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The Structural Basis for the Recognition of Diverse Receptor Sequences by TRAF2

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Summary

Many members of the tumor necrosis factor receptor (TNFR) superfamily initiate intracellular signaling by recruiting TNFR-associated factors (TRAFs) through their cytoplasmic tails. TRAFs apparently recognize highly diverse receptor sequences. Crystal structures of the TRAF domain of human TRAF2 in complex with peptides from the TNFR family members CD40, CD30, Ox40, 4-1BB, and the EBV oncoprotein LMP1 revealed a conserved binding mode. A major TRAF2-binding consensus sequence, (P/S/A/T)x(Q/E)E, and a minor consensus motif, PxQxxD, can be defined from the structural analysis, which encompass all known TRAF2-binding sequences. The structural information provides a template for the further dissection of receptor binding specificity of TRAF2 and for the understanding of the complexity of TRAF-mediated signal transduction.

Introduction

Following activation by tumor necrosis factor (TNF) and related cytokine ligands, members of the TNF receptor (TNFR) superfamily can elicit a wide spectrum of cellular responses, including proliferation, differentiation, and apoptosis (for review, see Smith et al., 1994). A few TNFRs, including Fas and TNF-R1, use the death domains in their intracellular regions to signal cell death (Chinnaiyan et al., 1995; Hsu et al., 1995), culminating in caspase activation. In contrast, the majority of TNFRs recruit the TNFR-associated factor (TRAF) family of intracellular adapter molecules to promote cell survival by the activation of downstream protein kinase cascades and, ultimately, transcription factors in the NF-κB and AP-1 family (for review, see Arch et al., 1998). These transcription factors can then turn on numerous genes involved in immune, inflammatory, and acute phase responses, as well as in sustaining proliferation during tumorigenesis. The constitutive recruitment of TRAFs by the EBV oncoprotein LMP1 is an important component for EBV to potentiate growth and transformation (Devergne et al., 1996; Kaye et al., 1996).

TRAF2 was isolated biochemically from the TNF-R2 signaling complex (Rothe et al., 1994) and is the prototypical member of the TRAF family (TRAF1 through TRAF6) (Hu et al., 1994; Rothe et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Regnier et al., 1995; Cao et al., 1996; Ishida et al., 1996a, 1996b; Nakano et al., 1996). It can interact directly with and mediate the signal transduction of a number of TNFRs, including CD30, CD40, CD27, Ox40, 4-1BB, ATAR, and the EBV protein LMP1. TRAF2 can also interact indirectly with TNF-R1 via the adapter molecule TRADD and thereby couples TNF-R1 to TRAF signaling as well (Hsu et al., 1996b). In addition, TRAF2 has been shown to interact with a number of intracellular proteins such as I-TRAF/TANK (Cheng and Baltimore, 1996; Rothe et al., 1996), RIP (Stanger et al., 1995; Hsu et al., 1996a), and the caspase inhibitors cIAPs (Rothe et al., 1995).

Of the other TRAF family members, TRAF1, 3, and 5 have similar patterns of receptor binding specificity as TRAF2. TRAF6 was identified by its involvement in the IL-1 receptor signaling pathway (Cao et al., 1996) and by using CD40 in a yeast two-hybrid screen (Ishida et al., 1996a). Since then it has also been shown to interact directly with several other TNFRs, such as RANK and the p75 NGF receptor (Darnay et al., 1999; Khursigara et al., 1999). Finally, TRAF4 is not known to interact with any receptors, which may be consistent with its proposed nuclear localization (Regnier et al., 1995).

Targeted gene disruption experiments have established the important biological roles of TRAFs. Mice deficient in TRAF2, TRAF3, or TRAF6 have increased perinatal and postnatal lethality (Xu et al., 1996; Yeh et al., 1997; Lomaga et al., 1999). TRAF2-deficient mice exhibited atrophy of immune organs, and cells in the hematopoietic lineage were highly sensitive to TNF-induced cell death (Yeh et al., 1997). A lack of TRAF3 in mice resulted in a depletion of peripheral white blood cells. Reconstituted hematopoietic lineages from fetal liver cells were also impaired in T cell–dependent immune responses (Xu et al., 1996). The disruption of TRAF6 in mice led to defective bone metabolism and impaired CD40 and IL-1 signaling (Lomaga et al., 1999), consistent with the receptors with which it interacts.

The extracellular domains of the TNFRs share extensive sequence homology and bind to a family of homologous TNF-like cytokines. These cytokines are intrinsically trimeric and activate the receptors by trimerizing the bound receptor molecules. This extracellular recognition has been demonstrated by the crystal structure of the complex between the ligand LTα and the TNF-R1 receptor (Banner et al., 1993).

The intracellular domains of TRAF-interacting TNFRs do not possess recognizable similarity in primary sequences. A series of studies have identified short linear TRAF-binding sites that are generally less than 20 residues in length, even though the entire intracellular domains of these receptors can contain close to 200 residues. Several apparently different consensus sequences have been proposed to bind to TRAF2, including the PxQx(T/S/D) (x = any amino acid) motif in LMP1, CD30, CD40, and CD27 (Devergne et al., 1996; Franken

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Table 1. Crystallographic Analysis

Protein construct	310–501	327–501	327–501	327–501	327–501	327–501
Peptide[a]	hCD40 (250–266) PVQETLHGCQPVTQED	hCD40 (250–254) PVQET	hCD30 (576–583) MLSVEEEG	hOx40 (262–266) PIQEE	m4-1BB (231–236) GAAQEE	hLMP-1 (204–210) PQQATDD
Space group	$P2_1$	R3 (rhom. setting)	$P2_1$	C2	C2	$P2_1$
Cell dimensions	59.4 Å, 81.1 Å, 77.2 Å, 96.8°	111.4 Å, 103.7°	91.3 Å, 110.4 Å, 91.3 Å, 120.0°	136.4 Å, 85.6 Å, 125.4 Å, 118.9°	135.9 Å, 85.6 Å, 124.1 Å, 119.1°	56.2 Å, 76.8 Å, 66.9 Å, 93.2°
X-ray source	CHESS A1	CHESS A1	BNL X4A	CHESS A1	BNL X4A	BNL X4A
Z_a (protein)[b]	3	8	6	6	6	3
Z_a (peptide)[b]	2	8	3	6	5	2
Resolution	2.7 Å	2.0 Å	2.0 Å	2.0 Å	2.5 Å	2.0 Å
R_{merge}	4.4% (25.5%)	5.5% (18.0%)	6.1% (19.9%)	3.8% (12.7%)	6.1% (29.6%)	5.5% (15.0%)
Completeness	98.6% (99.4%)	100.0% (99.9%)	96.5% (93.8%)	99.8% (100%)	90.2% (85.2%)	99.7% (100%)
Number of water	104	616	827	715	239	391
R (R_{free})	22.1% (26.7%)	21.9% (25.4%)	22.0% (24.4%)	22.5% (25.1%)	22.7% (27.0%)	20.6% (24.5%)

[a]Residue numbers refer to the precursor receptor sequences. h, human; m, mouse.
[b]Z_a (protein), number of protein molecules per crystallographic asymmetric unit; Z_a (peptide), number of peptides per asymmetric unit.

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). The binding sites for TRAF6, however, differ from all the motifs described above.

The primary sequence of TRAF proteins may be generally divided into an amino-terminal domain with RING and zinc finger motifs and a carboxy-terminal TRAF domain (Rothe et al., 1994). The TRAF domain can be further subdivided into a TRAF-N domain with propensities for coiled-coil structures and a highly homologous TRAF-C domain. While the amino-terminal domain is essential for the activation of downstream effectors, the TRAF domain is both necessary and sufficient for self-association and receptor interaction. We have recently reported the crystal structures of the TRAF domain of human TRAF2, both alone and in complex with a TNF-R2 peptide (Park et al., 1999). The structures revealed a conserved trimeric self-association of the TRAF domain that is mediated by both the β sandwich TRAF-C domain and the preceding coiled-coil region. Each TRAF domain trimer provides three symmetrical receptor binding sites for the TNF-R2 peptides, suggesting an avidity component in the recognition of receptors by TRAFs.

Since there is no apparent sequence homology between the ϕSxEE motif possessed by the TNF-R2 peptide and the other motifs, the TRAF domain structure in complex with the TNF-R2 peptide does not provide information on whether the same receptor binding site may accommodate the different sequence motifs or additional binding sites may exist on the surface of the TRAF domain. In this study, we determined crystal structures of the TRAF domain of human TRAF2 in complex with receptor peptides from each of the three proposed motifs. The structures revealed a common receptor binding site on the TRAF domain and a conserved mode of recognition, even though not a single residue is identical among the different TRAF binding sequences. Unified TRAF-binding motifs can be defined from the structural studies, which explain the specificity of all known TRAF binding sequences and therefore the structure basis for the recognition of diverse receptor sequences by TRAF2. In addition, these consensus motifs provide a template for the further dissection of receptor binding specificity of TRAF2 using structure-based mutagenesis experiments.

Results

Peptide Selection and Structure Determination

In order to obtain a complete understanding of TRAF receptor recognition, we chose representative sequences from each of the three proposed TRAF-binding motifs (Table 1). The six receptor peptides studied here include sequences from CD40 and LMP1 (with the PxQxT motif), from Ox40 and 4-1BB (QEE motif), and from CD30 (ϕSxEE motif). The lengths of the peptides were mostly based on binding and mutagenesis studies reported in the literature, including the peptide from CD30, LMP1, and the long and short peptides from CD40. The peptides from Ox40 and 4-1BB were selected by including a few residues N-terminal to the QEE motif. All the receptor peptides were chemically synthesized with both amino-terminal acetylation and carboxy-terminal amidation to mimic the environment in the native protein.

As most of the receptor binding sites in the apo-TRAF2 crystals were blocked by crystal packing interactions, soaking of the peptides into these preformed crystals is unlikely to produce complexes. Cocrystallization experiment was used with each of these receptor peptides. Molar excess of the peptides (from 2- to 16-fold) was incubated with the TRAF domain of human TRAF2. Two alternative TRAF domain constructs with different lengths of the coiled-coil domain were used in crystallizing the complexes (Table 1). The six receptor–peptide complexes produced six different crystal forms (Table 1), with different packing arrangements of the complexes in the crystals. X-ray diffraction data for the six complexes were collected with synchrotron radiation on cryoprotected crystals. The structures were determined by the molecular replacement method, and the peptides were located by Fo−Fc difference electron density maps after initial refinement with protein models alone (Table 1).

All crystal forms contain at least three TRAF domain molecules per crystallographic asymmetric unit (Table 1). As was observed with the TNF-R2 peptide, one or more of the receptor binding sites on the TRAF domain

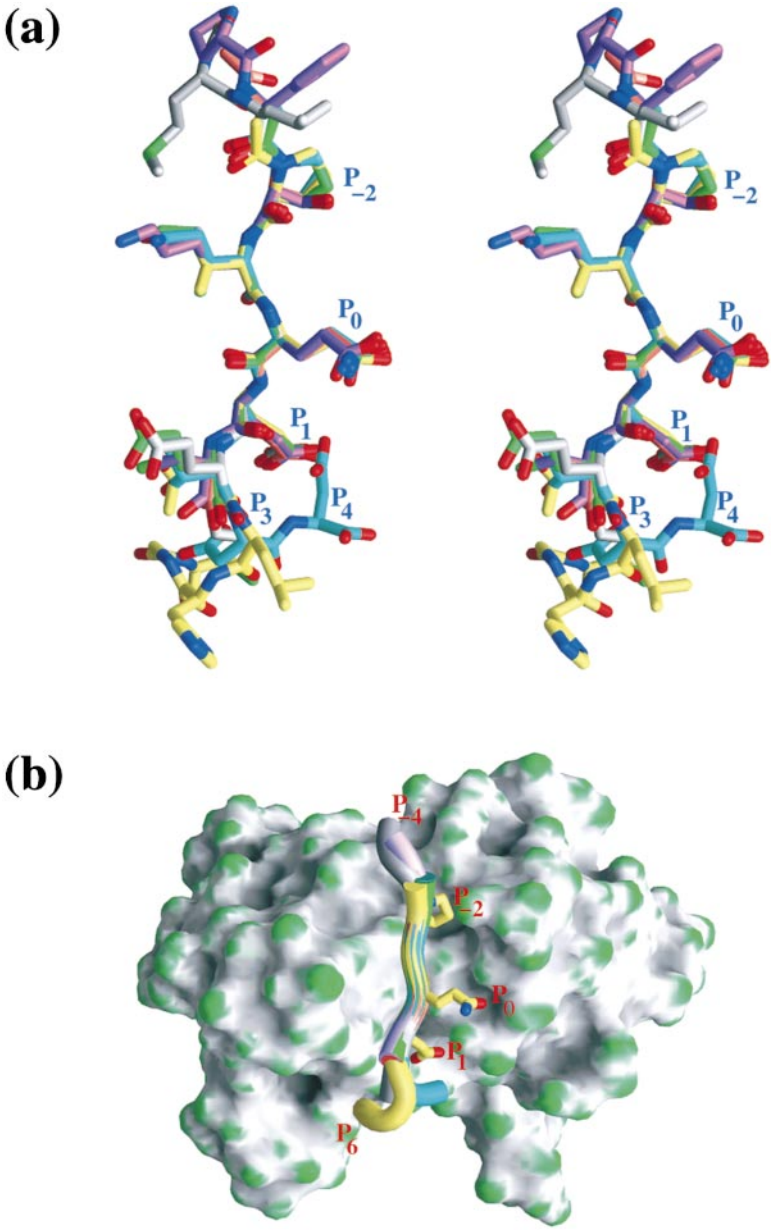

Figure 1. Conserved Recognition of Receptor Sequences by the TRAF Domain

(a) Stereo diagram of the structural superposition of receptor peptides. Nitrogen atoms, blue; oxygen atoms, red; sulfur atoms, green; carbon atoms, yellow (CD40), gray (CD30), green (Ox40), pink (4-1BB), cyan (LMP1), and purple (TNF-R2; PDB code 1ca9). Peptides from CD40, CD30, Ox40, 4-1BB, and LMP1 contain ordered acetyl groups at the amino termini.

(b) Surface contour representation of one protomer of the TRAF domain of structure, shown with the 3-fold axis vertical. The superimposed worm models of receptor peptides are shown in yellow (CD40), gray (CD30), green (Ox40), pink (4-1BB), cyan (LMP1), and purple (TNF-R2; PDB code 1ca9). The three key side chains (stick models) identified from the structural analysis, at positions P_{-2}, P_0, and P_1, are shown to reside in three distinct pockets on the TRAF domain surface.

trimer were not occupied by peptides from several of the receptors (Table 1). These unoccupied binding sites are involved in crystal packing interactions, consistent with the low affinity of TRAF2 for monomeric receptor peptides. On the other hand, fully occupied TRAF domain trimers were seen in the CD30, Ox40, 4-1BB, and one of the CD40 complex crystals, confirming that the three receptor binding sites of the TRAF domain trimer are capable of interacting with receptors simultaneously. A minimum of two copies of peptides were bound in each crystal, in which they assume essentially identical binding mode to the TRAF domain.

Conserved Binding Mode of the Different Receptor Peptides

The crystal structures of the TRAF domain of human TRAF2 in complex with six peptides from different receptors have been determined at up to 2 Å resolution. Together with the TNF-R2 peptide complex reported by us earlier (Park et al., 1999), these seven structures provide multiple observations in different crystal packing environments for the binding modes of receptor peptides from each of the three proposed TRAF-binding motifs—three structures for the PxQxT motif (one from LMP1 and two from CD40), two structures each for the ϕSxEE motif (CD30 and TNF-R2), and the QEE motif (Ox40 and 4-1BB). This collection of the different structures allowed a detailed comparison of the binding modes of the various receptor peptides.

Despite the high degree of sequence variations in the receptor peptides, the structures revealed that the peptides have a conserved binding mode and share a common binding site on the TRAF domain (Figure 1). Residues in this binding site are highly conserved among TRAF1, 2, 3, and 5, consistent with their overlapping specificity of receptor recognition (Arch et al., 1998).

TRAF4 and TRAF6, on the other hand, have nonconserved substitutions in the binding site, explaining the difference in receptor specificity. The six structures reported here, together with the two reported by us earlier, provide a total of 14 copies of the TRAF domain trimer, in the free state and in complex, with 1 to 3 copies of the receptor peptides. Structural comparisons among these different states showed that the TRAF domain trimer undergoes little conformational change upon receptor binding.

Structural superposition of the seven different structures of receptor peptide complexes indicated a conserved central core of four residues, giving rise to a root-mean-square (rms) distance of less than 0.1 Å among the main chain atoms of these residues (Figure 1). The structure-based sequence alignment of the receptor peptides (Figure 2) showed that the third position of this four-residue core is invariably occupied by either a Gln or Glu residue and possesses the highest degree of sequence conservation. We propose to denote this residue as the zero position (P_0) of the TRAF-binding motif. The conserved structural core of the receptor peptides, and therefore the TRAF-binding motif, then extends from the P_{-2} to the P_1 positions. This major TRAF-binding motif has the consensus sequence (P/S/A/T)x(Q/E)E (with the exception of the peptide from LMP1; see below), which can be identified in most of the known TRAF binding sequences from TNFRs (Figure 2). This structure-based consensus sequence also explains the three TRAF-binding motifs (PxQxT, ϕSxEE, and QEE) proposed from earlier biochemical and mutagenesis studies (Figure 2).

Outside this conserved core of P_{-2} to P_1 residues, the residue at P_2 shows some degree of conservation of the main chain conformation, whereas additional C-terminal residues (P_3 and beyond) have large conformational differences among the various peptides (Figure 1). At the N-terminal side, the P_{-3} residue appears to have reasonable conservation of the main chain conformation as well, although several of the peptides only have an acetyl group at this position (Figure 1). Therefore, a more relaxed definition of the TRAF binding core sequence would include the P_{-3} to the P_2 residue, covering six amino acids.

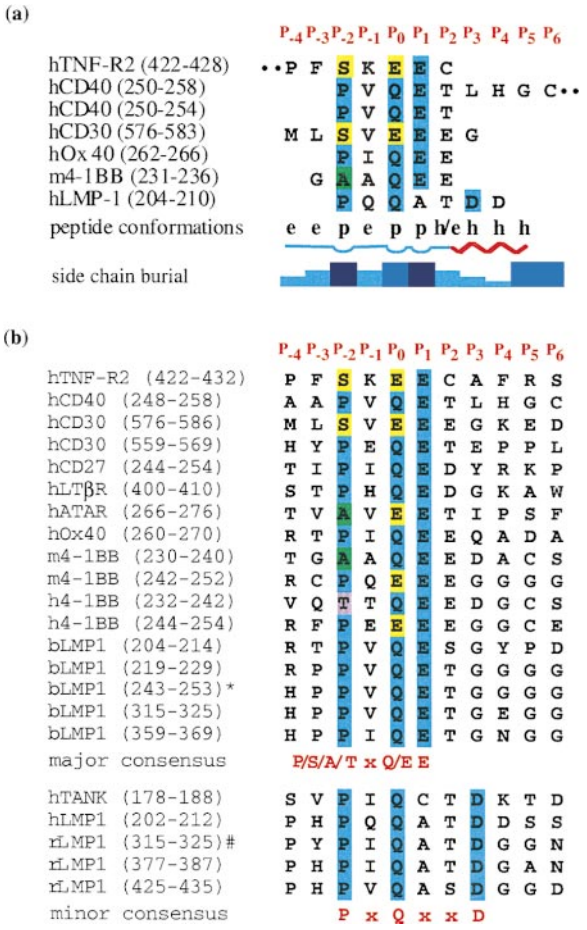

Figure 2. Structure-Based Receptor Peptide Alignment

(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. The main chain conformation of each residue is listed: β conformation (e), polyproline II helix conformation (p), and helical conformation (h), which are also represented graphically below the letter symbols. Positions recognized specifically by the TRAF domain are shaded respectively in colors. The percentage of surface burial for each side chain is represented by different shades of blue: dark blue (>90%), navy blue (50%–70%), and sky blue (20%–50%). The variation of surface burial at each position for the different peptides is inversely proportional to the height of the color bar: highest (<4%), second (6%–10%), third (15%–16%), and fourth (26%). There is only one observation for the P_5 and P_6 positions, which have 0% variations. 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. The consensus TRAF-binding motifs are shown in red. 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.

Interactions between Receptor Peptides and TRAF2

The amino-terminal part of the receptor peptides is extended, while the carboxy-terminal portion becomes helical at or after the P_2 position (Figure 2). Detailed analysis revealed that residues at P_{-2}, P_0, and P_1 possess the conformation of a polyproline II helix (PPII), even though they are mostly nonprolines in the peptides studied here. PPII is a slightly twisted β strand that allows a side chain periodicity of three per turn. A recent survey showed that a majority of proteins with known structures contain at least one PPII helix (Stapley and Creamer, 1999). The PPII conformation is also frequently used in protein–peptide interactions such as those seen in the peptide recognition by SH3 domains (Lim et al., 1994) and class II MHC molecules (Stern et al., 1994). This conformation allows the peptide chain to twist in order to maximize the interaction of its side chains with a protein surface. The PPII conformation for three out of the four core residues in the TRAF binding sequences allows the near complete side chain burial at both the P_{-2} and the P_1 residues (Figure 2).

The bound receptor peptides contact the TRAF-C domain exclusively, extending from the top to the bottom rim of the mushroom-shaped TRAF domain trimer. The peptide chains cut across four β strands (β6, β7, β4,

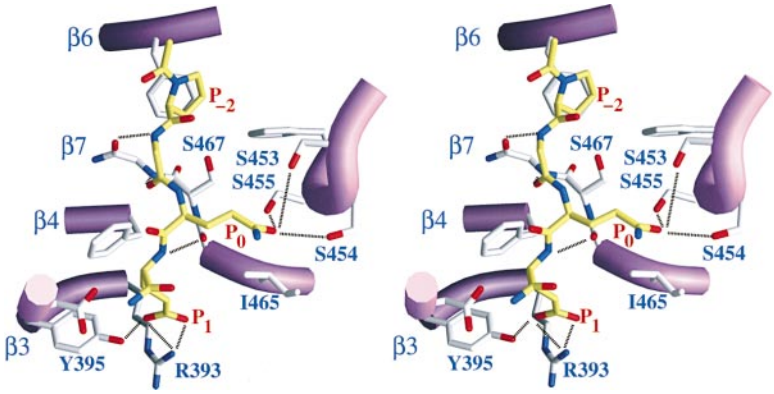

Figure 3. Detailed Interactions for the Major Consensus Motif (P/S/A/T)x(Q/E)E

Shown is the stereo diagram of the interaction between the TRAF domain and the CD40 peptide, with the molecular 3-fold axis vertical. The TRAF domain is represented by purple worm-and-stick models, with carbon atoms in gray. The peptide is shown as a stick model, 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. The conformations of the two CD40 peptides with different lengths (Table 1) are essentially identical. 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 interactions at the P_{-2}, P_0, and P_1 positions of the peptide.

and β3) on one side of the β sandwich structure of the TRAF-C domain. Although the direction of the peptide chains is essentially perpendicular to these β strands, the main chain atoms at the P_{-1} and the P_1 positions are able to form, in a highly twisted fashion, conserved antiparallel β edge hydrogen-bonding interactions with the β7 strand (Figure 3; Park et al., 1999). Additionally, the interaction between the amide of the P_2 residue and the carboxylate of D399 in TRAF2 is also conserved. These main chain interactions appear to be important factors in anchoring the core portion of the peptide to the TRAF domain.

The specificity of the TRAF2–TNFR interactions appears to be determined by the side chains at the P_{-2}, P_0, and P_1 positions within the TRAF-binding motif (Figure 3). They each engage into shallow but distinct pockets on the surface of TRAF2 (Figure 1). The P_{-2} position is most frequently occupied by Pro and less frequently by Ser and Ala (Figure 2). Pro makes extensive van der Waals contacts with the TRAF2 protein. In addition, the PPII conformation adopted by this residue may increase the preference for Pro at this position. The selectivity for Ser appears to derive from the hydrogen bond between its hydroxyl and the side chain of S467 in TRAF2, which may serve to anchor the Ser residue into a PPII instead of a typical β conformation. The side chains at this position are completely buried in a pocket on the TRAF domain surface. Model building of other amino acids at this position suggests that the size and the enclosure of the pocket may allow other small side chains such as Thr and Cys but may restrict the accommodation of larger side chains. Ala was observed at this position in the 4-1BB peptide, but the nature of the interaction may lower the affinity of the peptide, which is also consistent with the weaker electron density for the peptide in this structure.

Shape complementarity and hydrogen-bonding interactions appear to be the major determinants for the selectivity of Glu and Gln residues at the P_0 position (Figure 3). The aliphatic portion of the side chains packs against I465, while the hydrophilic tip is surrounded by the three hydroxyls of S453, S454, and S455 in TRAF2. The Gln side chain is within hydrogen-bonding distances to all three hydroxyl groups, perhaps making this one of the strongest anchoring points in the interaction. However, when this position is occupied by Glu (as in CD30 and TNF-R2), only one hydrogen bond may be possible, as the carboxylate side chain is positioned further away from the TRAF2 surface. Since there are no charged residues near the vicinity of the P_0 site, this difference may arise from the need for the negative charge in Glu to be more heavily solvated than its Gln counterpart.

The P_1 position in the major TRAF-binding motif is occupied by a Glu residue, although LMP1 has an Ala at this position (see below). The carboxylate moiety of the Glu residue makes bidentate ion pair interactions with the side chain guanidinium group of R393 and an additional hydrogen bond with the hydroxyl of Y395 (Park et al., 1999). These hydrogen-bonding interactions appear to require Glu specifically, as an Asp residue is too short to reach R393 and Y395 in TRAF2.

The side chains of the remaining residues of the receptor peptides are generally exposed on the surface of the complex, and these residues show little sequence conservation among the various TNFRs (Figure 2). The P_{-1} residue of the core motif appears to be mostly a spacer. The side chain of the P_2 residue is situated close to that of D399 in TRAF2. When P_2 is occupied by Thr, potential hydrogen-bonding interactions are observed with the side chain of D399. Somewhat surprisingly, several of the receptor peptides have an acidic residue (Asp or Glu), whereas none have a basic residue (Lys or Arg) at this position. It may be likely that this position may tolerate many other variations that have yet to be observed.

An Alternative Binding Mode Defined by the LMP1 Peptide

The TRAF-binding motif discussed so far has a conserved Glu residue at the P_1 position, which is involved in important ion pair and hydrogen-bonding interactions with TRAF2. This motif is consistent with the majority of known TRAF-binding receptor peptides (Figure 2). However, receptor peptides from LMP1 and TANK have an Ala and Cys, respectively, at the P_1 position (Figure 2), which can not participate in the ion pair and hydrogen-bonding interactions. The structure of the LMP1 peptide in complex with TRAF2 showed that the interactions with the R393 and Y395 residues are mediated by an

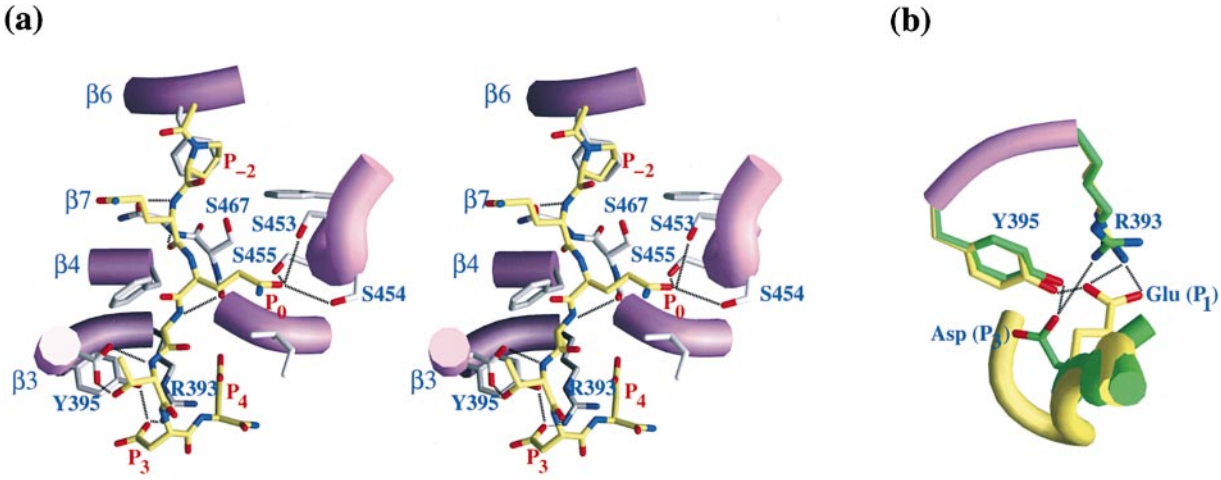

Figure 4. Detailed Interactions for the Minor Consensus Motif PxQxxD

(a) Shown is the stereo diagram of the interaction between the TRAF domain and the LMP1 peptide in an orientation similar to Figure 3. The TRAF domain is represented by purple worm-and-stick models, with carbon atoms in gray. The peptide is shown as a stick model, with carbon atoms in yellow. 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 interaction at the P_3 position of the peptide, which distinguishes the minor TRAF2-binding motif from the major TRAF2-binding motif.

(b) Structural comparison between the conformation of Glu at the P_1 position and that of Asp at the P_3 position, showing with 90° rotated around the horizontal axis in (a). The CD40 peptide is shown as a yellow worm, with the carbon atoms of the P_1 side chain (Glu) in yellow. The LMP1 peptide is shown as a green worm, with the carbon atoms of the P_3 side chain (Asp) in green. The R393 and Y395 side chains from TRAF2 are shown respectively, with carbon atoms in yellow and green for the CD40 complex and the LMP1 complex. Note the conformational change in the R393 side chain.

Asp at the P_3 position of this peptide (Figure 4). The side chain of R393 undergoes a small conformational change to accommodate this new interaction. This structural information, together with sequence analysis, suggests that there is a minor consensus sequence for TRAF-binding peptides—PxQxxD (Figure 2). All the naturally occurring sequences identified so far contain a Pro at the P_{-2} position and a Gln at the P_0 position. It is likely, however, that both positions would allow similar variations as seen in the major TRAF-binding consensus sequence.

The P_4 residue in the LMP1 peptide studied here is also an Asp, although this residue is not conserved among other TRAF binding sequences (Figure 2). The side chain is located near the P_1 Glu residue of the major TRAF-binding consensus sequence (Figure 1), suggesting that it might be able to compensate for a loss of the P_3 Asp residue. This is consistent with mutagenesis studies on LMP1 that showed that the Asp residues at both P_3 and P_4 positions need to be mutated to abolish TRAF binding (Devergne et al., 1996).

Discussion

Universal TRAF2-Binding Motifs

Our structural studies resulted in two TRAF2-binding consensus motifs: the major consensus motif (P/S/A/T)x(Q/E)E and a minor PxQxxD motif. There are three components for each of the motifs. The P_0 position is occupied by Gln or Glu in the major consensus and exclusively by Gln in the minor consensus. The P_{-2} position is occupied mostly by Pro, followed by Ser, Ala, and Thr in the major consensus, while the minor consensus only has Pro at this position. The structural similarity between the P_{-2} and P_1 regions of the receptor peptides suggests that the P_{-2} and P_0 positions will be subjected to the same selectivity for both consensus motifs. The distinguishing factor between the two motifs resides on the last residues: the Glu at the P_1 position for the major consensus and the Asp at the P_3 position for the minor consensus sequence. Structurally, similar interactions are seen between the acidic side chains of Glu at P_1 or Asp at P_3 and the conserved TRAF2 residues R393 and Y395.

All of the binding sequences identified so far for TRAF1, 2, 3, and 5 are consistent with the motifs defined from this study (Figure 2). In addition to receptors, the intracellular protein I-TRAF/TANK (Cheng and Baltimore, 1996; Rothe et al., 1996) possesses the PxQxxD minor consensus sequence and therefore is likely to bind to TRAFs via the same receptor binding site. The universal TRAF-binding motifs also explain the paradox between the inability to bind and the bearing of the previously identified PxQx(T/S) motif. These sequences include PPQLTEE (320–326) and PVQLSYY (379–385) in human LMP1 and other similar sequences in rhesus and baboon LMP1 (Devergne et al., 1996; Franken et al., 1996), in which the P_1 or P_3 position does not conform to either the major or the minor consensus sequences.

While more extensive mutations are required to determine the precise specificity of each position of the receptor peptides, existing alanine mutagenesis data are clearly consistent with the definition of important side chains in the binding motifs. In CD30, which contains two TRAF-binding sites, peptide binding and alanine mutagenesis has delineated a region between P_{-3} and P_2 for the binding of both TRAF binding sequences to TRAF2 (Boucher et al., 1997). Deletion mutagenesis in

CD40 has mapped the minimal TRAF binding sequence to the five-residue peptide between the P_{-2} and the P_2 positions as assayed by in vitro binding to immobilized receptor peptides (Pullen et al., 1998). In Ox40, triple alanine mutagenesis of residues at P_{-3}–P_{-1} or P_0–P_2 resulted in complete loss of TRAF interaction in yeast two-hybrid experiments, while similar mutations at P_3–P_5 only resulted in reduced signal (Arch and Thompson, 1998). Detailed alanine mutagenesis on LMP1 has indicated that the P_{-2} and P_0, as well as the P_3 and P_4, residues are the most important on TRAF binding and NF-κB activation (Devergne et al., 1996).

The conserved TRAF-binding motifs may be used to locate the precise binding region in proteins known to interact with TRAFs. Intracellular adapter molecules RIP and TRADD have been shown to interact with TRAF2 via the conserved TRAF-C domain (Hsu et al., 1996a, 1996b). Inspection of the RIP sequence revealed two potential TRAF-binding sites, one near the end of the amino-terminal kinase domain and the other within the linker region between the kinase and the carboxy-terminal death domain. These locations are consistent with the broader TRAF-binding regions identified earlier in RIP (Hsu et al., 1996a). The TRADD molecule, which is the direct signal transducer for TNF-R1 and couples the receptor to both caspase activation and TRAF signaling, does not appear to contain the same TRAF binding sequences identified from this study. It remains to be seen how TRAF2 recognizes TRADD.

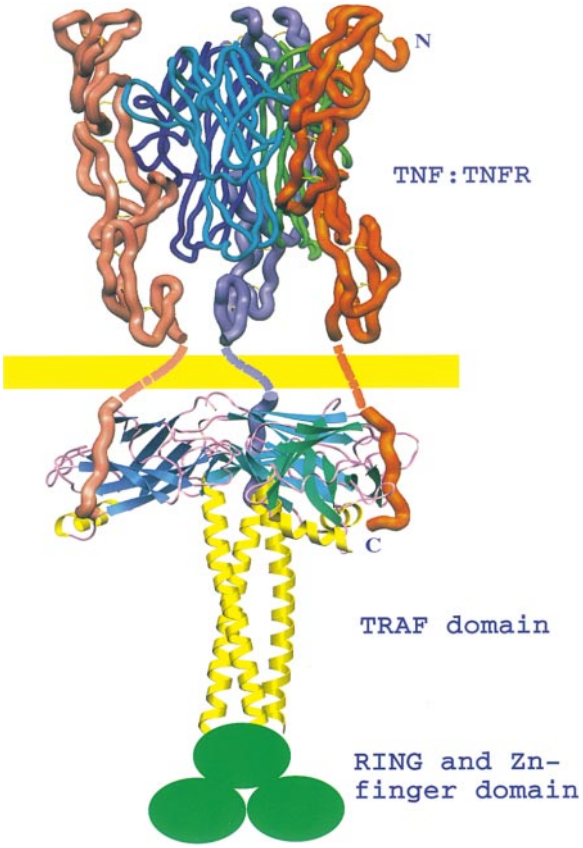

Figure 5. A Model for the Activated TRAF–Receptor Complex

The atomic coordinates for the complex between a TNF-like ligand and a TNFR were taken from PDB entry 1tnr and are shown as thin worms (green, light blue, and darker blue) for the ligand and thick worms (pink, orange, and blue) for the receptor. The TRAF domain structure from the CD40 complex is shown as ribbon traces, with the β strands in green, cyan, and dark blue for each of the protomers of the trimer. The receptor peptides (pink, orange, and blue) are shown as the CD40 peptide with an extended amino terminus from the CD30 peptide. Dotted lines are used to connect the extracellular and the intracellular portions of the receptor. The amino-terminal RING and zinc finger domains of the TRAF trimer are shown as green ovals. Cell membrane is represented in yellow.

The Extent of a TRAF-Binding Site

While the core of TRAF binding sequences may contain only four residues, those beyond the core may make further contacts with the TRAF domain of TRAF2. The total of seven different receptor peptide complexes may be used to estimate the maximum length of a TRAF binding sequence. The TRAF domain complex with TNF-R2 contains ordered residues starting from the P_{-4} position, even though additional residues exist at the amino terminus in the peptide used in the study (Park et al., 1999). Similarly, the peptide structure in the CD40 complex contains ordered residues only up to the P_6 position despite an extended carboxyl terminus in the CD40 peptide. These results then suggest that a complete TRAF2 binding sequence may contain 11 residues (from P_{-4} to P_6), which covers the entire span of one face of the TRAF domain surface (Figure 1). It should be kept in mind, however, that the conformations of end residues appear highly dependent on their side chain chemistry, and thus the actual lengths of the TRAF-binding regions may vary somewhat from receptor to receptor.

The natural lengths of the intracellular domains of the TNF receptors may range from 36 residues, as in Ox40, to 188 residues, as in CD30. Amino acid analyses using the PHD program (Rost and Sander, 1994) suggest that most of these receptors may exist in near random coil conformations with low secondary structure components. This observation further supports the hypothesis that the TRAF-binding sites of these receptors are primarily composed of linear sequences within the 11-residue extent estimated from our structural studies, rather than three-dimensional composites. However, secondary interactions may ensue after the central TRAF binding sequences are docked onto the TRAF domain surface. The flanking regions may also modulate the exposure and the dynamic behavior of the major TRAF2-binding determinants, thereby exerting influences on their functions. In addition, those regions outside the TRAF-binding site may mediate TRAF2-independent functions as shown for both CD40 and LMP1 (Izumi and Kieff, 1997; Pullen et al., 1998).

Geometry of TRAF2–Receptor Interactions

Each receptor peptide in all seven complexes binds symmetrically to the side of the mushroom-shaped TRAF domain trimer, extending from the top to the bottom rim of the mushroom cap. This directionality of the bound receptor peptides relative to the TRAF domain would bring the β sandwich portion (mushroom cap) of the TRAF domain trimer close to the membrane in the

activated signaling complex (Figure 5). This then allows the RING finger and zinc finger domains, which are amino-terminal to the coiled-coil domain in the full-length TRAF proteins, to be exposed to the cytosol for interaction with downstream effector molecules.

This geometry of the TRAF–receptor interaction requires minimal linker residues between the membrane and the TRAF-binding site. In keeping with this analysis, the 17-residue CD40 peptide used in this study has been shown to be capable of both TRAF interaction and wild-type-like NF-κB activation when linked immediately after the transmembrane region of the CD40 receptor (Cheng and Baltimore, 1996).

The presence of two identified TRAF-binding sites in CD30 and 4-1BB raises the possibility of variations regarding the stoichiometry of TRAF–receptor interaction. The first possibility relates to whether the two TRAF-binding sites in the trimerized receptors may recruit two TRAFs simultaneously. Assuming a minimal 4-residue TRAF binding sequence extending from P_{-2} to P_1, there would be 8 and 12 residues, respectively, between the two TRAF-binding sites in 4-1BB and CD30, leaving less than 50 Å maximal distance between the two TRAF-binding sites. This distance is unlikely to be sufficient to accommodate two TRAFs simultaneously, considering that the full-length TRAFs would have a size of at least 70 × 70 × 70 Å.

A second possibility is whether the two TRAF-binding sites within the same receptor molecule may interact with the neighboring protomers in a single TRAF domain trimer. The linear distance between the P_1 position of the first site to the P_{-2} position of the neighboring site in the TRAF domain trimer is 57 Å, which would require a minimum of 15, and preferably more than 20, residues between the two adjacent sites. This analysis excludes the possibility for 4-1BB and CD30 to carry out this intramolecular multivalent association. In 4-1BB, deleting either one of the binding sites resulted in reduced binding in yeast two-hybrid assays (Arch and Thompson, 1998). The larger signal observed in the yeast two-hybrid assay when both TRAF-binding sites are present may therefore be due to the increased probability of interaction by the presence of two sites. While each site in CD30 binds to TRAF proteins, it has not been tested whether the existence of two sites would increase the binding strength to TRAFs.

Multiple TRAF-binding sites have been identified in both rhesus and baboon EBV LMP1 (Franken et al., 1996). In the human virus, LMP1 contains one TRAF-binding site, and therefore the constitutive TRAF activation requires aggregation by the six-span transmembrane region. As there are sufficient spacings between some of the TRAF-binding sites in both rhesus and baboon LMP1, it may be predicted that some of these sites may bind to a single TRAF trimer simultaneously to mediate constitutive association and signaling, even in the absence of aggregation through their transmembrane helices.

TRAF-Mediated Signal Transduction

Our study has suggested a common mechanism by which TRAFs perceive the oligomerization state of the receptors. The interaction between TRAF2 and monomeric receptors is relatively weak (K_d = 0.04–1.5 mM; H. Y. and H. W., unpublished results), which ensures that TRAFs do not interact with nonactivated receptors. The dependence of TRAF recruitment on ligand-induced receptor trimerization or antibody-induced receptor dimerization has been shown for several TNFRs, including TNF-R2, CD40, and LTβR (Shu et al., 1996; Kuhne et al., 1997; VanArsdale et al., 1997), and is considered a common feature of the entire receptor family. Both affinity and specificity will be amplified exponentially by the avidity contribution from the oligomeric association, which is the signaling form of the interaction. This avidity dependence of the interaction makes it possible to transform a low-affinity and somewhat promiscuous interaction into a tight and highly specific one.

The ability of TRAFs to recognize diverse receptor sequences forms the basis for the wide spectrum of biological effects that TRAFs mediate. Both competitive and cooperative dynamics of TRAF signaling may result from the conserved recognition of receptors and other signaling molecules by TRAF proteins. This would relate the outcome of a particular receptor activation to the repertoire of TRAFs and TRAF-binding partners in a particular type of cell and at a certain stage of differentiation.

TRAF signaling may derive from induced proximity of downstream effectors. This mode of intracellular signaling has frequently been used for enzymatic activation of receptor tyrosine kinases (Ullrich and Schlessinger, 1990) and proposed for receptor-mediated caspase activation (Muzio et al., 1996, 1998). It has been suggested that TRAF proteins may couple to a MAP kinase cascade, and several MAP3K-like kinases, including NIK (Malinin et al., 1997) and ASK1 (Nishitoh et al., 1998), have been suggested to involve TRAF signaling. It remains to be demonstrated whether TRAFs are induced to trimerize by the recruitment to receptor tails, and thus activate downstream molecules, or use alternative processes for the downstream signaling.

Experimental Procedures

Protein Preparation and Crystallization

The TRAF domain proteins of human TRAF2 were prepared as described earlier (Park et al., 1999). Briefly, two constructs of the TRAF domain of TRAF2 (residues 315–501 and 310–501) were overexpressed in *Escherichia coli* and purified by nickel affinity chromatography (Qiagen) and gel filtration (Superose 12, Amersham Pharmacia). The protein containing residues 315–501 was treated with trypsin to generate the construct containing residues 327–501. The TRAF domain proteins (residues 310–501 and residues 327–501) were crystallized in the presence of molar excess receptor peptides (1:2 to 1:16 ratios). The reservoir conditions are 11% PEG4000, 0.2 M ammonium acetate, and 0.1 M citrate at pH 5.6 for the TRAF2 protein containing residues 310–501 and 8%–15% PEG4000 in 0.1 M MES at pH 6.0 for the TRAF2 protein containing residues 327–501.

Structure Determination and Analysis

All X-ray diffraction data were collected at synchrotron radiation sources on cryoprotected crystals and processed with the HKL package (Otwinowski and Minor, 1997). The structures were determined by molecular replacement calculations with the program REPLACE (Tong, 1993), using the trimeric atomic model of the apo-TRAF domain structure (PDB code 1ca4), and refined using program CNS in the presence of noncrystallographic symmetry restraints (Brünger et al., 1998). Difference Fourier maps calculated with the $|Fo|-|Fc|$ coefficients were used to locate the bound peptides. Sequence fitting and model building were carried out with the program

O (Jones et al., 1991). Molecular surface representations and stick models were produced with the program GRASP (Nicholls et al., 1991), and ribbon diagrams were generated using the program Setor (Evans, 1993).

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Protein Data Bank ID Codes

The ID codes for the structures reported in this article are 1CZY, 1CZZ, 1D00, 1D01, 1D0A, and 1D0J.