signaling.

TRAF2 is a RING-containing protein that was first identified from biochemical purification as a TNFR2-associated signaling protein (1). It interacts with many receptors in the TNF receptor superfamily and mediates the survival effects of these receptors (2,3). The TRAF family now consists of 7 mammalian members and is shown to participate in signal transduction of a large number of receptor families that also include the IL-1 receptors (IL-1R), the Toll-like receptors (TLR), T-cell receptors (TCR) and B-cell receptors (BCR) (4,5). Upon receptor activation, TRAFs are directly or indirectly recruited to the intracellular domains of these receptors. They subsequently engage other signaling proteins to activate the inhibitor of κ B (I κ B) kinase (IKK) and MAP kinases, leading ultimately to activation of transcription factors such as NF- κ B and AP-1 to induce immune and inflammatory responses and confer protection from apoptosis.

Like most TRAFs, TRAF2 contains an N-terminal domain with RING and four zinc fingers and a C-terminal TRAF domain that comprises a coiled coil domain and a conserved TRAF-C domain (1) (Figure 1A). Previous biochemical and structural studies have revealed that the TRAF domain forms a mushroom-shaped trimeric structure with the TRAF-C domain as the head for interaction with receptors and adaptor proteins and the coiled coil domain as the stalk for trimerization (6–10). The receptor-binding groove of TRAF2 shows sequence and structural similarity with those of TRAF1, 3 and 5, but is dissimilar to that of TRAF6.

TRAF proteins were originally thought as adapter proteins that connect activated receptors to downstream kinases for signal amplification. However, it was then shown that TRAFs might be RING-type ubiquitin ligases. This has been shown for TRAF6, which catalyzes K63-linked polyubiquitination both in vitro and in cells (11). Unlike K48-linked polyubiquitin chains that

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are hallmarks for proteasomal degradation, the K63 linkage is non-degradative and has been discovered to function as a signaling moiety in DNA damage repair processes and innate immunity pathways (12,13). Ubiquitination is accomplished in three steps, ATP-dependent attachment of ubiquitin to a ubiquitin activating enzyme (E1), transfer of ubiquitin from E1 to a ubiquitin conjugating enzyme (E2), and transfer of ubiquitin from E2 to Lys residues of substrates with the aid of a ubiquitin ligase (E3) (12,14,15).

Upon activation by the relevant signaling pathways after ligand stimulation, TRAF6 promotes K63-linked polyubiquitination of itself and downstream signaling proteins, a process that requires the heterodimeric E2 of Ubc13 and the ubiquitin E2 variant (Uev) known as Uev1A (12). The K63-linked polyubiquitin chains function as anchors to recruit the TAK1 kinase complex and IKK to activate both the MAP kinase pathway and the NF- κ B pathway (16,17). TAK1 directly phosphorylates MAP kinases while IKK-mediated phosphorylation of I κ B leads to its degradation to free NF- κ B for transcription.

It has been implicated that TRAF2 may act similarly to TRAF6 in stimulating K63-linked polyubiquitination in the TNF α and other related pathways. An assumed concept is that TRAF2 mediates K63-linked ubiquitination of RIP1, a kinase pivotal in TNF α -induced NF- κ B activation (18). Here we show, however, that the TRAF2 RING structure is very different from the TRAF6 RING structure we determined previously (19). The differences are due to multiple changes between TRAF2 and TRAF6, including amino acid differences at the critical Ubc13-interacting site, conformational differences and a unique 9-residue insertion between the RING and the first zinc finger in TRAF2. These structural differences prevent TRAF2 from interacting with Ubc13 and other related E2s. The structural observation should prompt a re-evaluation of the role of TRAF2 in TNF α signaling and may indicate that TRAF2-associated proteins such as cIAPs may be the ubiquitin ligases for NF- κ B signaling.

EXPERIMENTAL PROCEDURES

Protein Expression, Purification and Mutagenesis

The human TRAF2 construct RZ₁ (residues 1–133) was constructed with a C-terminal polyhistidine tag by cloning into the pET26b vector (Novagen) using PCR methods. The protein was expressed in BL21-CodonPlus®(DE3) cells and purified by Ni-affinity chromatography (Qiagen) followed by gel filtration chromatography (Superdex 200, GE Healthcare) in buffer containing 20 mM Tris-HCl at pH 7.5, 150 mM NaCl and 5 mM dithiothreitol (DTT). Mutagenesis was performed with the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) and mutant proteins were expressed and purified similarly.

Crystallization and Structure Determination

Crystallization of TRAF2 RZ₁ was performed using sitting drop vapor diffusion at 20 °C. The crystallization condition is 10% PEG 8000, 0.1M sodium Hepes at pH 7.5, and 8% ethylene glycol and the crystallization drops contain a 1:1 protein: precipitant ratio. The diffraction data were collected at beamline X4A of the Brookhaven National Lab and processed using HKL2000 (20). TRAF2 RZ₁ structure was determined by single wavelength anomalous diffraction (SAD) (21) of the intrinsic zinc atoms using the program SOLVE and RESOLVE (22). The crystals belong to space group P6₅22. Model building was performed in program WinCoot (23). Refinement was done using CNS 1.2 (24). Crystallographic statistics are shown in Table 1. All superpositions were performed with lsqman in the CCP4 suite (25). Structural presentations were generated using Pymol (DeLano Scientific).

Multi-angle light scattering (MALS) analyses

The molar masses of the WT and mutant TRAF2 RZ₁ were determined by MALS. The protein samples were injected into a Superdex 200 (10/300 GL) gel filtration column (GE Healthcare) equilibrated in a buffer containing 20 mM Tris at pH 7.5 and 150 mM NaCl. The chromatography system was coupled to a three-angle light scattering detector (mini-DAWN TRISTAR) and a refractive index detector (Optilab DSP) (Wyatt Technology). Data were collected every 0.5 s with a flow rate of 0.2 ml/min. Data analysis was carried out using ASTRA V.

In vitro ubiquitination assays

In vitro ubiquitination assays with GST-fusion proteins were performed essentially as described (26). TRAF2, TRAF3, TRAF5 and TRAF6 were expressed as GST fusion proteins in E coil and purified using glutathione-agarose beads (Sigma). The ubiquitination assay was performed in 20 μ l reaction volumes with the following components: glutathione-agarose bound GST-TRAFs, 20 mM Hepes at pH 7.2, 10 mM MgCl₂, 1 mM DTT, 59 μ M His-ubiquitin, 50 nM E1, 850 nM E2, 1 mM ATP, 30 μ M creatine phosphate, and 1 unit of creatine kinase. The mixture was incubated at 37 °C for 2 hours with gentle agitation. After incubation, 10 μ l of cold 20 mM Hepes (pH 7.4) was added to the reaction, and samples were centrifuged for 20 min at 4 °C. A volume (20 μ l) of the supernatant was collected. The beads were washed and both the beads and the supernatant were subjected to SDS-PAGE and immunoblotting with a ubiquitin-specific antibody (mouse anti-ubiquitin monoclonal (P4D1), Santa Cruz Biotechnology).

Transfection

TRAF2/TRAF5 double knockout MEFs were infected with empty retrovirus (pMX) or the indicated retrovirus expressing wild-type or mutant FLAG-tagged TRAF2 for 2 days and then selected for 2 weeks with puromycin (2 μ g/ml). Cells were plated in 10 cm dishes and starved overnight in incomplete medium. The cells were treated with TNF α (10 ng/ml) for the indicated times and harvested. Cell lysates were subjected to SDS-PAGE and immunoblotted with anti-FLAG (Sigma) and anti-phospho-I κ B α antibodies (Cell Signaling).

RESULTS

Structure of the RING and the First Zinc Finger (RZ₁) of TRAF2

The structure of TRAF2 RZ₁ (residues 1-133-His) was determined at 1.9 Å resolution using single wavelength anomalous diffraction of the intrinsic zinc atoms (Table 1, Figure 1B, 1C). The structure contains ordered residues from L14 to L133 plus seven residues from the C-terminal His-tag. The monomer structure seems rigid with extensive interactions of ~660 Å² buried surface area between the RING and the first zinc finger domains. The structure contains 6 α helices and 5 β strands. Among these, the RING region is composed of 3 α helices and 3 β strands while the zinc finger region contains 2 α helices and 2 β strands. A linker helix (α 4) connects the two domains.

A DALI structural homology search (27) showed that the RING domain is similar to many known RING structures but is most similar to the RING of TRAF6 (19) with Z-scores in the range of 7.1–7.6. A similar search using the first zinc finger of TRAF2 returned only the corresponding zinc finger of TRAF6 as a structural homologue with marginal Z-score values of 2.6–2.9. To determine whether the relative structural arrangement between the RING and the first zinc finger has been observed before, we performed a structural homology search using both the RING and the first zinc finger structure of TRAF2. In addition to the top hits of TRAF6 with Z-scores of 11.7–13.4, the RAG1 dimerization domain has a Z-score of 11.4 with TRAF2

and is the only known structure with a similar domain arrangement between a RING and a zinc finger. Interestingly, in addition to its role in RAG1 dimerization, the RING-zinc finger structure of RAG1 also acts as a ubiquitin ligase (28).

Residues coordinating the two zinc atoms in the RING domain comprise the canonical RING signature motif, CX₂CX₍₉₋₃₉₎CX₍₁₋₃₎HX₍₂₋₃₎CX₂CX₍₄₋₄₈₎CX₂C (where C is Cys, H is His and X is any amino acid) (29) (Figure 1D, 1E). Similarly, the zinc finger is a classical CCHC type finger (Figure 1D, 1E). Although the current structure contains only the RING and the first zinc finger domains of TRAF2, the zinc finger domains 2, 3 and 4 may be modeled based on the conserved sequence spacing and structural relationship between the zinc fingers in all TRAF proteins (19).

TRAF2 RZ₁ forms a dimer in the crystal and in solution

There is only one monomer of TRAF2 RZ₁ in the crystallographic asymmetry unit. However, it forms a crystallographic dimer that is very similar to the RING dimer of TRAF6 (Figure 2A, 2B). The dimerization interface is formed mostly by residues Q46, F91, N94, A95 and R98, which all bury more than 50 Å² surface area each. In particular, residue F91 buries 80 Å² surface area, the most amount among all the interfacial residues (Figure 2C, 2D).

TRAF2 RZ₁ (MW 15.4 kDa) eluted at 16.6 ml on a Superdex 200 gel filtration column (Figure 2E), a position that is consistent with a dimer of TRAF2 based on the elution positions of gel filtration standards. The Q46A and N94A mutants both eluted at around 16.8 ml, mostly like the wild type TRAF2. The R98A mutant eluted at 17.2 ml, a significant shift from the wild type position. The mutant that gave the most phenotype is F91A, which eluted at 17.4 ml, a position that is consistent with a monomer of TRAF2 RZ₁. Because gel filtration positions are affected by both size and shape and are not accurate indications of molecular mass, we further characterized the mutants using multi-angle light scattering (MALS) in line with gel filtration chromatography. While TRAF2 RZ₁ gave a measured molecular mass of 26.7 kDa (3% error), which is consistent with a dimer, the F91A mutant had a measured molecular mass of 16.1 kDa (5% error), which is consistent with mostly a monomer. The molecular mass measured by MALS for the R98A mutant is 25.1 kDa (3% error), showing that R98A only produced a weak defect in TRAF2 RZ₁ dimerization. These studies confirm that TRAF2 is a dimer both in the crystal and in solution and that this dimerization can be disrupted by a single mutation F91A at the observed interface.

To elucidate whether TRAF2 RING-mediated dimerization is important for its function, we reconstituted TRAF2 and TRAF5 double knockout MEFs with WT and dimerization defective mutants of TRAF2. Surprisingly, WT TRAF2, the F91A mutant, and the double mutant of F91A and R98A, all successfully rescued TNFα-induced IκBα degradation, suggesting that TRAF2 dimerization is not critical for TNF signaling (Figure 2F). This observation is in contrast to the requirement for TRAF6 dimerization in IL-1 signaling (19). In TRAF6, its dimerization is crucial for its ubiquitin ligase activity. In this regard, one might argue that the lack of importance of TRAF2 dimerization is consistent with its apparent lack of E3 activity (see below).

Overall structural differences between TRAF2 and TRAF6

We performed structural superposition with TRAF6 using the RING alone, the zinc finger domain alone, or both domains of TRAF2. The core of the RING domains of TRAF2 and TRAF6 are highly similar, with 56 aligned Cα positions and 1.3 Å R.M.S.D. There may be two regions of major differences (Figure 3A, 3B). The first difference is near the N-terminus in which an additional α helix (α1) of TRAF2 replaces the additional β strand (β1) in TRAF6. The second difference is at the TRAF2-unique insertion (Figure 1E). This insertion occurs

immediately after the last coordinating Cys residue of the RING and contains 9 residues. In the TRAF2 structure, this region forms an additional α helix ($\alpha 3$) followed by a loop. The zinc finger regions of TRAF2 and TRAF6 superimposed to 37 aligned C α positions and 1.4 Å R.M.S.D. A gross difference in length and direction exists between the helix $\alpha 5$ of TRAF2 and the corresponding helix in TRAF6 (Figure 3C). However, the zinc coordinating residues are minimally affected (Figure 3C).

Although the RING and the zinc finger domains are individually similar between TRAF2 and TRAF6, there is a difference in the junction between the RING and the zinc finger. If both the RING and the zinc finger of TRAF2 are used for structural superposition with TRAF6, only 76 C α positions can be aligned instead of the combined 93 (56+37) residues. The relative angular relationship between the RING and the zinc finger in TRAF2 is about 20° more acute than that in TRAF6 (Figure 3D, 3E). This difference is clearer when the zinc finger domain of TRAF2 is superimposed to the 3 consecutive zinc fingers of TRAF6 to represent the chain direction of TRAF2 in the second and third zinc fingers. The conformational difference may be due to the different inter-domain interaction; in particular, the interaction between the $\beta 1$ strand of the RING and the $\beta 2$ and $\beta 3$ strands of the zinc finger in TRAF6 is replaced by the interaction between the $\alpha 1$ helix of the RING and the same region of the zinc finger in TRAF2 (Figure 3A).

Multi-faceted differences underlie lack of Ubc13 interaction in TRAF2

We have shown earlier that unlike TRAF6, TRAF2 did not interact with Ubc13 in a yeast two hybrid assay (19). Indeed, while the TRAF6 RING and the first zinc finger construct is sufficient for Ubc13 interaction (19), the same region of TRAF2 did not interact with Ubc13 on a native PAGE assay (Figure 4A).

Structural analysis revealed that this lack of Ubc13 interaction might be a consequence of multiple factors. First, if a complex of TRAF2 with Ubc13 is constructed using the known TRAF6: Ubc13 interaction (19), two regions of the TRAF2 structure would be in steric clash with Ubc13 (<2Å in inter-atomic distances) (Figure 4B). One region is from the N-terminus, comprising residues Q16, G18 and K21. The other region is from the TRAF2-unique insertion and composed of residues A70 and H74. Second, core residues at the Ubc13-interacting site of TRAF6 are completely different in TRAF2 (Figure 4C, 1E). Specifically, residues E69, P71, I72, L74, M75, A101 and P106 are changed to L33, S35, A36, R38, N39, G65 and A70, respectively. These residues mainly exhibit side chain differences with fairly conserved main chain conformations. Third, both the sequence and conformation of residues near the N-terminus for Ubc13 interaction in TRAF6 are different in TRAF2 (Figure 4D). The difference in conformation is likely because of the two consecutive Pro residues (P62 and P63) in TRAF6 and no Pro residues in TRAF2 in this region. In addition, TRAF6 residues in direct contact with Ubc13, Q54 and D57, are replaced by P17 and S20, respectively.

TRAF2 failed to interact with a number of other E2s

Inability of TRAF2 to interact with Ubc13 is intriguing considering that TRAF2 is crucial for TNF α -induced NF- κ B activation. One possibility is that TRAF2 uses another E2 to perform K63-linked polyubiquitination. In this regard, at least UbcH5 has been implicated in mediating both K48 and K63-linked polyubiquitination (30). However, structurally UbcH5 is very similar to Ubc13 (Figure 4E) and the same steric hindrance between TRAF2 and Ubc13 should be true for UbcH5. This would then disallow TRAF2 to interact with UbcH5. To confirm our hypothesis and to test the ability of TRAF2 to cooperate with additional E2s, we performed *in vitro* polyubiquitination assays using full-length TRAF2 and eight other E2s (Figure 5A). While TRAF6 also promoted polyubiquitin chain synthesis by UbcH5a, UbcH5b, UbcH5c and UbcH6, TRAF2, as well as TRAF3 and TRAF5, failed to generate polyubiquitin chains in the

presence of any of the tested E2s. In contrast to the lack of E3 activity of TRAF2, both purified cIAP1 and cIAP2 can undergo autoubiquitination (Figure 5B).

DISCUSSION

Our current data support that TRAF2 per se is not a ubiquitin ligase. Nonetheless, we cannot rule out the possibility that TRAF2 may become an active E3 after undergoing post-translational modifications. In fact, it has been shown that residue T117 of TRAF2 is phosphorylated by PKC δ following TNF α stimulation and this phosphorylation is required for TRAF2 functional activity (31). However, when we generated the phospho-mimic mutant T117E, the phospho-disabling mutant T117A, or used recombinant PKC δ to phosphorylate wild type TRAF2 and these mutants, we could not observe any activity of these TRAF2 variants in promoting polyubiquitin chain synthesis or auto-ubiquitination by Ubc13/Uev1A (data not shown).

Is there a sequence signature that distinguishes RING type E3s from RING proteins with no E3 activities? Recently, a genome wide analysis of human E2: E3 interactions using yeast two-hybrid screens identified two sequence positions that correspond to A36 and I61 of TRAF2 as critical (32). In most E3s, these positions are Ile and Trp, respectively. However, this sequence conservation is not strict; for example, in TRAF6, these are Ile and Ser, respectively. Instead, it appears that sequence signatures for RING with E3 activities are complex, for which a better prediction might be achieved through 3-D homology modeling and free energy calculations for the E2: E3 pairs (32).

It seems more likely that TRAF2-associated proteins might be functioning as ubiquitin ligases for the TNF receptor superfamily pathways. TRAF2 has been known to be constitutively associated with cIAP1 and cIAP2, members of the inhibitor of apoptosis (IAP) family (33). Neither cIAP1 nor cIAP2 appears to be potent in caspase inhibition (34). Alternatively, cIAP1 and cIAP2 are RING-containing proteins and known to promote K48-linked polyubiquitination (35). It has also been shown that cIAP1 and cIAP2 facilitate cancer cell survival by functioning as ubiquitin ligases that promote RIP1 ubiquitination (36).

The sequences of the RING domains of cIAP1 and cIAP2 suggest that they might be able to interact with Ubc13 (19) and therefore might be able to promote K63-linked polyubiquitination as well. Indeed, we could show Ubc13/Uev1A-dependent auto-polyubiquitination of cIAP1 and cIAP2. The zinc-coordinating residues in cIAP1 and cIAP2 are critical for this polyubiquitination. These data suggest that cIAPs may be able to serve as the E3s for K63-linked polyubiquitination and NF- κ B activation in TRAF2 signaling pathways. Our in vitro observation is consistent with earlier studies showing the role of cIAPs in formation of the TNF receptor signaling complex and NF- κ B activation (37). More convincingly, when both cIAP1 and cIAP2 are absent, RIP1 polyubiquitination was diminished with reduced phosphorylation of IKK β (38).

In this context, one of the major roles of TRAF2 is to recruit cIAP1 and cIAP2. The interaction between TRAF2 and cIAPs is direct, as shown by both earlier yeast two hybrid studies (33) and our unpublished results using purified TRAF2 and cIAP2 BIR1 domain. In one likely scenario, the association of cIAPs with TRAF2 brings cIAPs to the TNF receptor signaling complex for activation of the canonical NF- κ B signaling pathway through RIP1 ubiquitination (38). In another more clearly defined scenario in the role of cIAPs in suppressing the noncanonical NF- κ B pathway by ubiquitinating the kinase NIK, TRAF2 is absolutely required for this activity of cIAPs. It is the bridge that brings cIAPs to its substrate NIK by interacting with TRAF3 that in turn associates with NIK (39). TRAF2 itself is also a substrate of cIAPs (40). In the absence of appropriate substrates or in the presence of IAP antagonists, cIAPs can

also undergo autoubiquitination and degradation (35,41,42). It is possible that in the multi-protein complex, TRAF2 functions to alter the substrate specificity and perhaps also the ubiquitination efficiency of cIAPs. The latter has also been proposed as the role of chain elongation factors or E4s (14). It is possible that cIAPs are just one of the E3s in the TRAF2 signaling pathways.

If TRAF2 is not an E3, but rather a platform for recruiting cIAP1 and cIAP2 via other domains of TRAF2, what is the function of its RING domain? It has long been observed that RING-deletion converts TRAF2 from a positive regulator of NF- κ B signaling to a dominant negative inhibitor (1). There may be at least a couple of possible answers for this question. TRAF2 RING may function as a K63-linked polyubiquitination acceptor site, rather than an E3. This site has been mapped to K31 in its RING domain (43). Perhaps another quite plausible possibility lies in the requirement of TRAF2 RING for the translocation of TNF receptor family signaling complexes to membrane rafts, a process required for their signaling (44). In any case, the structural incompatibility of the TRAF2 RING for interacting with Ubc13-like E2s should prompt a re-evaluation of its role in signal transduction.

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FIGURE 1.

Structure of the human TRAF2 RING and first zinc finger domains (RZ₁). (A) Domain organization of TRAF2. Z₁–Z₄: zinc finger domains 1–4; CC: coiled coil domain. (B) A ribbon diagram of TRAF2 RZ₁ colored in a rainbow mode from N- to C-termini. Secondary structure elements are labeled. (C) A region of SAD-phased electron density at 1.5 σ superimposed with the final model. (D) Ca trace of TRAF2 RZ₁ showing the zinc-coordinating residues. (E) Sequence alignment of TRAF2 from different species and with TRAF3, TRAF5 and TRAF6. Gray: zinc-coordinating residues; cyan: TRAF2-unique insertion; red: TRAF2 residues that would have been in clash with Ubc13; green: TRAF2 dimerization interface residues; yellow: residues in TRAF6 that contact Ubc13 and their equivalents in other TRAFs.

FIGURE 2.

TRAF2 dimerization in the crystal and in solution. (A) TRAF2 RZ₁ dimer shown in a ribbon diagram colored in a rainbow mode. (B) superposition of the TRAF2 RZ₁ dimer (red and cyan) with the TRAF6 RZ₁₂₃ dimer (PDB code 3HCS, magenta and green). (C) TRAF2 RZ₁ dimer shown with residues at the dimerization interface. The region that is shown in detail in (D) is blocked by a rectangle. (D) Details of the TRAF2 dimerization interaction. Critical residues are labeled on one side of the symmetrical interface. (E) Superposition of gel filtration profiles of WT TRAF2 RZ₁ (High: at higher concentration; Low: at lower concentration), and Q46A, F91A, N94A and R98A mutants of TRAF2 RZ₁. MALS measurement results for WT TRAF2 RZ₁, the F91A mutant and the R98A mutant, as well as elution positions of molecular weight

standards, are shown. (F) TRAF2 RING dimerization mutants rescued TNF α -induced I κ B α phosphorylation in TRAF2/TRAF5 double knockout MEFs.

FIGURE 3.

Comparison between TRAF2 RZ₁ (Cyan) and TRAF6 RZ₁₂₃ (magenta). (A) and (B) Superposition of TRAF2 and TRAF6 RING domains, showing the location of the unique insertion of TRAF2 in two roughly orthogonal orientations. (C) Superposition of the first zinc finger domains of TRAF2 and TRAF6. (D) and (E) Superposition of TRAF2 RZ₁ with TRAF6 RZ₁₂₃, showing the difference in relative orientation of the zinc finger domain relative to the RING domain. In (E), the Z₁-Z₃ domains of TRAF6 were superimposed onto the Z₁ of TRAF2 to show more clearly the relative rotation of ~20°.

FIGURE 4.

Lack of interaction between TRAF2 and E2s. (A) Native PAGE of TRAF2 RZ₁ with Ubc13 using 8–25% gradient PhastGel on a PhastSystem (GE Healthcare). A similar native PAGE of TRAF6 RZ₁₂₃ with Ubc13 is shown as a positive control. The gels were stained with coomassie blue. (B) TRAF2 residues (in red) that would have been in steric clash with Ubc13 if the interaction follows the mode of the TRAF6: Ubc13 interaction (PDB code 3HCU). (C) A close up view of the superposition of the RING domains of TRAF2 (cyan) and TRAF6 (magenta). TRAF6 residues in direct contact with Ubc13 and their equivalents for TRAF2 are shown in yellow for TRAF2 and in gray for TRAF6. (D) Conformational difference at the region immediately preceding the RING between TRAF2 and TRAF6. (E) Superposition of UbcH5b (PDB code 2CLW) onto the hypothetical TRAF2: Ubc13 complex, showing that the TRAF2 residues in clash with Ubc13 would have been in clash with Ubc5Hb as well.

FIGURE 5.

Ubiquitination assays. (A) Auto-ubiquitination and free chain polyubiquitin synthesis *in vitro* by TRAF2, TRAF3, TRAF5 and TRAF6 with a series of E2s. The indicated GST-fusion proteins bound to glutathione-agarose beads were subjected to an *in vitro* ubiquitination assay in the presence of the indicated E2s. Following the ubiquitination assay, a portion of the supernatant was subjected to SDS-PAGE and immunoblotted with anti-ubiquitin (middle panels). The beads were washed, subjected to SDS-PAGE, and immunoblotted with anti-ubiquitin (top panels). The membranes were stained with Ponceau S and one representative membrane is shown (bottom panel). G: GST; 6: GST-TRAF6; 2: GST-TRAF2; 3: GST-TRAF3; 5: GST-TRAF5. (B) cIAP1 and cIAP2 undergo RING-dependent auto-ubiquitination in the presence of the Ubc13/Uev1A E2 complex. C571R: RING mutant of cIAP1. C557R: RING mutant of cIAP2.

Table 1

Crystallographic statistics

TRAF2	
Constructs	
Structure determination	SAD
Data collection	
Beamlines	NSLS X4A
Space group	P6 ₅ 22
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	43.7, 43.7, 284.4
α , β , γ (°)	90.0, 90.0, 120.0
Resolution	1.9
R _{sym} (%)	7.0 (34.7)
I/ σ I	59.2 (4.0)
Completeness (%)	98.8 (90.1)
Redundancy	13.2 (5.6)
Refinement	
Resolution (Å)	37.8 – 1.9
No. unique reflections	23,319
R _{work} / R _{free} (%)	21.6 / 23.9
No. atoms	
Protein	940
Water and ion	95
Average B-factors (Å ²)	
Protein	36.5
Water and ion	47.8
R.M.S deviations	
Bond lengths / angles	0.004 Å / 1.15 °
Ramachandran Plot (%)	
Most favored / allowed	87.6 / 100.0

[†]The highest resolution shell (1.97–1.90 Å) is shown in parenthesis.