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Structural Studies of Human TRAF2

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The tumor necrosis factor (TNF) receptor (TNFR) superfamily effects a wide spectrum of cellular responses including proliferation, differentiation, and apoptosis (for review, see Smith et al. 1994). Current members of TNFRs include TNF-R1, TNF-R2, Fas, CD40, CD30, CD27, LTβR, Ox40, 4-1BB, RANK, p75 nerve growth factor (NGF) receptor, and a series of death receptors (DRs). Recent studies have implicated the TNFR-associated factors (TRAFs) as major signal transducers for many members of this receptor superfamily (for review, see Arch et al. 1998). The Epstein-Barr virus (EBV) oncogene product LMP1 also uses TRAFs for inducing long-term growth potentiation and cell transformation (Mosialos et al. 1995). In addition, TRAF signaling is important for receptors of the pro-inflammatory cytokine interleukin-1 (IL-1) (Cao et al. 1996b). The downstream signaling of TRAFs appears to involve the activation of kinases in the MAPK family, leading eventually to the activation of Rel (NF-κB) and AP-1 transcription factors (for review, see Arch et al. 1998). These transcription factors can switch on various genes involved in inflammatory, immune, and acute phase responses.

TRAF2 is a prototype for the six known TRAF family members. TRAF1 and TRAF2 were isolated from the TNF-R2 signaling complex by biochemical purification (Rothe et al. 1994). TRAF3 and TRAF5 were identifed by virtue of their interactions with several members of the TNF receptors and LMP1 (Hu et al. 1994; Cheng et al. 1995; Mosialos et al. 1995; Ishida et al. 1996a; Nakano et al. 1996). The overexpression of TRAF4 in breast carcinoma cells resulted in its isolation and cloning (Regnier et al. 1995). TRAF6 was identified independently as a component of the IL-1 and CD40 signaling pathway (Cao et al. 1996b; Ishida et al. 1996b).

TRAFs exhibit differential receptor-binding specificity. TRAF1, 2, 3, and 5 directly bind to a common set of TNFRs including TNF-R2, CD40, CD30, CD27, LTβR, Ox-40, 4-1BB, and LMP1 (for review, see Arch et al. 1998). TRAF2 indirectly associates with TNF-R1 through the adapter molecule TRADD (Hsu et al. 1996). TRAF4 has not been shown to interact with any receptor, consistent with its proposed nuclear localization. TRAF6 has been shown to interact directly with CD40, RANK, and the p75 NGF receptor (Ishida et al. 1996b; Darnay et al. 1999; Khursigara et al. 1999) and indirectly with IL-1 receptors through the adapter kinase IRAK (Cao et al. 1996a,b).

The TRAF-binding sites on various receptors have been mapped to short linear sequences in their intracellular regions. Three types of motifs have been proposed for TRAF1 2, 3, and 5: the PxQx (T/S/D) (x = any amino acid) motif in LMP1, CD30, CD40, and CD27 (Devergne et al. 1996; Franken et al. 1996; Gedrich et al. 1996; Aizawa et al. 1997; Boucher et al. 1997; Brodeur et al. 1997; Sandberg et al. 1997; Akiba et al. 1998), the ΦSxEE (Φ = large hydrophobe) sequence in TNF-R2 and CD30 (Rothe et al. 1994; Boucher et al. 1997), and the QEE motif in 4-1BB and Ox40 receptors (Arch and Thompson 1998). These motifs are highly diverse and do not share any apparent sequence similarity. The binding motif for TRAF6 differs from those described above.

Most TRAF proteins share a common domain organization (Fig. 1). The amino-terminal domain contains RING finger and several zinc finger motifs. The carboxy-terminal TRAF domain is subdivided into a TRAF-N domain and a highly conserved TRAF-C domain. The TRAF-N domain is variable in length and sequence among the six TRAFs but contains characteristic hydrophobic heptad repeats for coiled-coil structures. The amino acid identity within the TRAF-C domain ranges between 32% and 67% for pairs of TRAFs. Whereas the amino-terminal domain is required for downstream signaling, the TRAF domain is responsible for self-association and for interaction with upstream receptors.

The cytokine ligands for TNFRs are trimeric in nature and activate the receptors by trimerizing the bound receptor molecules, but it is not clear how trimerization initiates the intracellular TRAF-signaling pathway. To elucidate the structural basis for this signal transduction, we determined the crystal structures of the TRAF domain of human TRAF2, both in its free form and in complex with a peptide from TNF-R2 (Park et al. 1999). The structural study revealed the trimeric nature of the TRAF domain, providing an avidity-based model for TRAF-receptor interaction. To further decipher the mechanism for recognizing diverse receptor sequences by TRAF2, we determined additional complexes of the TRAF domain of human TRAF2 with receptor sequences from each of the three proposed TRAF-binding motifs (Ye et al. 1999). These receptors include CD40, CD30, Ox40, 4-1BB, and LMP1. A conserved mode of receptor recognition was revealed, conferring a common mechanism for TRAF2 to relay the signal transduction of the family of TNFRs.

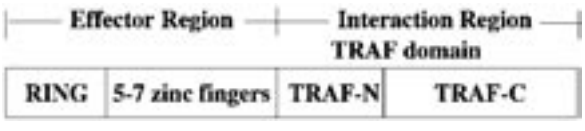

Figure 1. Domain organization of TRAFs.

PROTEIN EXPRESSION AND OLIGOMERIZATION CHARACTERIZATION

A combination of sequence analysis and experimental dissection were used to identify suitable constructs for overexpression and crystallization. As the entire TRAF domain (residues 276–501) did not yield soluble protein, we removed an amino-terminal portion of the TRAF-N domain that has low propensity for forming coiled-coil structures (Fig. 2). These shorter constructs (residues 310–501 or residues 315–501) were successfully expressed at 20°C in *Escherichia coli*. Further proteolysis of the construct containing residues 315–501 gave rise to a TRAF2 protein with residues 327–501. Although the TRAF2 proteins containing either residues 310–501 or residues 327–501 can be crystallized, the latter often gave superior crystals in terms of diffraction quality.

The oligomerization states of the TRAF domain constructs were characterized by a series of experiments. Initial experiments were performed on the construct containing residues 310–501. The gel-filtration profile of the proteins is consistent with an oligomeric structure. Chemical cross-linking with glutaraldehyde produced a single band with an approximate molecular mass of 65 kD on an SDS-PAGE, suggesting that the protein is a trimer in solution. This observation was confirmed by dynamic light scattering measurement, which gave an average molecular size of 60.4 kD, and by equilibrium sedimentation, which showed a homogeneous species of 63.3 kD. Characterizations using similar methods have also established that the TRAF domain constructs containing residues 315–501, 327-501, and 332–501 were also trimeric, whereas further shortening of the coiled-coil domain (residues 342–501) produced monomeric species. These experiments established the requirement of at least a portion of the coiled-coil domain for the trimer formation.

The cytokine ligands of TNFRs are also intrinsically trimeric. The crystal structure of the complex between LTα and the extracellular domain of TNF-R1 revealed that each LTα trimer has three receptor-binding sites. The interaction leads to a ligand-induced indirect trimerization of receptor molecules without direct contacts between the receptors (Banner et al. 1993). The trimeric nature of the TRAF domain of human TRAF2, as established in this study, is likely to provide an optimal mode of recognition for trimerized receptor molecules.

FOLD DESCRIPTION

As predicted from sequence analysis, each protomer of the TRAF domain trimer of human TRAF2 consists of two domains: an amino-terminal coiled-coil domain and a carboxy-terminal TRAF-C domain (Fig. 3). The coiled-coil domain (up to residue 347) is composed of a single α-helix (α_N) which associates with symmetry-related helices from the other two protomers of the trimer to form a parallel coiled-coil structure. Residues in this domain appear to be quite flexible and sensitive to crystal packing environments. They therefore exhibit varying degrees of deviation from a proper threefold symmetry.

The TRAF-C domain (residues 352–501) forms an eight-stranded antiparallel β-sandwich, with strands β1, β8, β5, and β6 in one sheet and β2, β3, β4, and β7 in the other. The strand directions of the two opposing sheets bear a crossing angle of approximately 45°. Strands β2 and β7 are highly twisted and each contains a β-bulge in the middle of the strands. These features appear to be im-

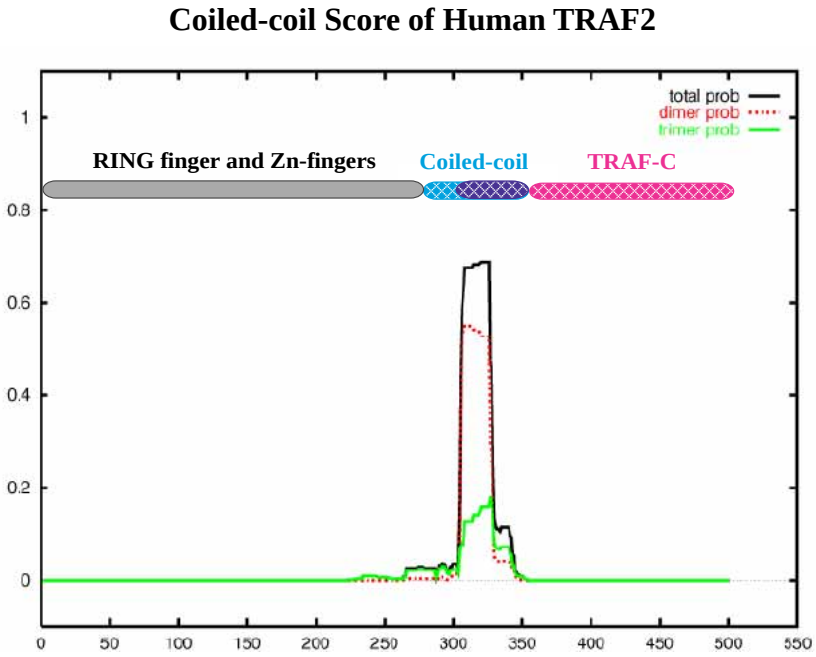

Figure 2. Prediction of coiled-coil structure of human TRAF2 by the MultiCoil program (Wolf et al. 1997). (*Red line*) Dimeric coiled-coil score; (*green line*) trimeric coiled-coil score; (*black line*) total coiled-coil score. The TRAF domain construct containing residues 310–501 is schematically shown by the horizontal bars in *dark blue* and *magenta*.

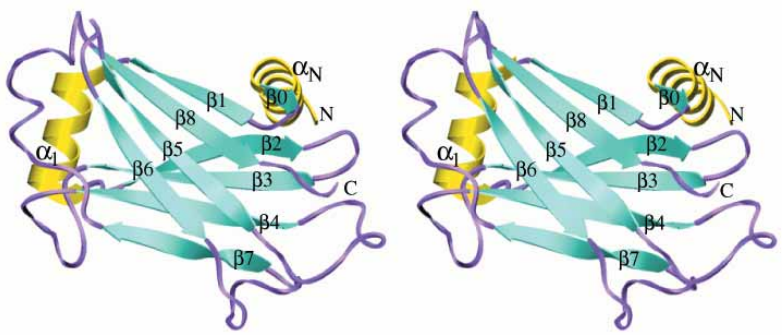

Figure 3. Stereo ribbon diagram of the TRAF domain protomer of human TRAF2 in the peptide-free structure. (*Cyan*) β-strands; (*yellow*) α-helix; (*magenta*) loops. (Reprinted, with permission from Park et al. 1999 [copyright Macmillan] http://www.nature.com.)

portant for both structural architecture and receptor interaction (see below). There is a three-turn α-helix (α_1) in the crossover connection between strands β1 and β2. A short β-strand (β0, residues 348–350) precedes the TRAF-C domain and runs parallel to the portion of β2 strand immediately after the β-bulge.

Visual inspection of the SCOP structure database (Murzin et al. 1995) and automatic structural similarity search using the Dali program (Holm and Sander 1995) failed to identify any known structure with a similar β-sandwich fold as the TRAF-C domain. It appears then that this domain represents a novel fold for an eight-stranded antiparallel β-sandwich. The sequence conservation of the TRAF-C domain among the TRAF family members suggests strongly that this structure is preserved throughout the TRAF family.

STRUCTURE OF THE TRAF DOMAIN TRIMER

The TRAF domain trimer of human TRAF2 has the shape of a mushroom with the TRAF-C domain as the cap and the coiled-coil domain as the stalk (Fig. 4). The mushroom cap has a dimension of between 50 and 80 Å wide and obeys proper threefold symmetry. The length of the stalk is related to the protein constructs in each crystal and is approximately 50 Å long for the five heptad repeats in the complex with TNF-R2. The coiled-coil region is more flexible and may deviate from threefold symmetry as it projects away from the mushroom cap. There is a solvent-accessible hole at the center of the top face of the mushroom, directly opposite the protruding coiled-coil.

The trimer interface is formed by residues in the coiled-coil domain as well as those in the TRAF-C domain (Fig. 4). The TRAF-C domain alone buries 1920 $Å^2$ surface area upon trimerization. This gives 640 $Å^2$ per protomer and is relatively poor compared to some other tight interactions (Janin et al. 1988). This is, however, in agreement with earlier studies that showed the inadequacy of the TRAF-C domain alone for TRAF self-oligomerization (Takeuchi et al. 1996). Inclusion of the coiled-coil domain increases the buried surface area, making oligomerization possible. Our solution studies suggest that a minimum of approximately two heptad repeats (residues 334–347), which increases the surface area burial to 1060 $Å^2$ per protomer, may be required for trimer formation. Interestingly, the coiled-coil domain of TRAF4 contains only three heptad repeats, the shortest among TRAFs. Further shortening of the coiled-coil domain by trypsin digest (residues 342–501) produced monomers in solution, whereas lengthening of the coiled-coil region may be expected to enhance the stability of the trimeric state of TRAF proteins.

Hydrophobic residues at positions a and d of the heptad repeats mediate the coiled-coil association in the TRAF domain structure, as seen in other coiled-coil

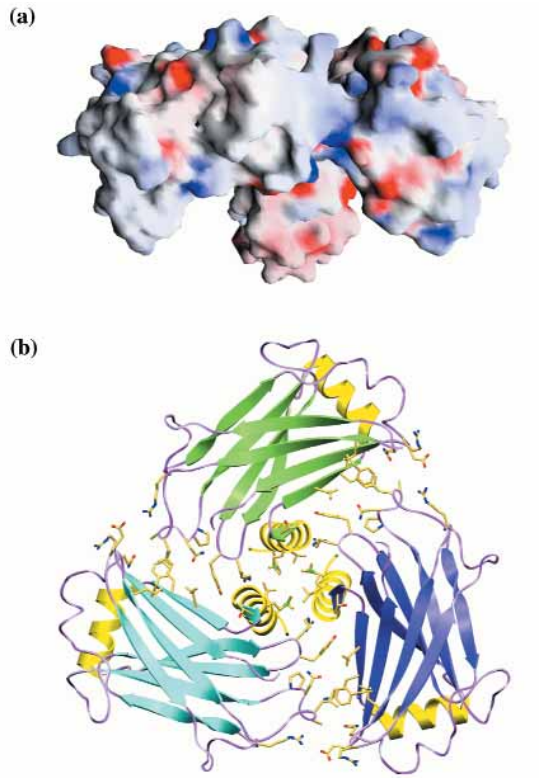

Figure 4. Structure of the TRAF domain trimer in the peptide-free form. (*a*) Surface representation of the TRAF domain trimer, showing the mushroom-shaped molecule with the threefold axis in the vertical direction. Surface color coding is according to electrostatic surface potential. (*b*) Ribbon diagram of the TRAF domain trimer, looking down the threefold axis. Residues involved in trimerization are shown as stick models.

structures (Harbury et al. 1993). Superposition of the coiled-coil structure with those from other proteins, including hemagglutinin (PDB entry 2viu) (Fleury et al. 1998) and gp41 (PDB entry 1aik) (Chan et al. 1997), showed significant differences in the relative positioning and twisting of the helices, reflecting differences in the buried side chains. There is a hydrogen bond between a main-chain carbonyl at the end of the coiled-coil domain (residue 346) and the side-chain guanidinium group of R385 in the TRAF-C domain of a neighboring protomer, making it the only interaction between the two domains at the trimerization interface.

The TRAF-C domains interact with each other by packing one end of the β-sandwich (the β2β3, β4β5, and β6β7 connections) against an edge and a face of the β-sandwich (β0, β1, β8, β5β6, and β7β8 connections) of the neighboring protomer (Fig. 4). Residues that participate in the formation of this interface are mixed in nature, with hydrophobic residues (such as I355, Y386, A420, L421, and F491) that cluster at the center of each interface. Besides the hydrophobic interactions, the side chain of K357 forms a hydrogen bond with the main-chain carbonyl of residue 417. In addition, there is a salt bridge between the side chains of residues R458 and D487. Both polar and nonpolar contacting residues show significant conservation among the TRAF family members.

The significant sequence conservation at the trimerization interface suggests that other TRAF molecules may be able to form similar homotrimers as well. In addition, heterotrimers of TRAF proteins may also be possible with this conserved interface. Hetero-oligomerizations between many TRAF proteins have been observed in vitro (Pullen et al. 1998). TRAF2 and TRAF1 have been shown to interact in vivo (Rothe et al. 1994). In the presence of TRAF1, TRAF2 appears to prefer interaction with TRAF1 rather than itself. TRAF1, which lacks the zinc-binding motifs in the amino-terminal region, has been shown to inhibit apoptotic signals via an unknown mechanism (Speiser et al. 1997). The simultaneous recruitment of both TRAF1 and TRAF2 to the receptors through hetero-oligomerization may therefore enrich the signal transduction from these receptors.

Although the energetics of TRAF trimerization appears to depend on both the TRAF-C domain and the coiled-coil domain, the preference of trimerization versus other types of oligomerization may rely on the TRAF-C domain. This speculation comes from the observation that the coiled-coil domains of TRAFs do not appear to contain characteristic sequences for trimerization. In fact, predictions often gave higher scores for dimerization instead of trimerization for the coiled-coils in TRAFs (see Fig. 2). It is therefore likely that TRAF trimerization is initiated from the TRAF-C domain and further stabilized by the coiled-coil domain. Such a hypothesis may be verified by producing TRAF proteins containing only the coiled-coil domains.

INTERACTION OF TRAF2 WITH TNF-R2

Using deletion mutagenesis and peptide mapping, the TRAF-binding site on TNF-R2 has been located to a nine-residue peptide near the carboxyl terminus of the receptor (420′-QVPFSKEEC-428′, primed numbers used to denote receptor residues) (Boucher et al. 1997). This peptide was chemically synthesized with amino-terminal acetylation and carboxy-terminal amidation for cocrystallization with the TRAF domain of TRAF2.

In this complex structure, each TNF-R2 peptide binds symmetrically to a shallow surface depression on the side of the mushroom-shaped TRAF domain trimer, extending from the top to the bottom rim of the mushroom cap (Fig. 5). This orientation of the peptide suggests that the mushroom cap may be placed proximal to the cell membrane for close interaction with receptors, and the stalk may be pointing toward the inside of the cell for the amino-terminal domain to interact with downstream molecules.

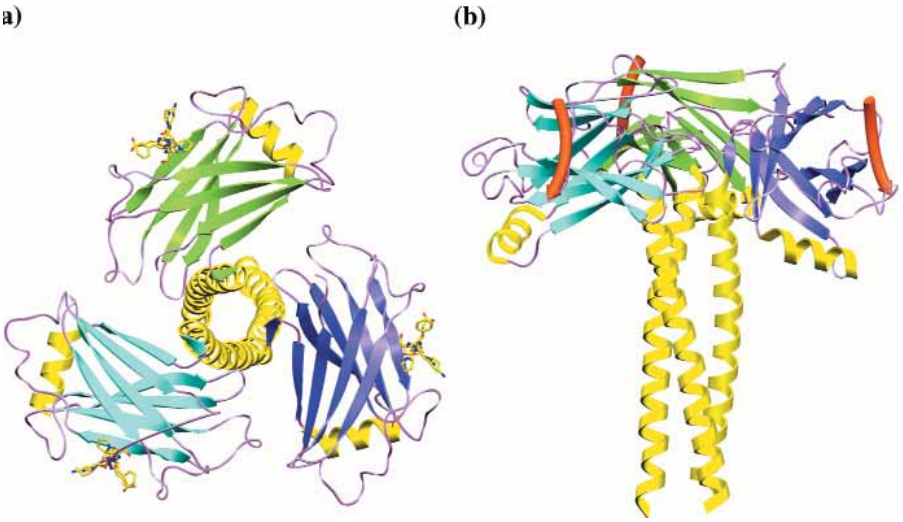

Figure 5. Ribbon drawing of the trimeric TRAF domain in complex with the TNF-R2 peptide, looking down the threefold axis (*a*) and with the threefold axis vertical (*b*). The β-strands in each protomer are shown, respectively, in *cyan*, *green*, and *purple*. The peptide is shown as stick models in *a* and as orange circular ribbons in *b*. One copy of the TNF-R2 peptide is generated by symmetry.

The receptor-binding site on TRAF2 is situated away from the trimerization interface so that each receptor peptide contacts one TRAF domain exclusively. The conformation of the receptor-binding site remains essentially unchanged upon binding, and there is no significant rearrangement of the quaternary organization of the trimer.

A major portion of the receptor peptide (residues S424′–E427′) assumes a polyproline II helix (PPII) main-chain conformation when bound to TRAF2. PPII is analogous to a slightly twisted β-strand that allows a side-chain periodicity of three per turn. Although originally named after the conformation assumed by a polyproline helix, this conformation does not require proline and is quite abundant in protein structures (Stapley and Creamer 1999). It is frequently observed in protein-peptide interactions such as the peptides bound to MHC and SH3 proteins (Lim et al. 1994; Stern et al. 1994). The main-chain twisting appears to allow peptides to maximize side-chain contacts with proteins.

The receptor-binding site on TRAF2 is formed by the exposed faces of strand β6 in the first sheet and strands β7, β4, and β3 in the second sheet (Fig. 6). A portion of the peptide chain (K425′-E427′) runs antiparallel and adjacent to the latter half of strand β7 (residues 466–468), immediately after the β-bulge in this strand. This leads to three main-chain hydrogen bonds between the peptide and TRAF2, extending the four-stranded second β-sheet by one strand. In addition, the main-chain amide group of C428′ is within hydrogen-bonding distance to the carboxylate group of D399 in TRAF2. The formation of a β-sheet is a major feature of many protein-peptide interactions, such as the substrate recognition by proteases (Tong et al. 1998) and the peptide recognition by the PTB and PDZ domains (Kuriyan and Cowburn 1997).

Residues P422′–C428′ of the receptor peptide show interactions with the TRAF2 protein (Fig. 6), burying 500 $Å^2$ surface area upon binding. Residues Q420′–V421′ are ordered only in one copy of the peptide, where it is stabi-

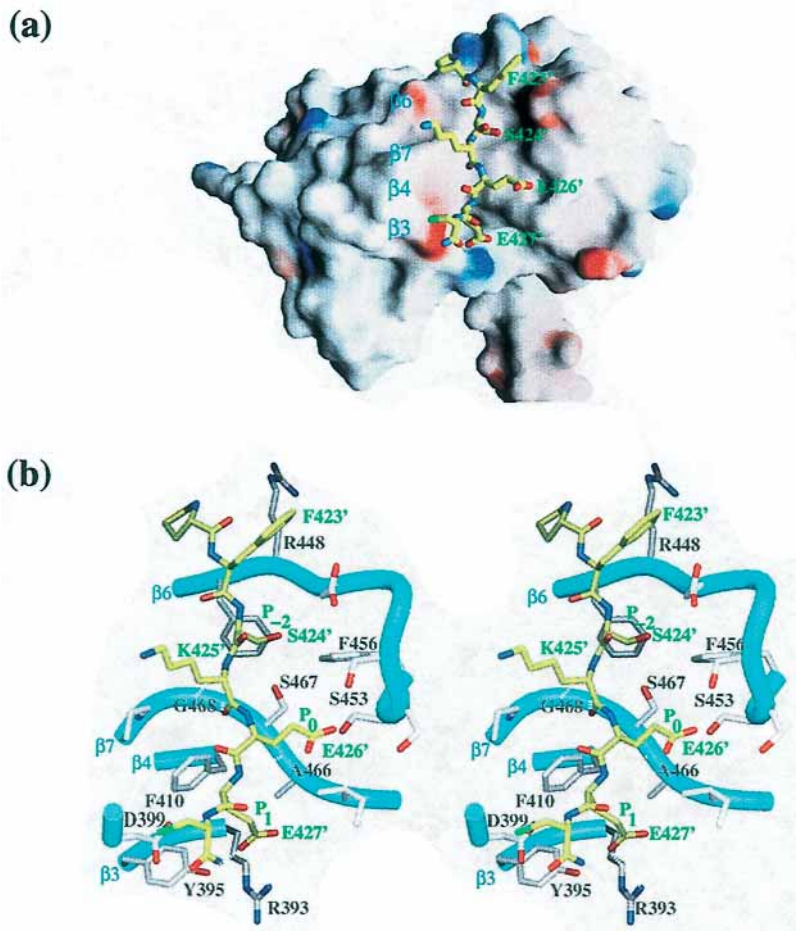

Figure 6. TRAF2–TNF-R2 interaction. (*a*) Molecular surface of a TRAF domain protomer of TRAF2 and the bound TNF-R2 peptide (stick model), showing with the threefold axis in the vertical direction. Surface color coding is according to electrostatic surface potential, scaled from –30 to +30 kTe^{-1}, with *blue* for positive and *red* for negative. Selected residues in the receptor peptide and the underlying secondary structural elements of TRAF2 at the binding site are labeled. (*b*) Stereoview of the detailed interaction between the TNF-R2 peptide (carbon atoms shown in *yellow*) and TRAF2 (carbon atoms shown in *gray*). The main chain of the TRAF2 structure is shown in *cyan* as backbone worms. Selected residues in the peptide (primed numbers in *green*) and the protein (in *gray*) are labeled. The peptide residues are also labeled by their positions in the TRAF-binding motif (see text). Hydrogen bonds and salt bridge are shown by black dotted lines. (Modified, with permission, from Park et al. 1999.)

lized by crystal packing. Although most of the receptor residues are bound to shallow pockets on the surface of the TRAF domain, there is considerable shape complementarity between TRAF2 and the TNF-R2 peptide, resulting in many van der Waals contacts. Among these interactions, approximately 50% are polar-nonpolar contacts, 35% are nonpolar-nonpolar contacts, and 15% are polar-polar contacts.

Residues S424′, E427′, and possibly E426′ of the receptor peptide are recognized specifically by TRAF2 (Fig. 6). The side chain of S424′ binds to a small pocket on the surface of the protein and becomes completely buried. The hydroxyl of S424′ is within hydrogen-bonding distance to the hydroxyl group of S467 (in strand β7) of TRAF2. The side-chain carboxylate group of E427′ forms an ion pair with R393 (β3) and is also hydrogen-bonded to the side-chain hydroxyl of Y395 (β3). The aliphatic portion of the E427′ side chain is mostly buried, leaving 13% surface exposure for the entire side chain. The terminal carboxylate group of residue E426′ sits close to the hydroxyls of three serine residues (S453, S454, and S455), even though only one potential hydrogen bond may be possible between the carboxylate and the hydroxyls. These residues of the receptor therefore appear to be the major determinants of binding specificity. Additionally, the main-chain hydrogen-bonding interactions between the peptide and TRAF2 are also mediated through these residues. They are then likely to serve as anchoring points during binding.

The receptor-binding surface, including the key residues R393, Y395, and S467, is highly conserved among TRAF1, 2, 3, and 5, explaining the overlapping receptor specificity of these TRAFs. TRAF4 and TRAF6 both show drastic mutations at these residues, such as the R393S, Y395F, and S467F mutations in TRAF4 and the Y395H and S467F mutations in TRAF6. Whereas TRAF4 appears to locate in the nucleus and has never been shown to interact with any receptors, TRAF6 binds to sequences distinct from those described for other TRAFs.

INTERACTIONS OF TRAF2 WITH OTHER RECEPTORS

Although the TRAF-binding TNF-R2 peptide bears an apparent consensus of ΦSxEE, many other TRAF-binding sequences contain the PxQx(T/S/D) or the QEE motif. It is not clear from the TNF-R2 complex whether these additional sequences may also utilize the same receptor-binding site on TRAF2. Additional crystal structures of the TRAF domain of TRAF2 in complex with sequences from CD40 and LMP1 (PxQxT motif), Ox40 and 4-1BB (QEE motif), and CD30 (ΦSxEE motif) revealed a conserved mode of receptor recognition by TRAF2.

Structural superposition of six receptor peptides (TNF-R2, CD40, LMP1, CD30, Ox40, and 4-1BB) gave rise to a core of four residues (Fig. 7). The third position of the four-residue core is highly conserved in sequence with glutamic acid in TNF-R2 and CD30 and glutamine in CD40, LMP1, Ox40, and 4-1BB. We denote this position as the zero position (P_0) of TRAF2-binding sequences with the core residues extending from P_{-2} to P_1.

The structure-based alignment of all known TRAF-binding sequences allowed the unification of TRAF-binding motifs (Fig. 8). Two alternative TRAF-binding motifs were defined. The major TRAF-binding motif has the consensus of (P/S/A/T)x(Q/E)E, which encompasses most of the TRAF-binding sequences. The minor TRAF-binding motif was observed in the LMP1 complex, bearing a consensus of PxQxxD.

The major TRAF-binding motif is dictated by the side chains at positions P_{-2}, P_0, and P_1 (Fig.7). The side chains at the P_{-2} position are buried into a small pocket on the surface of TRAF2. They are most frequently occupied by proline, followed by serine and other amino acid with small side chains. Important conformational differences exist between the two observed side chains at the P_0 position. The glutamine side chain at this position is within hydrogen-bonding distance to the three hydroxyls from S453, S454, and S455 of TRAF2 whereas the glutamic acid side chain is positioned somewhat farther away from the TRAF2 surface so that only one hydrogen bond may be possible. The glutamic acid at the P_1 position makes specific interactions to the R393 and Y395 side chains of TRAF2, as seen in the TNF-R2 complex.

The minor TRAF-binding motif is determined by the P_{-2}, P_0, and P_3 positions (Fig. 7). Although the only observed residues at the P_{-2} and P_0 positions of this motif are proline and glutamine, respectively, the structural similarity with the major TRAF-binding motif at these positions suggests that they are subjected to the same side-chain selectivity. The differences between the two motifs reside at the P_1 and P_3 positions. In the minor motif, the P_1 position is not occupied by glutamic acid. Instead, the P_3 position is aspartic acid, which compensates for the salt bridge and hydrogen-bonding interactions with R393 and Y395 of TRAF2.

IMPLICATION IN TRAF SIGNAL TRANSDUCTION

TRAF molecules have intrinsic low affinities to monomeric receptor peptides with dissociation constants in the range of 0.04–1.5 mM (H.Ye et al., in prep.). This suggests that TRAF molecules do not associate with receptors in the unliganded state. TRAF signaling is initiated as the inherently trimeric ligands of the TNF superfamily bind and oligomerize the extracellular domains of TNFRs, which brings the intracellular domains of three receptor molecules into proximity for the simultaneous interaction with TRAFs.

Our structural studies provide an avidity-based explanation for the response of TRAF molecules to receptor trimerization and therefore the transduction of signals across the cellular membrane. This avidity-based enhancement of TRAF-receptor interactions is in agreement with experimental observations. For example, ligand-induced TRAF recruitment to receptors or the cell surface has been observed for several TNFRs including TNF-R2, CD40, and LTβR (Shu et al. 1996; Kuhne et al. 1997; Van Arsdale et al. 1997).

There are many possibilities regarding how TRAF recruitment to receptors might activate downstream effectors. First of all, if TRAFs are induced to trimerize upon receptor binding, this trimerization may then bring the associated kinase molecules into proximity for autophosphorylation and activation. Second, following receptor binding, gross conformational changes may occur in the relative orientation between the amino-terminal zinc-containing domain and the TRAF domain to allow the binding and activation of downstream effectors. Third, if

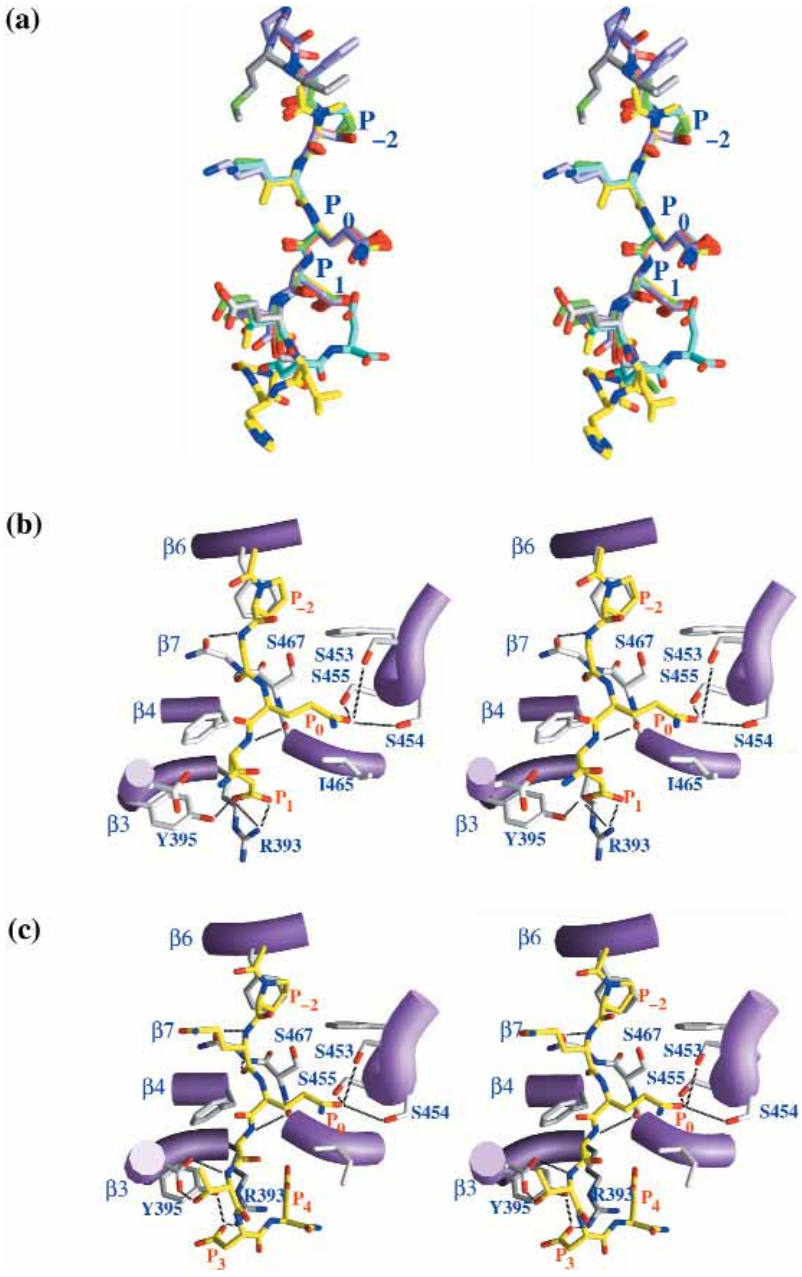

Figure 7. Structural comparison of several TRAF-binding sequences. (*a*) Stereoview of the structural superposition of receptor peptides. (*Blue*) Nitrogen atoms; (*red*) oxygen atoms; (*green*) sulfur atoms. Carbon atoms: *yellow* (CD40), *gray* (CD30), *green* (Ox40), *pink* (4-1BB), *cyan* (LMP1), and *purple* (TNF-R2, PDB entry 1ca9). Peptides from CD40, CD30, Ox40, 4-1BB, and LMP1 contain ordered acetyl groups at the amino termini. (*a*, Reprinted, with permission, from Ye et al. 1999 [copyright Cell Press].) (*b*) Stereo plot of the interaction observed in the major TRAF-binding motif of CD40. The TRAF domain is shown as *purple* worm and stick models with carbon atoms in *gray*. The peptide is shown as stick models with carbon atoms in *yellow*. The side chain at the P_{-1} position and the entire chain after the amide of the P_2 position are omitted for clarity. Atoms within hydrogen-bonding distances are connected with black dotted lines. The β-strands in the path of the peptide and selected residues in the TRAF domain are labeled. Note the difference between the glutamine at the P_0 position and the glutamic acid at the P_0 position (Fig. 6). (*c*) Stereo plot of the interaction observed in the minor TRAF-binding motif of LMP1. Note the differences at the P_1 and P_3 positions between the major and minor TRAF-binding motifs. (Modified from Ye et al. 1999.)

(a)

	P_{-4}	P_{-3}	P_{-2}	P_{-1}	P_0	P_1	P_2	P_3	P_4	P_5	P_6
hTNF-R2 (422-428)	••	P	F	S	K	E	E	C			
hCD40 (250-258)				P	V	Q	E	T	L	H	G	C••
hCD40 (250-254)				P	V	Q	E	T				
hCD30 (576-583)	M	L	S	V	E	E	E	G			
hOx 40 (262-266)				P	I	Q	E	E			
m4-1BB (231-236)			G	A	A	Q	E	E			
hLMP-1 (204-210)				P	Q	Q	A	T	D	D	

(b)

	P_{-4}	P_{-3}	P_{-2}	P_{-1}	P_0	P_1	P_2	P_3	P_4	P_5	P_6
hTNF-R2 (422-432)	P	F	S	K	E	E	C	A	F	R	S
hCD40 (248-258)	A	A	P	V	Q	E	T	L	H	G	C
hCD30 (576-586)	M	L	S	V	E	E	E	G	K	E	D
hCD30 (559-569)	H	Y	P	E	Q	E	T	E	P	P	L
hCD27 (244-254)	T	I	P	I	Q	E	D	Y	R	K	P
hLTβR (400-410)	S	T	P	H	Q	E	D	G	K	A	W
hATAR (266-276)	T	V	A	V	E	E	T	I	P	S	F
hOx40 (260-270)	R	T	P	I	Q	E	E	Q	A	D	A
m4-1BB (230-240)	T	G	A	A	Q	E	E	D	A	C	S
m4-1BB (242-252)	R	C	P	Q	E	E	E	G	G	G	G
h4-1BB (232-242)	V	Q	T	T	Q	E	E	D	G	C	S
h4-1BB (244-254)	R	F	P	E	E	E	E	G	G	C	E
bLMP1 (204-214)	R	T	P	V	Q	E	S	G	Y	P	D
bLMP1 (219-229)	R	P	P	V	Q	E	T	G	G	G	G
bLMP1 (243-253)*	H	P	P	V	Q	E	T	G	G	G	G
bLMP1 (315-325)	H	P	P	V	Q	E	T	G	E	G	G
bLMP1 (359-369)	H	P	P	I	Q	E	T	G	N	G	G
major consensus			P/S	A/T	x	Q/E	E				
hTANK (178-188)	S	V	P	I	Q	C	T	D	K	T	D
hLMP1 (202-212)	P	H	P	Q	Q	A	T	D	D	S	S
rLMP1 (315-325)#	P	Y	P	I	Q	A	T	D	G	G	N
rLMP1 (377-387)	P	H	P	I	Q	A	T	D	G	A	N
rLMP1 (425-435)	P	H	P	V	Q	A	S	D	G	G	D
minor consensus			P	x	Q	x	x	D			

Figure 8. Structure-based alignment of TRAF-binding sequences. (*a*) Structure-based sequence alignment of the receptor peptides used in the crystals. Only ordered residues are shown. Additional residues at each termini that were disordered in the structures are represented by double dots. Positions recognized specifically by the TRAF domain are shaded, respectively, in colors. Residue numbers in parentheses are those of the receptor precursors. (*b*) Global alignment of known TRAF-binding sequences based on the structural information. Residues between sites P_{-4} and P_6 are shown, with color shadings at the P_{-2}, P_0, P_1, and P_3 positions. (*Red*) Consensus TRAF-binding motifs. (*h*)human; (*m*) mouse; (*b*) baboon; (*r*) rhesus. (*) This sequence repeated in baboon LMP1 at residues 267–277 and 291–301. (#) This sequence repeated in rhesus LMP1 at residues 340–350. (Reprinted, with permission, from Ye et al. 1999 [copyright Cell Press].)

TRAFs are associated with inhibitory molecules such as I-TRAF/TANK in the cytosol, the receptor association might displace these molecules and allow the binding of downstream effectors. In addition, if the downstream kinase cascade is to be carried out near the membrane, the cell surface localization of TRAFs through receptor binding may facilitate such kinase cascade.

Our structures do not provide any direct support to any of these possible mechanisms of TRAF downstream signaling. Further structural studies, in conjunction with other research methods, are required to determine the basis of downstream signaling by the TRAF proteins.

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