ASSEMBLY OF POST-RECEPTOR SIGNALING COMPLEXES FOR THE TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY

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Abstract

The tumor necrosis factor (TNF) receptor (TNFR) superfamily comprises more than 20 type-I transmembrane proteins that are structurally related in their extracellular domains and specifically activated by the corresponding superfamily of TNF-like ligands. Members of this receptor superfamily are widely distributed and play important roles in many crucial biological processes such as lymphoid and neuronal development, innate and adaptive immunity, and maintenance of cellular homeostasis. A remarkable dichotomy of the TNFR superfamily is the ability of these receptors to induce the opposing effects of gene transcription for cell survival, proliferation, and differentiation and of apoptotic cell death. The intracellular signaling proteins known as *TNF receptor associated factors* (TRAFs) are the major signal transducers for the cell survival effects, while the death-domain-containing proteins mediate cell death induction. This review summarizes recent structural, biochemical, and functional studies of these signal transducers and proposes the molecular mechanisms of the intracellular signal transduction.

I. Introduction

A. *Remarkable Dichotomy: Survival and Death*

The tumor necrosis factor (TNF) receptor (TNFR) superfamily comprises more than 20 type-I transmembrane proteins that are structurally related in their extracellular domains and specifically activated by the corresponding superfamily of TNF-like ligands (Locksley *et al.*, 2001). Members of this receptor superfamily are widely distributed and play important roles in many crucial biological processes such as lymphoid and neuronal development, innate and adaptive immunity, and maintenance of cellular homeostasis. Agents that manipulate the signaling of these receptors are being used or showing promise towards the treatment and prevention of many human diseases (Ashkenazi and Dixit, 1998; Leonen, 1998; Newton and Decicco, 1999).

A remarkable dichotomy of the TNFR superfamily is the ability of these receptors to induce the opposing effects of gene transcription for cell survival, proliferation, and differentiation and of apoptotic cell death (Gravestein and Borst, 1998; Locksley *et al.*, 2001; Smith *et al.*, 1994). Some members of the superfamily—such as TNF-R2, CD40, CD30, Ox40, 4-1BB, LTβR, and TRANCE-R (also known as RANK)—induce mostly survival effects, while others—such as Fas, DR4 and DR5—are mostly pro-apoptotic. In addition, receptors such as TNF-R1 and DR3 induce

either cell survival or cell death in different cellular contexts. This functional divergence within the receptor superfamily is a consequence of the varied intracellular domains, leading to the assembly of different intracellular signaling complexes (Fig. 1).

B. Identification of TNF: Historical Perspective

Anecdotal but convincing associations of tumor necrosis or regression with bacterial infections have been noted throughout history and all over the world. In particular, pioneering clinicians in the late 19th century began adopting the idea of provoking acute skin infections, such as erysipelas, for the treatment of various kinds of tumors including sarcomas, cancers of the bone and connective tissues, breast cancer, ovarian cancer, Hodgkin's disease, and melanoma (Coley, 1893).

Our understanding on the underlying mechanism of this novel cancer treatment was significantly advanced by the discovery in 1975 that bacterial endotoxin induced the production and release of an anti-tumor activity from host cells like macrophages. This activity caused hemorrhagic necrosis of transplanted tumors in mice and killed transformed cell lines (Carswell *et al.*, 1975). The promise of TNF as a cancer cure prompted many laboratories to search the molecular identity of TNF, which eventually led to the purification, characterization and cloning of TNF (Beutler and Cerami, 1986; Pennica *et al.*, 1984; Shirai *et al.*, 1985; Wang *et al.*, 1985).

However, it was soon discovered that TNF is a pleiotropic cytokine important in host defense against pathogens and capable of inducing cell survival, proliferation, and differentiation as well as cell death (Fiers, 1991; Goeddel *et al.*, 1986). These collections of effects are mediated by the two receptors of TNF, TNF-R1, and TNF-R2 (Lewis *et al.*, 1991). In fact, TNF does not generally provoke cell killing as in its anti-tumor activity but more often promotes gene transcription and cell activation. The opposing cell death and cell survival functions of TNF have since become the major functional characteristics of the TNF and the TNFR superfamily.

C. Intracellular Signaling Pathways: TRAFs and DD Proteins

Upon receptor activation, different intracellular signaling complexes are assembled for different members of the TNFR superfamily, depending on their intracellular domains and sequences (Fig. 1). Receptors that do not contain a structural module known as the death domain (DD) in their intracellular domains are survival receptors, which directly recruit adapter

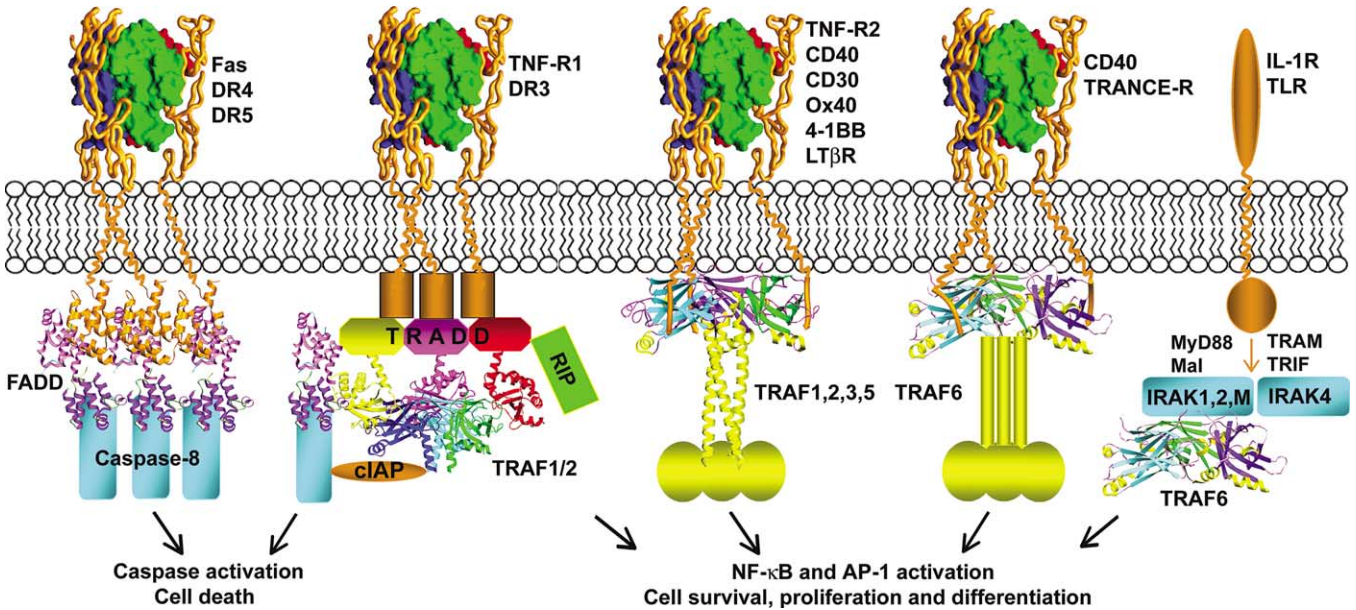


FIG. 1. Intracellular signaling pathways for the TNFR superfamily and the IL-1R/TLR superfamily. Proteins with known structures are shown as ribbon drawings. Hypothetical transmembrane helices are built to connect extracellular rand intracellular domains of these receptors (shown in orange). The amino terminal domains of TRAFs are shown as yellow spheres.

proteins known as the *TNF receptor associated factors* (TRAFs) (Arch *et al.*, 1998; Chung *et al.*, 2002; Rothe *et al.*, 1994). Six mammalian TRAFs (TRAF1-6) have been identified so far, out of which, TRAF1, 2, 3, 5, and 6 participate in the signal transduction of the TNFR superfamily (Cao *et al.*, 1996b; Cheng *et al.*, 1995; Ishida *et al.*, 1996a,b; Mizushima *et al.*, 1998; Mosialos *et al.*, 1995; Nakano *et al.*, 1996; Regnier *et al.*, 1995; Rothe *et al.*, 1994; Sato *et al.*, 1995). The Epstein-Barr virus oncoprotein LMP1 also constitutively recruits TRAFs to promote growth and transformation (Mosialos *et al.*, 1995). Within the TRAF family, TRAF1, 2, 3, and 5 are considered TRAF2-like because they are recruited to a shared set of receptor family members by recognizing the same sequences on these receptors (Arch *et al.*, 1998).

TRAF6, on the other hand, has a unique sequence specificity that does not overlap with that of other TRAFs (Darnay *et al.*, 1999; Pullen *et al.*, 1998). It directly interacts with a subset of the TNFR superfamily such as CD40 and TRANCE-R. In addition to signal transduction for the TNFR superfamily, TRAF6 is also a major signal transducer for the interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily. The intracellular domains of IL-1Rs and TLRs contain a structural module known as the *TIR domain*, which recruit a family of TIR-domain containing intracellular signaling proteins including MyD88, Mal/TIRAP, TRIF, and TRAM (O'Neill *et al.*, 2003) and several other adapter proteins such as Tollip and SARM (Burns *et al.*, 2000; O'Neill *et al.*, 2003). In turn, these signaling complexes recruit Ser/Thr kinases, IRAK1, IRAK2, IRAK-M, and IRAK4 (Cao *et al.*, 1996a; Muzio *et al.*, 1997; Suzuki *et al.*, 2002; Wesche *et al.*, 1999), which directly interact with TRAF6 to activate downstream signal transduction.

The downstream effectors of TRAF signaling are transcription factors in the nuclear factor κ-B (NF-κB) and activator protein-1 (AP-1) family (Ghosh and Karin, 2002; Shaulian and Karin, 2002), which can turn on numerous genes involved in many aspects of cellular and immune functions. While the carboxyl terminal TRAF domain, containing a coiled-coil TRAF-N domain and a conserved TRAF-C domain, is both necessary and sufficient for TRAF self-association and receptor interaction, the amino terminal domain, containing RING and zinc-finger motifs, is important for downstream functions (Rothe *et al.*, 1994).

TRAF2 and TRAF6 apparently utilize different molecular pathways for NF-κB and AP-1 activation (Chung *et al.*, 2002; Wu and Arron, 2003). However, in both cases, it has been shown that a unique form of non-degradative polyubiquitination plays an important role in TRAF downstream signaling. *In vitro* biochemical reconstitution showed that TRAF6, as a RING domain protein, directly supported the synthesis of these unique polyubiquitin

chains, together with a ubiquitin conjugating enzyme system (Deng *et al.*, 2000; Wang *et al.*, 2001a). Similarly, negative regulation of NF-κB activity by a TRAF2-interacting deubiquitination enzyme specific for non-degradative polyubiquitin chains implicated the role of ubiquitination in TRAF2-mediated NF-κB activation (Brummelkamp *et al.*, 2003; Kovalenko *et al.*, 2003; Trompouki *et al.*, 2003).

Receptors that contain an intracellular DD are known as death receptors, which are exemplified by Fas and TNF-R1 (Ashkenazi and Dixit, 1998; Nagata, 1997). Fas is an effective prototypical cell killing receptor. The intracellular DD of Fas directly recruits a DD-containing protein known as Fas-associated DD (FADD) via DD-DD interactions (Chinnaiyan *et al.*, 1995). FADD also contains a death effector domain (DED), which further recruits the DED-containing pro-caspase-8 or pro-caspase-10 to elicit caspase activation and apoptosis (Boldin *et al.*, 1996; Muzio *et al.*, 1996; Wang *et al.*, 2001b).

TNF-R1-like death receptors, on the other hand, possess the intrinsic capability of both cell death and cell survival induction. The underlying mechanism for this duality lies on the recruitment of a multifunctional protein, TNF receptor-associated DD (TRADD), via DD-DD domain interactions, by TNF-R1 (Hsu *et al.*, 1996b). The amino terminal domain of TRADD (TRADD-N) recruits TRAF2 (Hsu *et al.*, 1996b), while the carboxyl terminal DD of TRADD can recruit FADD and a DD-containing Ser/Thr kinase, receptor-interacting protein (RIP), via DD-DD interactions (Hsu *et al.*, 1996a,b; Stanger *et al.*, 1995). Both TRAF2 and RIP contribute to survival signaling (Kelliher *et al.*, 1998; Yeh *et al.*, 1997), while FADD recruits and activates caspases to induce apoptosis. TNF-R1-induced apoptosis appears to involve the switch from an initial plasma membrane bound complex consisting of TNF-R1, TRADD, RIP1, and TRAF2 to a cytoplasmic complex consisting of TRADD, RIP1, FADD, and caspase-8 (Micheau and Tschopp, 2003). The control between the survival and death pathways from TNF-R1 is likely to be rather complex and may involve cellular inhibitors of apoptosis (cIAPs), FLICE-inhibitory proteins (FLIPs), and c-Jun N-terminal kinase (JNK) (Deng *et al.*, 2003; Irmler *et al.*, 1997; Micheau and Tschopp, 2003; Wang *et al.*, 1998).

D. Structural and Functional Studies of Intracellular Signaling Pathways

During the past few years, a large number of crystal and NMR structures (Table I), structure-based mutations (Table II) and thermodynamic data (Table III) have become available. These studies have led to a much more advanced understanding towards the molecular basis of signaling transduction of the TNFR superfamily.

TABLE I
Experimental Structures of Intracellular Signaling Proteins

Protein	Binding partner and sequence[a]	Method	Resolution	Protein, partner[b]	PDB code, reference
TRAF2 (327–501)			2.2 Å	6	1CA4 (Park *et al.*, 1999)
TRAF2 (310–501)	TNF-R2 (420–428) QVPFSKEEC	Co-crystallization	2.3 Å	6, 2	1CA9 (Park *et al.*, 1999)
TRAF2 (310–501)	CD40 (250–266) PVQETLHGCQPV TQEDG	Co-crystallization	2.7 Å	3, 2	1CZZ (Ye *et al.*, 1999)
TRAF2 (327–501)	CD40 (250–254) PVQET	Co-crystallization	2.0 Å	8, 8	1D00 (Ye *et al.*, 1999)
TRAF2 (327–501)	CD40 V251I mutant (249–254) YPIQET	Co-crystallization	2.0 Å	8, 8	1QSC (McWhirter *et al.*, 1999)
TRAF2 (327–501)	CD30 (576–583) MLSVEEEG	Co-crystallization	2.0 Å	6, 3	1D01 (Ye *et al.*, 1999)
TRAF2 (327–501)	Ox40 (262–266) PIQEE	Co-crystallization	2.0 Å	6, 6	1D0A (Ye *et al.*, 1999)
TRAF2 (327–501)	m4-1BB (231–236) GAAQEE	Co-crystallization	2.5 Å	6, 5	1D0J (Ye *et al.*, 1999)
TRAF2 (327–501)	LMP1 (204–210) PQQATDD	Co-crystallization	2.0 Å	3, 2	1CZY (Ye *et al.*, 1999)
TRAF3 (341–568)			2.8 Å	2	1FLK (Ni *et al.*, 2000)
TRAF3 (341–568)	CD40 (247–266) TAAPVQETLHGC QPVTQEDG	Soaking	3.5 Å	2, 2	1FLL (Ni *et al.*, 2000)
TRAF3 (377–568)	TANK (178–195) SVPIQCTDKTDK QEALFK	Soaking	2.9 Å	1, 1	1L0A (Li *et al.*, 2002)

(*continued*)

TABLE I (*continued*)

Protein	Binding partner and sequence[a]	Method	Resolution	Protein, partner[b]	PDB code, reference
TRAF3 (377–568)	TANK (171–191) IATDTQCSVPIQCT DKTDKQE	Soaking	3.5 Å	1, 1	1KZZ (Li *et al.*, 2002)
TRAF3 (377–568)	LTβR (385–408) PYPIPEEGDPGPPG LSTPHQEDGK	Soaking	3.5 Å	1, 1	1RF3 (Li *et al.*, 2003)
TRAF6 (346–504)			2.4 Å	1	1LB4 (Ye *et al.*, 2002)
TRAF6 (346–504)	CD40 (230–238) KQEPQEIDF	Co-crystallization	1.8 Å	1, 1	1LB6 (Ye *et al.*, 2002)
TRAF6 (346–504)	TRANCE-R (342–349) QMPTEDEY	Co-crystallization	2.0 Å	1, 1	1LB5 (Ye *et al.*, 2002)
TRAF2 (327–501)	TRADD-N (7–163)	Co-crystallization	2.0 Å	1, 1	1F3V (Park *et al.*, 2000)
TRADD-N (1–169)		NMR			1F2H (Tsao *et al.*, 2000)
Fas DD (202–319)		NMR			1DDF (Huang *et al.*, 1996)
FADD DD (89–183)		NMR			1FAD (Jeong *et al.*, 1999)
TNFR-1 DD (316–426) R347A		NMR			1ICH (Telliez *et al.*, 2000)
FADD DED (1–83) F25Y		NMR			1A1W (Eberstadt *et al.*, 1998)

[a]m: mouse; otherwise from human.
[b]Number of protein and partner molecules per crystallographic asymmetric unit.

TABLE II
Structure-Based Mutational Studies

TRAF	Receptor/ adapter, motif position	K_d[a]	Method[b]	Reference
TRAF3	CD40 (Q263A) P_{11}	−	SPR	(Ni *et al.*, 2000)
	CD40 (T254A) P_2	−−		
TRAF2, 3, 5	LTβR (P387A) P_{-3}	+, − −	GST-pulldown	(Li *et al.*, 2003)
	LTβR (P389A) P_{-1}	+		
	LTβR (D393A) P_3	+		
	LTβR (E390A/ E391A) $P_{0/1}$	−, − − −		
	LTβR (E390A/ E391A/D393A) $P_{0/1/3}$	− − −		
TRAF2, 3	TANK (Q182A) P_0	− −	GST-pulldown	(Li *et al.*, 2002)
	TANK (T184A) P_2	− −		
	TANK (D185A) P_3	− −		
	TANK (D188A) P_6	−		
	TANK (F194A) P_{12}	−		
TRAF6	CD40 (P237A) P_{-2}	+	GST-pulldown and NF-κB activation	(Ye *et al.*, 2002)
	CD40 (P237Q) P_{-2}	− −		
	CD40 (E239Q) P_0	− −		
	CD40 (D242A) P_3	−		
	CD40 (Q235A) P_{-4}	+		
	TRANCE-R (E342A, E375A, E449A) P_0	−−	NF-κB activation	
	TRANCE-R (E342A, E375A) P_0/P_0	+		
	TRANCE-R (E342A, E449A) P_0/P_0	+		
	TRANCE-R (E375A, E449A) P_0/P_0	+		
	IRAK (E706A) P_0	−	NF-κB activation	
	IRAK (E587A, E706A) P_0/P_0	−−		
	IRAK (E544A, E587A, E706A) $P_0/P_0/P_0$	−−−		
TRAF6 (R392A)	IRAK	−−	TRAF6 dominant negative effect on NF-κB activation	
TRAF6 (F471A)		−−−		
TRAF6 (Y473A)		−−−		
TRAF2	TRADD (Y16A)	32	SPR	(Park *et al.*, 2000)
	TRADD (Y16A, F18A)	>641		

(*continued*)

TABLE II (*continued*)

TRAF	Receptor/ adapter, motif position	K_d[a]	Method[b]	Reference
	TRADD (H65A)	9.1		
	TRADD (S67A)	9.2		
	TRADD (Q143A)	1.3		
	TRADD (D145K)	2.2		
	TRADD (R146A)	17		
	TRADD (L147A)	3.2		
TRAF2 ($S454A^{TRAF1}$)	TRADD	0.6	SPR	(Park *et al.*, 2000)
TRAF2 ($T401M^{TRAF3}$)		18		
TRAF2 ($L471K^{TRAF4}$)		U.D.		
TRAF2 ($L471R^{TRAF5}$)		U.D.		
TRAF2 ($D450K^{TRAF6}$)		5.8		
TRAF2 ($S467F^{TRAF4,6}$)		5.4		

[a] +: no effect; −: decreased; −−: greatly decreased; −−−: drastically decreased; numbers: relative K_d to wild type interactions; U. D.: undetectable.
[b] SPR: surface plasmon resonance.

II. Domain and Oligomeric Structures of TRAFs

A. *TRAF-C Domains: Anti-Parallel β-Sandwiches with a Unique Topology*

The structure of a TRAF-C domain was first revealed from the crystal structure of the TRAF domain of human TRAF2 (Fig. 2A, E), alone and in complex with a receptor peptide from TNF-R2 (Park *et al.*, 1999). The main structural architecture of the TRAF-C domain comprises an eight-stranded anti-parallel β-sandwich, with strands β1, β8, β5, and β6 in one sheet and β2, β3, β4, and β7 in the other. Visual inspection of the SCOP structure database (Murzin *et al.*, 1995) and an automatic structural similarity search with the Dali program (Holm and Sander, 1995) showed that the TRAF-C domain represent a novel fold for an eight-stranded anti-parallel β-sandwich. However, the topology observed

TABLE III
Thermodynamic Characterizations of TRAF-Receptor Interactions

TRAF	Receptor/ adapter and sequence[a]	K_d[b]	Method[c]	Reference
TRAF2 (310–501)	CD30 (573–583) SDVMLSVEEEG $\Delta H = -14.0 \pm 0.8$ kcal/mol; $-T\Delta S = 8.03$ kcal/mol; $\Delta C_p = -245$ cal/mol·K	40 μM	ITC	(Ye and Wu, 2000)
	CD40 (250–266) PVQETLHGCQPVTQEDG $\Delta H = -9.5 \pm 1.0$ kcal/mol; $-T\Delta S = 3.87$ kcal/mol	60 μM		
	Ox40 (262–266) PIQEE $\Delta H = -13.0 \pm 0.9$ kcal/mol; $-T\Delta S = 7.22$ kcal/mol	50 μM		
	TNF-R2 (420–428) QVPFSKEEC	0.5 mM		
	m4-1BB (231–236) GAAQEE	1.0 mM		
	LMP1 (204–210) PQQATDD	1.9 mM		
TRAF2 (327-501)	TRADD (7–163)	7.8 μM	SPR	(Park *et al.*, 2000)
TRAF3 (341-568)	TANK (178–195) SVPIQCTDKTDKQEALFK	23.9 μM	ITC	(Li *et al.*, 2002)
TRAF6 (333-508)	CD40 (216–245) KKVAKKPTNK APHPKQEPQEINFPDDLPGS	59.9 μM	ITC	(Ye *et al.*, 2002)
	CD40 (230–238) KQEPQEIDF	84.0 μM		
	mTRANCE-R (337–345) RKIPTEDEY	78.0 μM		
	mTRANCE-R (370–378) FQEPLEVGE	770.0 μM		
	mTRANCE-R (444–452) GNTPGEDHE	763.0 μM		
	IRAK (539–548) PPSPQENSYV	518.1 μM		
	IRAK (582–590) PNQPVESDE	79.0 μM		
	IRAK (701–710) RQGPEESDEF	54.3 μM		
	IRAK-2 (523–532) SNTPEETDDV	66.2 μM		
	IRAK-M (475–483) PSIPVEDDE	142.2 μM		

[a] m: mouse; otherwise from human.
[b] K_d: dissociation constant; ΔC_p: heat capacity change with temperature.
[c] ITC: isothermal titration calorimetry; SPR: surface plasmon resonance.

for this domain may be reached by circular permutations of the β strands in Cu, Zn superoxide dismutase (PDB entry 2SOD) (Tainer *et al.*, 1982) and the C2 domain from synaptotagmin I (PDB entry 1rsy) (Sutton *et al.*, 1995).

The TRAF-C domain of TRAF2 contains several additional structural features. In particular, strands β2 and β7 are highly twisted with a β-bulge in each strand. Preceding the β1 strand, residues 348-350 form a parallel β structure (β0) with strand β2, immediately after the β-bulge in this strand. The side chains of residues in β0 partly cover one edge of the β-sandwich. Therefore the twisting of β2 appears to play a structural role in the TRAF-C domain. Since the β7 strand contains the primary receptor peptide interaction site, the β-bulge and the twist in this strand may also play important structural and biological roles. A three-turn helix is present in the crossover connection between strands β1 and β2. Comparison among the 48 independent copies of the TRAF-C domain of TRAF2 (McWhirter *et al.*, 1999; Park *et al.*, 1999; Ye *et al.*, 1999) showed that with the exception of the flexible β7-β8 loop (up to 3–4 Å in Cα distance), the structures are highly conserved in different crystal packing environment and they superimpose with r.m.s.d. of around 0.3–0.6 Å. Structural comparison of TRAF-C domain structures in the absence and presence of receptor peptide interactions has revealed that the TRAF-C domain is fairly rigid in its overall architecture and does not undergo large conformational changes upon receptor peptide binding.

Subsequently, structures of the TRAF-C domains of TRAF3 (Li *et al.*, 2002; Ni *et al.*, 2000) and TRAF6 (Ye *et al.*, 2002) were determined and shown to exhibit a similar structural architecture. TRAF3 structures are extremely similar to TRAF2 (Fig. 2B), with r.m.s.d. in Cα positions of around 0.5–0.8 Å, similar to the variations observed within the different TRAF3 structures. This structural conservation is consistent with the shared receptor binding specificity of TRAF2 and TRAF3. In comparison to TRAF2, TRAF3 contains an insertion at the β5-β6 loop that makes this loop somewhat more flexible and a deletion at the β7-β8 loop that makes the loop more ordered and defined.

In contrast to TRAF3, structural differences between TRAF2 and TRAF6 are much more pronounced (Fig. 2C), resulting in r.m.s.d. of 1.1–1.2 Å for 127 aligned Cα positions within 3.0 Å. While the central β-sheet superimposes well, significant differences are observed in almost all loop regions, including the α1 helix within the β1-β2 loop, the β3-β4 loop (one residue insertion), the β5-β6 loop (three residue insertion), the β7 strand (one residue insertion) and the β7-β8 loop (one residue deletion). Relative to TRAF2, the β3-β4 loop of the TRAF-C domain of TRAF6 exhibits an

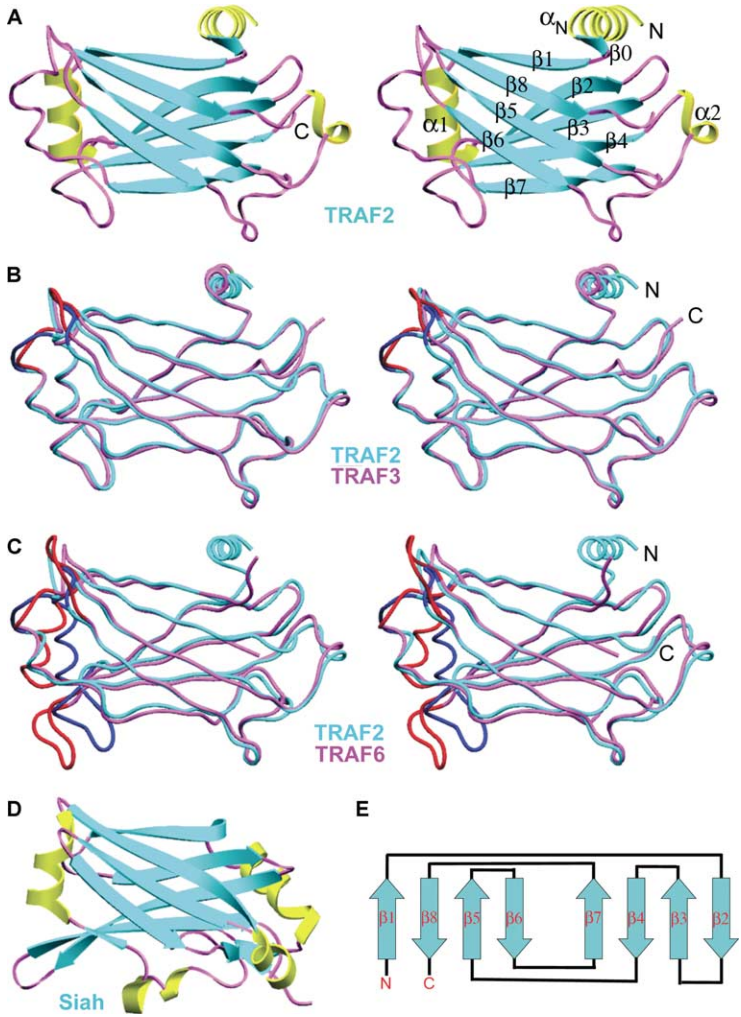


FIG. 2. TRAF domain structures. (A) Stereo drawing of the TRAF domain of TRAF2 with labeled secondary structures. (B) Superposition of the TRAF domain of TRAF2 (cyan) and TRAF3 (magenta). Regions with large differences between the two structures are shown in blue for TRAF2 and red for TRAF3. (C) Superposition of the TRAF domain of TRAF2 (cyan) and TRAF6 (magenta). Regions with large differences between the two structures are shown in blue for TRAF2 and red for TRAF6. (D) Ribbon drawing of Siah. (E) Topology of TRAF-C domains.

up to 12 Å movement in Cα positions, so that it no longer interacts with the peptides in the TRAF6 complexes (see Section V). The remaining loops exhibit 2–5 Å movement in Cα positions. Due to the deletion in β7-β8 loop, this loop is more similar to TRAF3 than to TRAF2. The β6-β7 loop is disordered in the absence of receptor binding. Interestingly, the β2-β3 and the β4-β5 loops involved in TRAF trimerization are conserved between TRAF6 and TRAF2, demonstrating that on the structural level TRAF6 can form similar trimers.

Sequence analysis showed that a diverse set of proteins with unrelated functions to TRAFs appear to contain the TRAF-C domain. These include meprins, a family of extracellular metalloproteases (Uren and Vaux, 1996), MUL, the product of the causative gene in Mulibrey Nanism syndrome, USP7 (HAUSP), an ubiquitin protease, and SPOP, a POZ (poxvirus and zinc finger) domain-containing protein (Zapata *et al.*, 2001). Because of its similarities with meprins, TRAF-C domain was also dubbed meprin- and TRAF-homology (MATH) domain (Uren and Vaux, 1996).

Although sharing no significant sequence homology, a recent crystal structure of seven in absentia homolog (Siah), a member of the E3 ubiquitin ligase RING domain proteins, surprisingly revealed that its substrate-binding domain (SBD) is dimeric and adopts an eight-stranded anti-parallel β-sandwich fold that is highly similar to the TRAF-C domain (Polekhina *et al.*, 2002) (Fig. 2D, E). The Siah structure also reveals two novel zinc fingers in a region with sequence similarity to TRAFs. In addition, it appears that the SBD of Siah potentiates TNF-mediated NF-κB activation, suggesting potential functional similarities as well between Siah and TRAFs.

B. Conserved Trimeric Structures of TRAF Domains: Energetics and Specificity

A most striking structural feature of the TRAF domain, comprising a coiled coil TRAF-N domain followed by the TRAF-C domain, is the formation of a mushroom-shaped trimer with the TRAF-C domain as the cap and the coiled-coil domain as the stalk (McWhirter *et al.*, 1999; Park *et al.*, 1999; Ye *et al.*, 1999) (Fig. 3A, B). The trimer obeys perfect or near perfect threefold symmetry. The diameter of the mushroom cap ranges between 50 to 80 Å while the stalk is approximately 50 Å long for 5 heptad repeats (residues 311–347).

Both the coiled coil domain and the TRAF-C domain mediate TRAF domain trimerization. The three-stranded parallel coiled coil structure is stabilized by hydrophobic residues at positions A and D of the

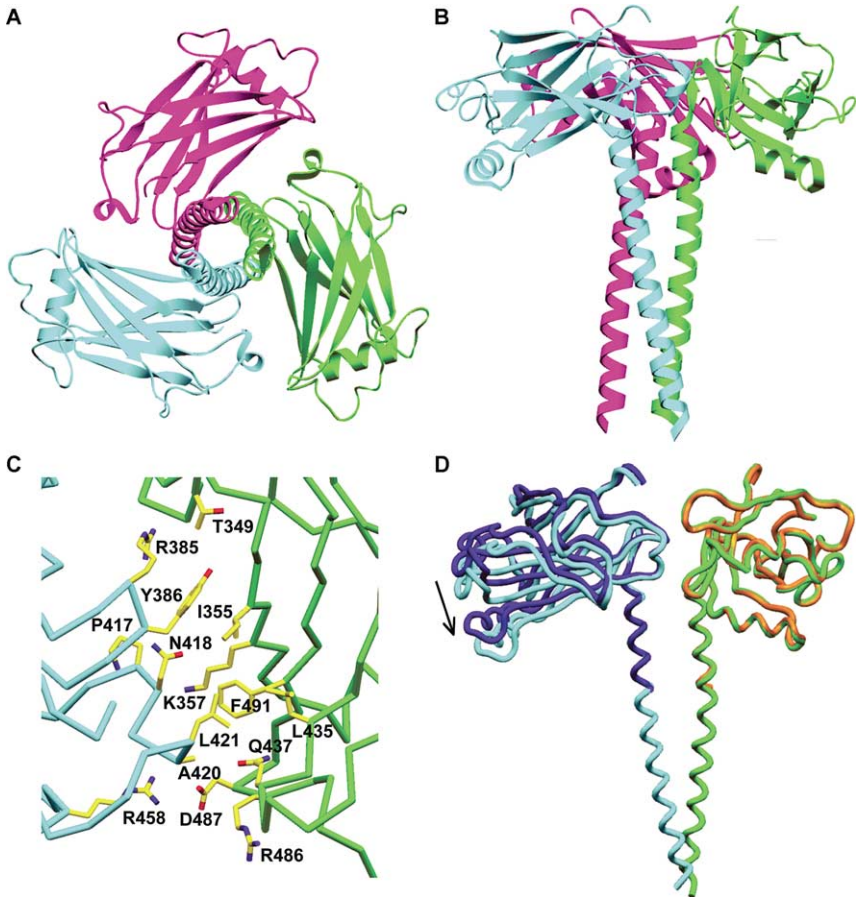


FIG. 3. TRAF trimerization. (A, B) Trimeric structure of the TRAF domain of TRAF2, shown with the three-fold axis into the page and vertical, respectively. (C) Detailed interaction between the TRAF-C domains in the trimer. (D) Observed structural variation among TRAF domain trimers. When superimposed onto one of the protomers in the trimer, a neighboring protomer may exhibit a different tilt of the mushroom cap-shaped TRAF-C domain.

heptad repeats of the coiled coil. The trimeric interface of the TRAF-C domain is formed by packing one end of the β-sandwich (the β2-β3, β4-β5 and β6-β7 connections) against an edge and a face of the β-sandwich (β0, β1, and β8 strands, β5-β6 and β7-β8 connections) of the neighboring protomer (Fig. 3C). Most residues that participate in

the formation of this interface are rather hydrophobic (such as I355, Y386, A420, L421 and F491). Hydrophilic interactions are also observed at this trimer interface, involving the side chains of K357, R385, R458, and D487.

The coiled coil domain plays an important role in stabilizing TRAF domain trimerization. Each TRAF-C domain buries roughly 640 $Å^2$ surface area upon trimerization (Park *et al.*, 1999), which is rather small compared to other stable protein-protein interactions, suggesting that the TRAF-C domain alone is not sufficient for trimerization (Janin *et al.*, 1988). Consistent with this analysis, solution studies on several TRAF domain constructs of TRAF2 showed that minimally three heptad repeats (residues 327–347), which increase the surface area burial to 1060 $Å^2$, are required for trimer formation (Park *et al.*, 1999). Interestingly, the coiled-coil domain of TRAF4 contains only three heptad repeats, the shortest among TRAFs. In comparison, the coiled-coil domain of TRAF2 appears to contain up to 14 heptad repeats, which could stretch to 140 Å long.

Structural and computational analyses suggest that the TRAF-C domain is the major specificity determinant of TRAF domain trimerization. Amino acid residues contributing to trimerization of the TRAF domain of TRAF2 are largely conserved among the TRAF family members (Park *et al.*, 1999), suggesting that all TRAFs may be able to form similar homotrimers as well. This hypothesis has been experimentally verified for the TRAF domains of TRAF1, TRAF2, TRAF3, and TRAF6 (Ni *et al.*, 2000; Park *et al.*, 1999; Pullen *et al.*, 1999b; Ye *et al.*, 2002). Formation of heterotrimers, as has been shown for TRAF1 and TRAF2, may also be possible (Rothe *et al.*, 1994). On the other hand, the coiled coil domains, which may be important as an energetic determinant of trimerization, do not contain conserved signature sequences characteristic of trimeric coiled coils (Harbury *et al.*, 1993). Prediction of coiled coil structures using the Multicoil program (Wolf *et al.*, 1997) showed the preference of TRAF1, TRAF2, and TRAF6 for dimeric, rather than trimeric, coiled coils. Therefore it appears that the TRAF-C domain, rather than the coiled coil domain, determines the observed specificity of TRAF trimerization.

Structural comparison among 18 different TRAF2 trimers and 6 TRAF3 trimers revealed that the trimeric structure is highly conserved. However, slight variations in the relative disposition of the protomers in the TRAF domain trimer are observed. These structural differences are exemplified by a flexing of the head of the mushroom relative to the stalk, on the order of 2–6° (Fig. 3D) and are unlikely to have functional implications. The coiled coil domains are more flexible, especially near the ends remote to the TRAF-C domains.

III. TRAF2-Receptor Interactions: Establishment of the Paradigm

A. *Conserved Recognition of Diverse Receptors*

The first glimpse of a TRAF2-receptor interaction was provided by the crystal structure of the TRAF domain of TRAF2 in complex with a receptor peptide from TNF-R2 (Park *et al.*, 1999). Each peptide is bound symmetrically to a shallow surface depression on the side of the mushroom-shaped trimer, extending from the top to the bottom rim of the mushroom cap (Fig. 4A, B). The peptide contacts one TRAF domain exclusively, with no contacts to the other two molecules of the trimer. This mode of interaction is distinct from the interaction between TNF and the extracellular domain of its receptor, where each receptor binds at the interface between neighboring protomers in the TNF trimer. Therefore, TRAF2-receptor interactions do not rely structurally on TRAF2 trimerization, but rely energetically on avidity-mediated affinity enhancement afforded by TRAF2 and receptor trimerization.

Because TRAF2 interacts with many different receptors, a major structural question is the molecular basis of this diversity. Towards understanding this question, a total of eight crystal structures of the TRAF domain of TRAF2 in complex with diverse receptor peptides have been determined (McWhirter *et al.*, 1999; Park *et al.*, 1999; Ye *et al.*, 1999) (three structures are with CD40) (Fig. 4C). These different structures encompass the three TRAF2 binding motifs proposed previously from biochemical and functional studies, the PxQx(T/S/D) (x = any amino acid) motif in LMP1, CD30, CD40 and CD27 (Aizawa *et al.*, 1997; Akiba *et al.*, 1998; Boucher *et al.*, 1997; Brodeur *et al.*, 1997; Devergne *et al.*, 1996; Franken *et al.*, 1996; Gedrich *et al.*, 1996; Sandberg *et al.*, 1997), the FSxEE (F = large hydrophobe) sequence in TNF-R2 and CD30 (Boucher *et al.*, 1997; Rothe *et al.*, 1994) and the QEE motif in 4-1BB and Ox40 (Arch and Thompson, 1998). The 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, four structures for the PxQxT motif (LMP1 and CD40), two for the FSxEE motif (CD30 and TNF-R2), and two for the QEE motif (Ox40 and 4-1BB). This collection of the different structures allowed a unified understanding and detailed comparison of the binding modes of the various receptor peptides.

Despite the high degree of sequence variation in the receptor peptides the structures surprisingly revealed that the peptides have a conserved binding mode and share a common binding site on the TRAF domain.

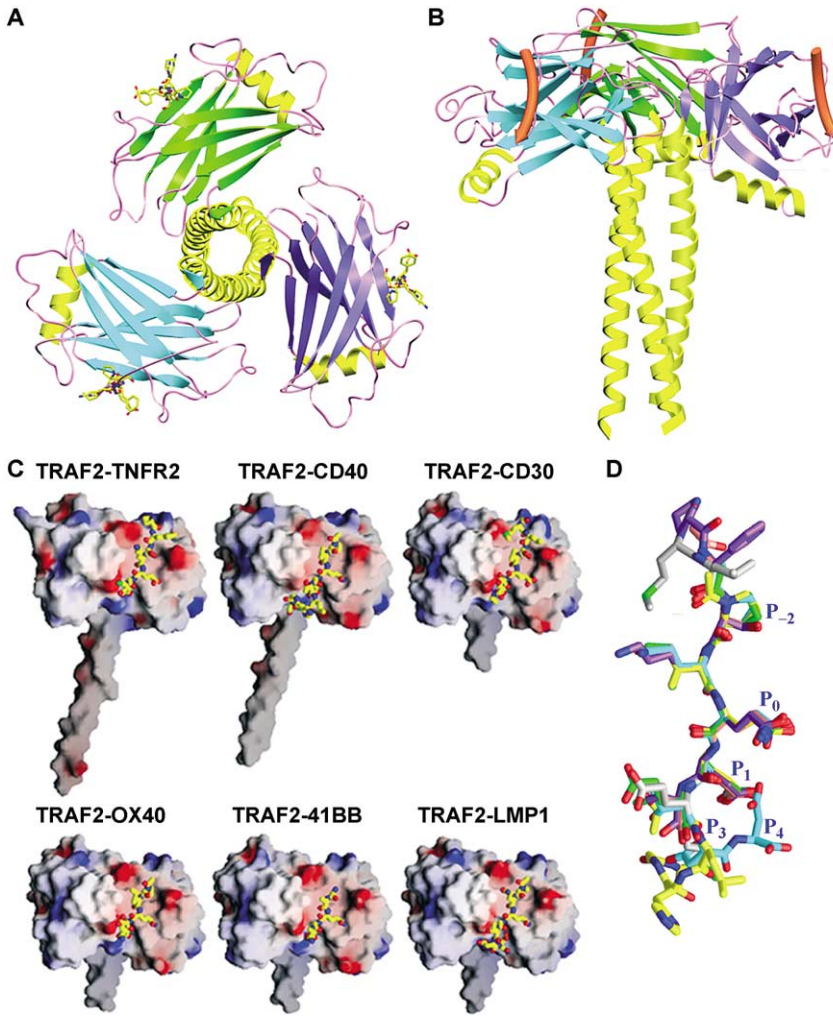


FIG. 4. TRAF2-receptor interactions. (A) Ribbon diagram of a TRAF2-receptor complex, looking down the three-fold axis. The bound receptor chains are shown as stick models. (B) Ribbon diagram of a TRAF2-receptor complex with the threefold axis vertical. The bound receptor chains are shown as arrows. (C) Surface electrostatic representation of TRAF2-peptide complexes. (D) Superposition of bound receptor peptides, showing the structural conservation of the main chain conformations and the side chain conformations at P_2, P_0, and P_1 positions. Parts of this figure were modified from Ye *et al.* (1999).

Structural superposition of the 7 different structures of receptor peptide complexes indicated a most highly conserved central core of four residues, giving rise to an r.m.s.d of less than 0.1 Å among the main chain atoms of these residues (Fig. 4D). The structure-based sequence alignment of the receptor peptides 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. This residue was denoted 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.

The receptor peptides assume an essentially extended main chain conformation in the complex and cut across four β-strands (β6 in the first sheet, β7, β4, and β3 in the second sheet) on one side of the β-sandwich structure of the TRAF-C domain. Although the direction of the peptide chain is essentially perpendicular to these β strands, a portion of the peptide chain (P_{-1} to P_1) runs anti-parallel and adjacent to the latter half of strand β7 (residues 466–468), immediately after the β-bulge in this strand. This leads to three anti-parallel β-edge main chain hydrogen bonds between the peptide and the β7 of TRAF2, extending the four-stranded second β-sheet by one strand (Fig. 5A). In addition, the main chain amide group of the following residue is within hydrogen-bonding distance to the carboxylate group of D399 in TRAF2.

The formation of a β-sheet has been frequently observed in protein-peptide interactions, such as substrate recognition by certain serine proteases (Tong *et al.*, 1998) and peptide recognition by the PTB and PDZ domains (Kuriyan and Cowburn, 1997). Detailed analysis further revealed that the central portion of the receptor peptide (P_{-2}, P_0, and P_1 positions) is more twisted than a regular β-strand to possess the polyproline II (PPII) helix conformation. 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. As a consequence, large proportions of the side chains at the P_{-2}, P_0, and P_1 positions of the receptor peptides are buried at the TRAF2 interface. Therefore, in the case of TRAF2-receptor interactions, the main chain hydrogen bonds and the PPII conformation maximize both main chain and side chain interactions with the TRAF2 surface.

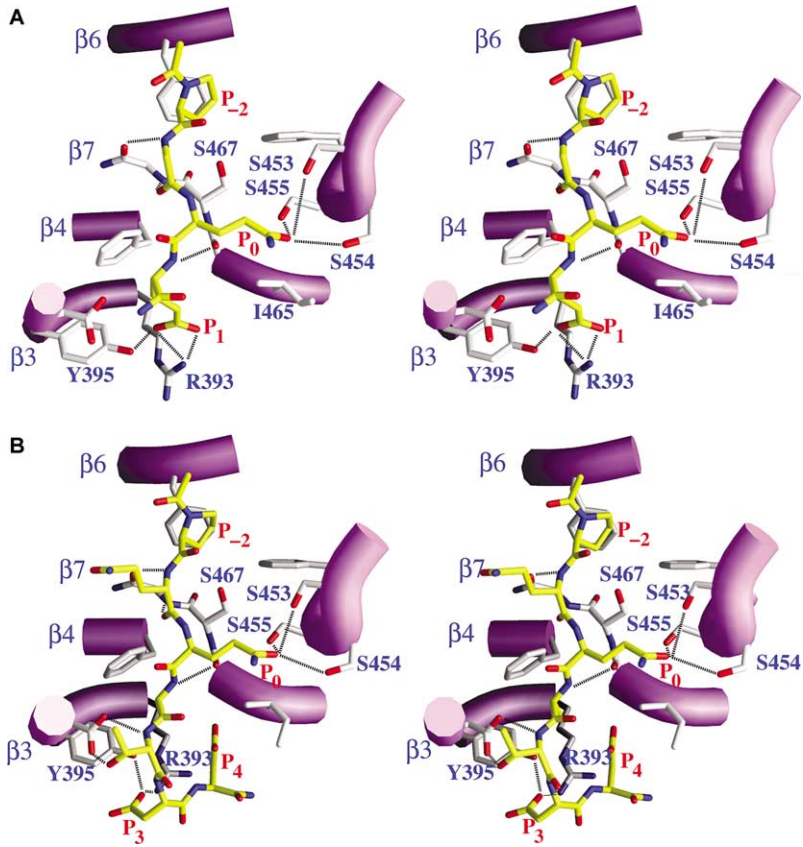


FIG. 5. Detailed TRAF2-receptor interactions. (A) Interactions seen in the major TRAF2-binding motif. (B) Interactions seen in the minor TRAF2-binding motif. TRAF2 structures are shown as magenta worms and white stick models. The bound receptors are shown as yellow stick models. Modified from Ye *et al.* (1999).

B. Key Residues and the Universal Major TRAF2 Binding Motif

The major structural determinants of TRAF2-receptor peptide interactions appear to reside on the side chains at the P_{-2}, P_0, and P_1 positions within the TRAF2 binding sequences (Fig. 5A). They each engage into distinct pockets on the surface of TRAF2. The P_{-2} side chains are completely buried by the TRAF domain surface. The most frequently occupied residues at this position are Pro and Ser, both of which make extensive van der Waals contacts with TRAF2. In addition, Ser forms a hydrogen bond between its hydroxyl and the side chain of S467 in TRAF2. Model building

of other amino acids at this position suggests that the size and the enclosure of the pocket may allow other non-charged medium-sized side chains such as Thr, Cys and Ile but may restrict the accommodation of larger side chains such as Gln. In 4-1BB, this position is an Ala, which would be expected to fit less well, consistent with the weaker TRAF2 binding affinity and the weaker electron density in this structure.

Shape complementarity and hydrogen bonding interactions are the major determinants for the selectivity of Glu and Gln residues at the P_0 position. The aliphatic portion of the side chains pack 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 a 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 most TRAF2 binding sequences is occupied by a Glu residue, although LMP1 has an Ala at this position (see Subsection C). The carboxylate moiety of the Glu residue makes bi-dentate ion-pair interactions with the side chain guanidinium group of R393, and an additional hydrogen bond with the hydroxyl of Y395. These hydrogen-bonding interactions appear to require Glu specifically, as an Asp residue is too short to reach R393 and Y395 in TRAF2.

The sequence and structural conservations at the P_{-2}, P_0, and P_1 positions define a major TRAF2 binding motif that bears the consensus sequence of px(Q/E)E, in which Pro is shown in lower case because it can be substituted for other medium sized non-charged residues. Most of the binding sequences identified so far for TRAF1, 2, 3, and 5 are consistent with the motif, thereby explaining the recognition of diverse receptor sequences by TRAF2 (Fig. 6).

C. The Minor TRAF2 Binding Motif

The major TRAF binding motif has a conserved Glu residue at the P_1 position, which is involved in important ion-pair and hydrogen-bonding interactions with TRAF2. However, the receptor peptide from LMP1 has an Ala at the P_1 position (Fig. 6), which cannot participate in the ion-pair and hydrogen-bonding interactions. The structure of the LMP1 peptide in complex with TRAF2 (Ye *et al.*, 1999) showed that the interactions with the

	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 (386-410)	Y	P	I	P	E	E	G	D	P	G	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
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
Major Motif			p	x	Q/E	E					
Minor Motif			p	x	Q/E	x	x	D			

FIG. 6. Sequence alignment of TRAF2 binding sequences, illustrating the two TRAF2-binding motifs. h: human; m: mouse; b: bovine; r: rat. Modified from Ye *et al.* (1999).

R393 and Y395 residues are mediated by an Asp at the P_3 position of this peptide (Fig. 5B). 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 another TRAF2 binding consensus sequence motif, px(Q/E)xxD, in which the P_1 Glu is replaced by a P_3 Asp (Fig. 6). 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.

In addition to LMP1, the intracellular protein, TANK (also known as I-TRAF) (Cheng and Baltimore, 1996; Rothe *et al.*, 1996), possesses the minor TRAF2 binding consensus motif (Fig. 6) and may interact with TRAFs similarly as seen in the TRAF2-LMP1 complex. This hypothesis is

consistent with the crystal structure of the TRAF3-TANK peptide complex (see Section IV) (Li *et al.*, 2002).

D. Extent and Variations

Outside the conserved core of P_{-2} to P_1 residues, residues at P_2 show 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 (Fig. 4D). 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. Therefore a more relaxed definition of the TRAF binding core sequence would include the P_{-3} to the P_2 residue, covering 6 residues. In addition, both amino and carboxyl terminal extensions of the core make further contacts with the TRAF domain of TRAF2. At the amino terminal end, 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. At the carboxyl terminal end, the TRAF2-CD40 complex contains ordered residues up to the P_6 position. These results suggest that a complete TRAF2 binding sequence may contain eleven residues (from P_{-4} to P_6), which covers the entire span of one face of the TRAF domain surface. Residues at the variable positions within or outside the length of the TRAF2 binding motifs are generally exposed on the surface of complex, explaining the tolerance to substitutions. 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 from receptor to receptor.

While it appears that all TRAF2 binding sequences identified so far bear either the major or minor TRAF2 binding motif, the presence of the motifs may not be sufficient for the interactions and that residues at other positions may be important as well in specific cases. For example, the side chain of the P_2 residue is situated close to that of D399 in TRAF2. When P_2 is a Thr, as in CD40, potential hydrogen bonding interactions are observed with the side chain of D399. Mutational studies have shown that this residue is important for TRAF2 interaction (Pullen *et al.*, 1999a). In addition, given the low affinity of these TRAF2-receptor interactions, it will not be too surprising if other variations of the TRAF2-interaction motifs are present.

The natural lengths of the intracellular domains of the TNF receptors may range from 36 residues as in Ox40 to several hundred residues as in TRANCE-R. 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 TRAF2 binding sites of these receptors are primarily composed of linear sequences rather than three-dimensional composites. However, secondary interactions may ensue after the central TRAF2 binding sequences are docked onto the TRAF domain surface. The flanking regions may also modulate the exposure and the dynamic behavior of the TRAF2 binding determinants, thereby exerting influences on their functions.

E. Conservation of Receptor Interaction in TRAF1, 2, 3, and 5

All residues of TRAF2 that recognize the TRAF2 binding motifs are conserved among TRAF1, 2, 3, and 5, explaining the overlapping receptor-binding specificity among these TRAFs. In contrast, these residues are not conserved in TRAF4 and TRAF6. In TRAF4, three important TRAF2 residues, R393, Y395, and S467, are changed to Ser, Phe, and Phe, respectively. Curiously, TRAF4 has never been shown to interact with any receptor. In TRAF6, Y395, and S467 of TRAF2 are changed to His and Phe, respectively. The S467F change introduces the bulky phenyl ring on the surface, which may disallow the binding of receptor peptides in the same manner as observed here. This analysis is consistent with the unique receptor-binding specificity of TRAF6.

IV. TRAF3-Receptor Interactions: Similarities and Differences with TRAF2

The surface of TRAF2 used for interacting with the core regions of the receptor peptides is highly conserved among TRAF1, 2, 3, and 5, suggesting that these TRAFs would recognize receptors in a similar fashion. Crystal structures of TRAF3 in complex with peptides from CD40, TANK, and LTβR (Li *et al.*, 2002; Ni *et al.*, 2000) (Fig. 7A, B, C) have mostly confirmed this hypothesis, as seen in the crystal structures of TRAF3 in complex with TANK and LTβR. However, added insights and sometimes surprising observations have also been revealed by these structures.

One important insight derived from these structures is that, residues beyond, especially carboxyl terminal to, the core motifs, may also directly contact TRAF3 and play an additional energetic and functional role in TRAF3 interaction (Fig. 7A, B). In the TRAF3 complex with CD40 and LTβR, the peptides assume a reverse turn conformation that folds back onto the side of the TRAF domain. In the TRAF3 complex with TANK, the

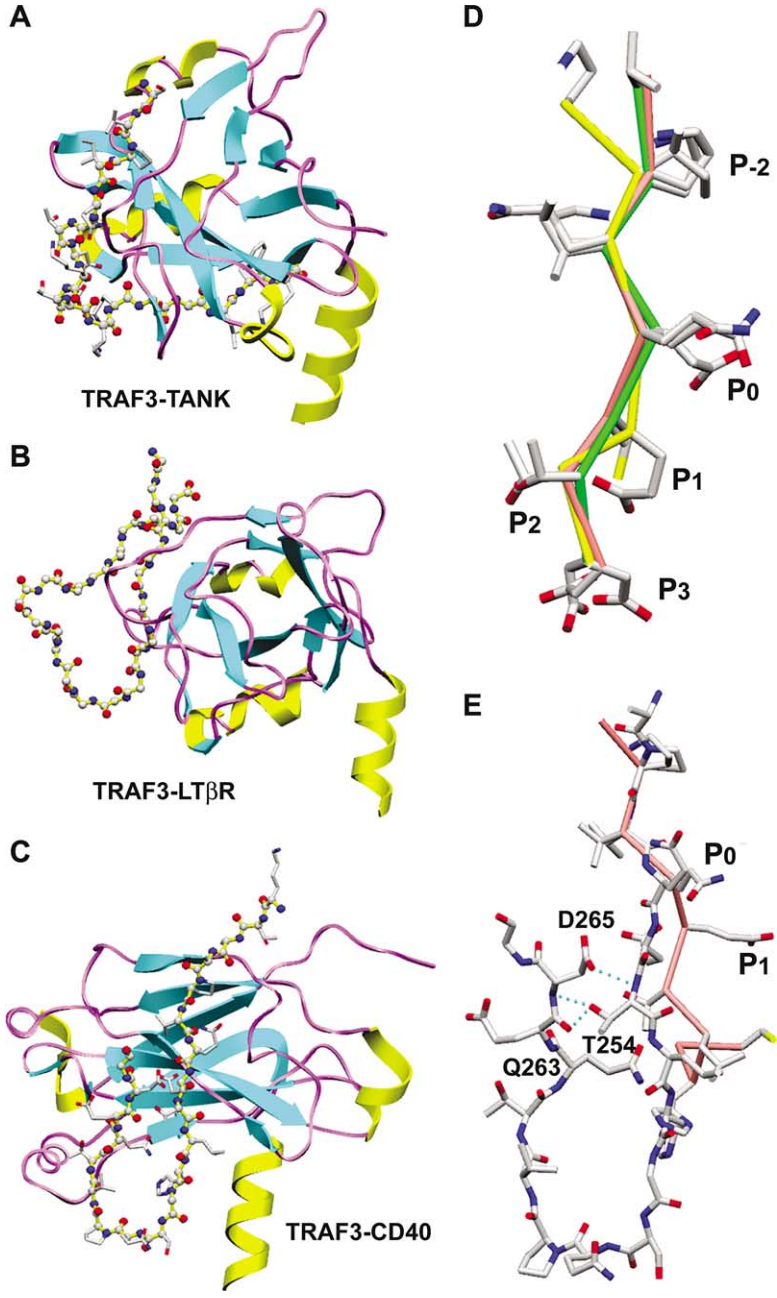
peptide chain continues to the bottom of the TRAF domain, making a boomerang-like shape on the surface of the TRAF domain.

Interestingly, both TANK and LTβR interacted with TRAF3 in a similar manner as seen in the TRAF2-LMP1 complex (Fig. 7D), which belong to the minor TRAF binding motif that we classified based on structural observations. TANK, bearing a sequence motif of PIQCTD, was predicted to interact similarly as in TRAF2-LMP1 complex. In the two independent structures of the TRAF3-TANK complexes, both peptides superimpose well with the receptor peptide conformations in the core regions of the TRAF2-LMP1 complexes and both showed the same hydrogen-bonding interactions between the P_3 Asp and R393 and Y395 of TRAF3. However, the side chain conformations of the P_0 residues are different between the two observations with one in a similar position to interact with TRAF3 via hydrogen bonding interactions and the other not. Mutational studies of TANK confirmed the critical role of the P_0, P_2, and P_3 residues and supported additional roles from the more remote residues, P_6 and P_{12} in TRAF3 interaction (Table II).

On the other hand, it was not apparent at all that the LTβR sequence (IPEEGD) could fall into the TRAF2 binding motif because the P_{-2} position is occupied by the Ile, not the neighboring Pro in this sequence. In fact, this LTβR sequence is a composite of the major and minor motifs since P_1 is a Glu and P_3 is an Asp. In the structure, both P_1 Glu and P_3 Asp interact with the conserved R393 and Y395 residues. Mutational studies showed that triple mutations at P_0, P_1, and P_3 of the LTβR sequence completely abolish the interactions with both TRAF2 and TRAF3.

The biggest surprise is from the structure of TRAF3 in complex with a CD40 peptide (Fig. 7C). A very similar peptide was also used in the crystallization of TRAF2 in complex with CD40 (Ye *et al.*, 1999). What is surprising was not that the CD40 peptide forms a hairpin loop on the surface of TRAF3 to make additional contacts with the TRAF domain, but the lack of conserved interactions of the P_0 and P_1 positions of the peptide with TRAF3 (Fig. 7E), despite high degree of structural conservation of TRAF3 with TRAF2, due to a significant shift in the main chain of the CD40 peptide. Instead, T254 at the P_2 position makes intra-chain hydrogen bonds with the main chain of E264 and D265 at the adjacent strand and Q263 of CD40 interacts with Y395 and D399 of TRAF2. Competitive binding studies using surface plasmon resonance (SPR) confirmed the importance of T254 and Q263 in TRAF3 binding.

It is likely that the weak affinity between TRAFs and receptor peptides made it possible for these interactions to be influenced by flanking residues in the receptor peptides and even by crystal packing. The lack of peptide interaction due to packing constraint has been seen in several


A
TRAF3-TANK
B
TRAF3-LTβR
C
TRAF3-CD40
D
P-2
P0
P1
P2
P3
E
P0
D265
P1
T254
Q263

of the crystal forms of TRAF2. For TRAF3, both the CD40 and TANK peptides also have a number of packing interactions in their crystals. This adds another layer of complexity in dissecting and extrapolating the modes of interactions in physiological settings.

V. TRAF6-Receptor Interactions: Distinct Specificity

In contrast to the shared receptor binding specificity among TRAF1, 2, 3, and 5, TRAF6 has a unique receptor interaction specificity that is not shared by other members of the TRAF family. In addition, TRAF6 is the only TRAF family member that also participates in the signal transduction of the IL-1R/TLR superfamily by its specificity for IRAKs.

In keeping with the distinct specificity, crystal structures of TRAF6 in complex with peptides from CD40 and TRANCE-R revealed striking differences in the mode of peptide binding (Ye *et al.*, 2002). Although a similar region of the TRAF domain is used for receptor interaction, the receptor chain cuts across the TRAF domain in a dramatically different trajectory that is 40° away from the TRAF2 direction (Fig. 8A, B). As a result, side chains of TRAF6 binding peptides interact with surface pockets on TRAF6 that are completely different from those on TRAF2 (Fig. 8C).

Among the numerous structural differences between TRAF6 and TRAF2, the insertion of P468 in the β-bulge of the β7 strand makes possible for TRAF6 binding peptides to form more extensive main chain hydrogen bonds with this strand of the TRAF-C domain (residues 234–238 of CD40 and 344–349 of TRANCE-R with residues P468-G472 of TRAF6) (Fig. 8D). Rather than the more twisted PPII helix conformation for the core region of TRAF2 binding peptides, the TRAF6 binding peptides assume a typical β conformation. The peptides no longer interact with the β3-β4 loop in the TRAF6 complexes, due to a 12 Å movement in the position of this loop (Fig. 8B).

Similar to the nomenclature used for TRAF2 binding peptides, residues E235 of CD40 and E346 of TRANCE-R were designated as the P_0 position of TRAF6 binding peptides, because they occupy a similar, although not identical location to the P_0 residue (Q/E) in the TRAF2 binding motif

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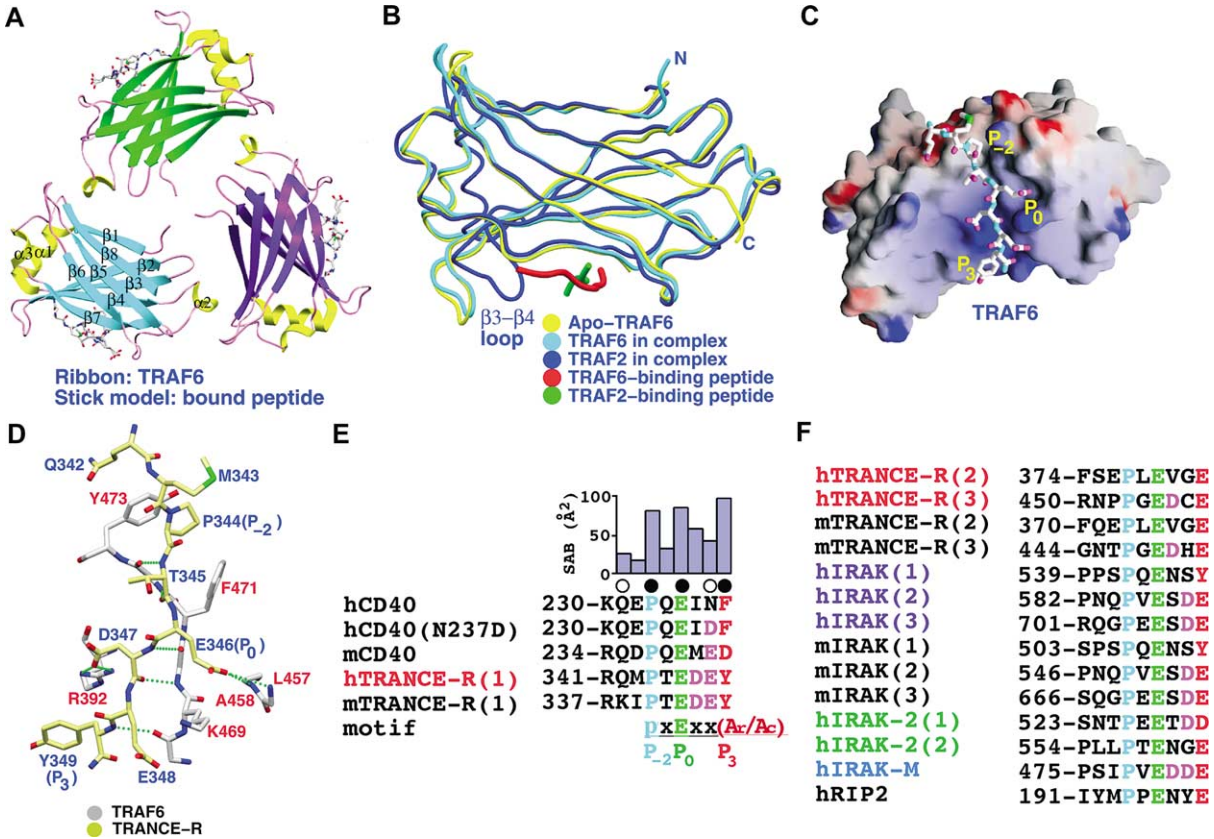
Fig. 7. TRAF3-receptor interactions. (A, B, C) Ribbon diagrams of TRAF3-TANK, TRAF3-LtβR and TRAF3-CD40 complexes. (D) Superposition of the TANK peptide (green) and the LTβR peptide (yellow) with the LMP1 peptide (pink) in the TRAF2-LMP1 complex. Only the core residues are shown. (E) Details of the CD40 conformation in the TRAF3-CD40 complex. Shown in pink is the superimposed CD40 conformation seen in the TRAF2-CD40 complex.


A
β1
β8
α3
α1
β2
β6
β5
β3
β4
α2
β7
Ribbon: TRAF6
Stick model: bound peptide
B
N
C
β3–β4
loop
Apo–TRAF6
TRAF6 in complex
TRAF2 in complex
TRAF6–binding peptide
TRAF2–binding peptide
C
P−2
P0
P3
TRAF6
D
Q342
M343
Y473
P344(P−2)
T345
F471
D347
E346(P0)
L457
R392
A458
K469
Y349
(P3)
E348
TRAF6
TRANCE–R
E
SAB (Å2)
100
50
0
hCD40 230-KQEPQEINF
hCD40(N237D) 230-KQEPQEIDF
mCD40 234-RQDPQEMED
hTRANCE-R(1) 341-RQMPTEDEY
mTRANCE-R(1) 337-RKIPTEDEY
motif pxExx(Ar/Ac)
P−2P0 P3
F
hTRANCE-R(2) 374-FSEPLEVGE
hTRANCE-R(3) 450-RNPPGEDCE
mTRANCE-R(2) 370-FQEPLEVGE
mTRANCE-R(3) 444-GNTPGEDHE
hIRAK(1) 539-PPSPQENSY
hIRAK(2) 582-PNQPVESDE
hIRAK(3) 701-RQGPEESDE
mIRAK(1) 503-SPSPQENSY
mIRAK(2) 546-PNQPVESDE
mIRAK(3) 666-SQGPEESDE
hIRAK-2(1) 523-SNTPEETDD
hIRAK-2(2) 554-PLLPTENGE
hIRAK-M 475-PSIPVEDDE
hRIP2 191-IYMPPENYE

(corresponding Cα distance 2.3 Å). The P_0 position is near the point of intersection between the two classes of peptides. Based on this naming system, peptide residues from P_{-4} to P_3 are in direct contact with TRAF6. Analyses of surface area burial and specific side chain interactions suggest that the P_{-2}, P_0, and P_3 residues contribute most to the structural interaction, each of which interacts with a specific pocket on the surface of TRAF6 (Fig. 8D).

The P_{-2} Pro residue interacts with the TRAF6 surface pocket formed by hydrophobic residues F471 and Y473 of TRAF6. The carboxylate of the P_0 Glu residue is recognized by hydrogen bonding with main chain amide nitrogen atoms of L457 and A458 and the aliphatic portion of the side chain exhibits a close fit with the TRAF6 surface. In addition, the carboxylate of the P_0 residue may form a favorable charge-charge interaction with the side chain of K469, although the interaction is not within hydrogen bonding distance. The P_3 residue, F238 of CD40 or Y349 of TRANCE-R, is adjacent to several aromatic and basic residues of TRAF6, including H376, R392, H394, and R466. An amino-aromatic interaction is observed between Y349 of TRANCE-R and R392 of TRAF6. Structurally, a similar interaction should be possible for F238 of CD40, or for an acidic residue, which is present in mouse CD40.

Interestingly, the peptide interaction sites of TRAF6 are quite analogous to those of TRAF2. The P_{-2} pocket forming residues are replacement of S467 and C469, respectively, which form the P_{-2} pocket in TRAF2 that is about 3 Å away from the corresponding TRAF6 pocket. The P_0 pocket residues L457 and A458 correspond to S454 and S455 of TRAF2, the side chains of which provide the hydroxyls for hydrogen bonding with the Glu/Gln side chains at the P_0 position. Similarly, residues R392 and H394 are structural correspondent of R393 and Y395 of TRAF2, two critical residues of the TRAF2 P_1 pocket. Evolutionarily speaking, TRAF6 is one of the oldest TRAF family members. The previous observations suggest an evolutionary mechanism in which the same mutations that abolish the interaction with TRAF6 also create new specificity for TRAF2.

FIG. 8. TRAF6-receptor interactions. (A) TRAF6 trimeric model. (B) Superposition of TRAF6 with TRAF2, with bound receptor peptides. (C) Electrostatic surface of TRAF6 in complex with the TRANCE-R peptide. (D) Detailed interactions between TRAF6 and TRANCE-R. (E) Sequence alignment of CD40 and TRANCE-R and the corresponding surface area burial (SAB) and the TRAF6-binding motif. (F) TRAF6-binding sequences identified based on the structurally defined motif. Modified from Ye *et al.* (2002).

Structure-based sequence alignment of TRAF6 binding sites in human and mouse CD40 and TRANCE-R led to the definition of a TRAF6 binding consensus sequence pxExx(Ar/Ac) (p shown in lowercase because of tolerance for other small to medium sized residues, Ar for aromatic and Ac for acidic residues) (P_{-2} to P_3) (Fig. 8E, F). Similarly as observed for TRAF2 binding peptides, the P_{-2} position is likely able to accommodate conserved changes to small and medium-sized hydrophobic residues. Consistent with this analysis, mutational studies on CD40 showed that the Pro residue can be changed to Ala without showing qualitative differences in TRAF6 interaction and signaling, while a change to Gln abolishes interaction and signaling (Table II). The P_0 position could similarly accommodate Gln as well as Glu, but a change to Ala drastically reduced interaction and signaling. Similarly, removing the side chain of the P_3 residues drastically reduced TRAF6 interaction and signaling. In addition, it appears that the P_1 and P_2 positions may have a preference for acidic residues due to their complementarity to the basic TRAF6 surface formed in particular by the side chains of R392 and K469 at this region. Isothermal titration calorimetry (ITC) measurements showed that peptides with acidic residues at these positions possess higher affinity to TRAF6 (Table III).

VI. Thermodynamics of TRAF-Receptor Interactions

A. *Weak Affinity and Avidity*

TRAF recruitment requires ligand-induced changes in receptors that allow simultaneous interactions of each TRAF trimer with three receptor intracellular domains. This observation implicates that monomeric TRAF-receptor interactions are of low affinity so that the interactions do not occur in the absence of receptor activation. A number of quantitative biophysical characterizations with isothermal titration calorimetry (ITC) and surface plasma resonance (SPR) have provided solid support to this view (Table III).

Measurements of TRAF2-receptor peptide interactions using ITC showed that TRAF2 interacts with CD40, CD30, and Ox40 peptides with dissociation constants in the range of 40–60 μM and with TNF-R2, 4-1BB, and LMP1 with dissociation constants in the 0.5–1.9 mM range (Ye and Wu, 2000). Similarly, TRAF3 interacts with TANK with a dissociation constant of 24 μM (Li *et al.*, 2002). In the case of TRAF6, a range of dissociation constant between 50–770 μM was measured by ITC (Ye *et al.*, 2002). In all cases, the heat release during the titration of receptor peptides into a

TRAF solution exhibited excellent agreement with ideal binding, indicating the presence of a single type of binding site and the lack of cooperativity in the interaction.

The quantitative measurements between TRAFs and receptor peptides likely represent TRAF-receptor interactions in the context of the full-length intracellular domains. The ability of the short five-residue peptide of Ox40 to confer an affinity to TRAF2 as high as the longer peptides is consistent with the structural observation that a core of a few residues appears to dominate the interaction with TRAF2 (Park *et al.*, 1999; Ye *et al.*, 1999), although it is likely that residues beyond the core contribute further to the interactions. In the case of CD40, characterization by SPR of the interaction between TRAF2 and monomeric full-length cytoplasmic domain of CD40 gave rise to a dissociation constant of 30 μM (Pullen *et al.*, 1999b), similar to the 60 μM dissociation constant derived from the ITC measurement on a CD40 peptide.

The measured TRAF-receptor interaction affinities are much lower than most protein-protein or protein-peptide interactions involved in signal transduction (Kuriyan and Cowburn, 1997), suggesting that TRAF recruitment is entirely dependent on affinity enhancement through avidity. However, the exact magnitudes of this enhancement by trimerization are not really clear and may depend on the exact separation, geometry and conformational state of the oligomerized receptors. In an artificial experiment using trimeric coiled coil to trimerize the intracellular domain of CD40, a 12-fold higher affinity was observed using SPR experiments. However, it may be expected that in optimal cases, the avidity effect should be exponential.

B. Favorable Enthalpy, Unfavorable Entropy, and Induced Fit

ITC measurements on TRAF2-receptor interactions have revealed invariably favorable enthalpies and unfavorable entropies, indicating that all these interactions are energetically driven by exothermic enthalpy. The enthalpy of the interaction showed a relative large negative linear dependence with temperature, as measured for the TRAF2-CD30 interaction at 10, 20 and 30 °C (Table III).

Structurally, TRAF2-receptor interactions possess both hydrophilic and hydrophobic components. For example, in the TRAF2-CD30 interaction, approximately 750 Å^2 of hydrophobic surface area and 400 Å^2 polar surface area are buried at the interface. Therefore the favorable enthalpy may arise from the significant amount of polar interactions, including main chain hydrogen bonds, side chain hydrogen bonds and salt bridges in the complex. On the other hand, the relatively large negative heat

capacity change is consistent with the presence of significant hydrophobic component in the interactions.

The large dependence of enthalpy with temperature is indicative of a specific interaction, even though that the affinities of these monomeric interactions between TRAF2 and receptors are rather low. As suggested from thermodynamic studies of protein-DNA interactions, a non-specific weak complex held together by electrostatic forces often exhibits little temperature dependence of enthalpy (Ladbury, 1995).

The observed unfavorable entropy appears to contradict the presumably favorable solvation entropy from the burial of significant hydrophobic areas at the TRAF2-receptor interfaces. It is likely that the unfavorable entropy may be largely due to conformational restraints on the receptor peptides upon TRAF2 interaction. Secondary structure predictions of cytoplasmic tails of most TRAF-interacting TNFRs suggest that these receptor tails do not have a pre-formed well-ordered three-dimensional structure. Rather, linear peptides from localized regions of the receptors are responsible for TRAF2 interaction. Therefore it is likely that the peptides, or full-length receptors, are flexible before docking onto the protein and penalized by conformational entropy. This suggests the involvement of conformational changes and induced fit in the interaction between TRAF2 and receptor peptides.

VII. TRAF2-TRADD Interaction: A Novel Mode of TRAF Signaling

A. The TRADD-N Domain

The TRAF2-TRADD interaction is mediated by the TRAF domain of TRAF2 and the N-terminal domain of TRADD (TRADD-N). The TRADD-N domain has so far only been found in mammalian TRADD proteins. It folds into an α-β sandwich with a four-stranded β-sheet and six α-helices, each forming one layer of the structure (Park *et al.*, 2000; Tsao *et al.*, 2000) (Fig. 9A). The β-sheet is entirely anti-parallel and slightly twisted with a strand order of β2, β3, β1, and β4. There are two helices each in the β1-β2 and β3-β4 crossover connections while the β2-β3 connection is hairpin-like. The remaining two helices (E and F) are near the carboxy-terminus of the domain; the loop in between (EF loop) partly covers one end of the exposed face of the β-sheet.

The basic topology of the TRADD-N domain resembles the family of ferredoxin-like α-β sandwiches (Orengo and Thornton, 1993), which are often present as domains in larger structures such as the palm domain of

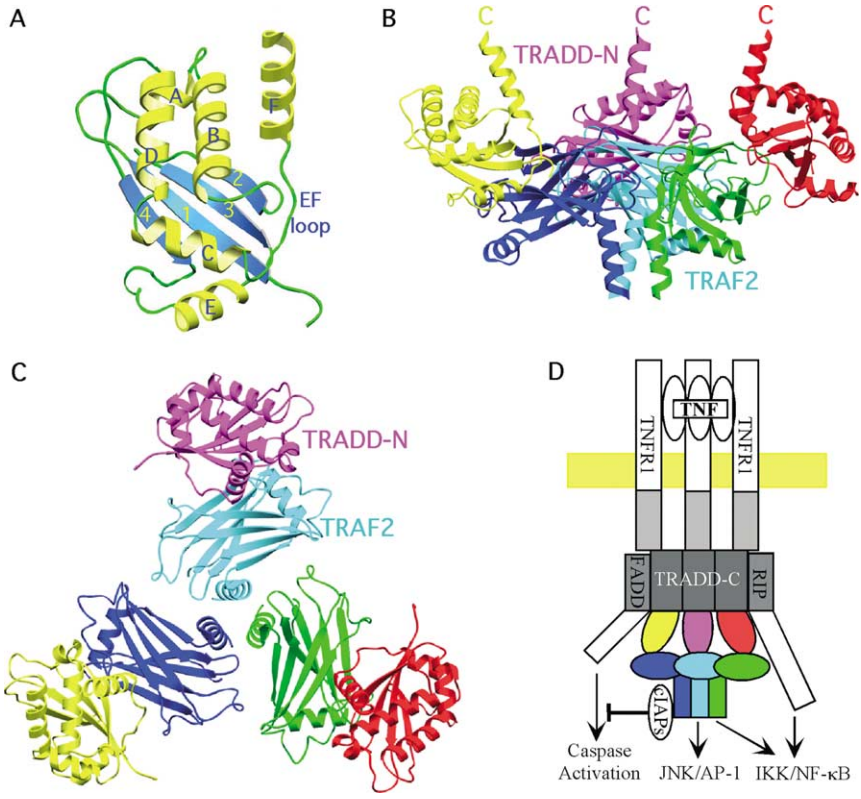


FIG. 9. TRAF2-TRADD interaction. (A) Ribbon drawing of the TRADD-N domain. (B, C) Ribbon diagrams of the TRAF2-TRADD complex. (D) Schematic representation of the TNF-R1 signaling complex. Reproduced from Park *et al.* (2000).

many polymerases and the dimerization domain of carboxypeptidases. However, the TRADD-N structure is much more elaborate than a typical ferredoxin-like fold, as represented by extra helices in β1-β2 and β3-β4 connections and two additional helices near the carboxy-terminus.

B. TRADD-TRAF2 Interface: Interactions and Energetics

The trimeric TRAF domain of TRAF2 imposes the threefold symmetry to the stoichiometrically bound TRADD-N (Fig. 9B, C). When viewed from the side of the mushroom-shaped trimeric structure of TRAF2,

TRADD-N interacts with TRAF2 at the upper rim of the mushroom cap and adds a wing-like structure to the mushroom. The carboxyl terminus of TRADD-N projects up to the membrane-proximal direction of the complex, indicating the possible location of the carboxyl terminal death domain of TRADD. In this orientation, TRADD can be recruited to TNF-R1 via its death domain and forms the central platform for recruiting other signaling molecules such as FADD, RIP and TRAF2 (Fig. 9D).

The TRAF2-TRADD interaction occupies a similar surface at the edge of the β-sandwich of TRAF2, indicating the competitive nature of TRAF2-TRADD and TRAF2-receptor interactions. Each TRADD-N contacts one protomer of the TRAF domain. The binding is at the β-sandwich domain exclusively, away from the coiled-coil domain. The interaction between TRAF2 and TRADD buries a total of 1500 $Å^2$ surface area, in contrast with the smaller protein-peptide contacts in TRAF2-receptor interactions. There are small local conformational adjustments in the Cα positions of TRAF2 (0.5–1.0 Å) within or immediately adjacent to the TRADD binding site. We suspect that TRADD-N (especially the EF loop) may undergo larger conformational rearrangement upon TRAF2 binding based on the relative instability of TRADD-N in its isolated state.

The actual molecular contacts of TRAF2 with TRADD and with receptors are entirely different, and TRADD does not possess TRAF2 binding motifs. The interface between TRADD-N and the TRAF domain of TRAF2 possesses dual ''ridge into groove'' contacts, in which both TRADD-N and TRAF2 contain reciprocal elevations and depressions (Fig. 10A). Two distinct and adjacent regions may be defined (Fig. 10B). Region I is mediated by the exposed shallow face of the β-sheet of TRADD and a surface protrusion of TRAF2 formed by β7, the following loop and the connection between β3 and β4. Many residues, such as Y16, F18, H65, and S67 and I72 of TRADD and T401, H406, L471 and P474 of TRAF2, collectively contribute to this interaction (Fig. 10C). Region II is mediated by a highly charged prominent ridge formed by TRADD residues 143–149 in the EF loop and a surface depression of TRAF2 presented by strand β6 and the following loop. Residues 145–147 of TRADD form anti-parallel main chain hydrogen bonds with residues 448–450 of TRAF2 in the connection between β6 and β7. Many side chain hydrogen bonds and salt bridges exit at this interface including the hydrogen bonds between R146 of TRADD and D445 of TRAF2, between R76 of TRADD and D450 of TRAF2, between Q143 of TRADD and S454 of TRAF2, and between D145 of TRADD and main chain of G468 of TRAF2 (Fig. 10D). Water molecules abound at Region II of the interaction and at the boundary between the two regions (Fig. 10E).

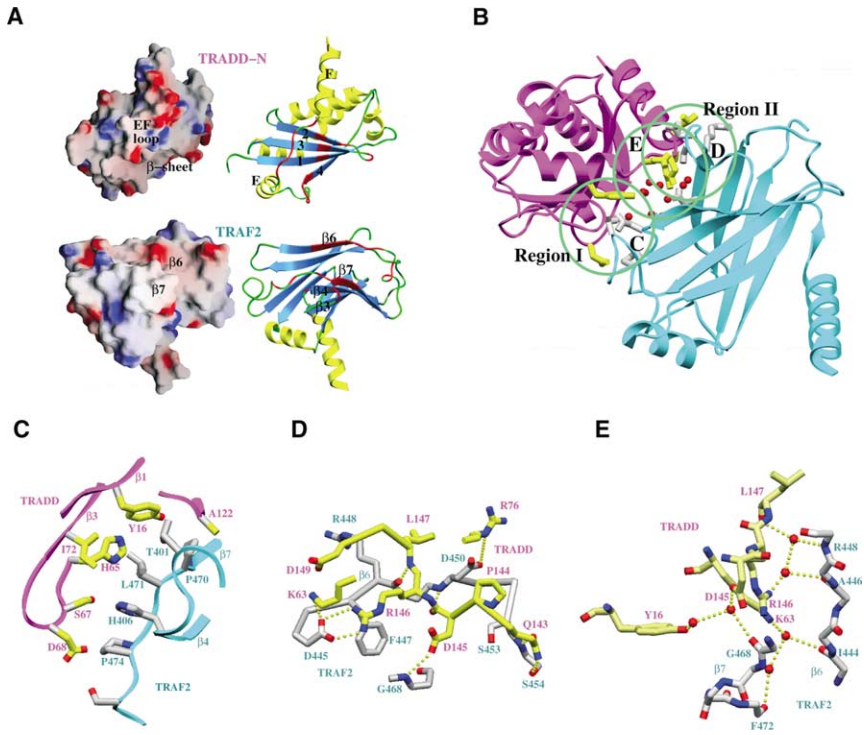


FIG. 10. Detailed TRAF2-TRADD interaction. (A) Interaction surfaces and their locations on the individual structures (in red). (B) Molecular interactions at the two regions of the interactions. (C, D, E) Details of region I, region II and water-mediated interactions, respectively.

Residues in the two regions showed differential effects on the binding affinity, in a manner unrelated to their surface area burial at the interface (Table II). In general, alanine substitutions of residues in Region I (Y16, F18, H65, and S67) had much more drastic effects, despite their smaller surface area contribution. This may be explained by their complete solvent inaccessibility in the complex and the largely hydrophobic nature of the contact. Residues in Region II were selected for their large surface area burial and their abilities to form direct and water-mediated hydrogen bonds or salt bridges at the interface (Q143, D145, R146 and L147). Rather unexpectedly, mutations on Q143, D145 and L147 had minimal effects on the binding energy ($\leq$3-fold). The only residue that exhibits a large effect is R146, which forms buried bi-dentate hydrogen bonds and

a salt bridge to residue D445 of TRAF2. These structure-based mutational studies reveal that Region I is the primary energetic determinant of the interaction, while Region II contributes to specificity and perhaps long range attraction forces to facilitate association.

C. Higher Affinity and Distinct Specificity: More Efficient Signaling and Suppression of Apoptosis

SPR measurements revealed that the TRAF2-TRADD interaction possesses a dissociation constant of 7.8 μM, which is significantly stronger than direct TRAF2-receptor interactions ($K_d = 40$ μM–1.9 mM) (Ye and Wu, 2000). The higher affinity of the TRAF2-TRADD interaction suggests that TRADD might be a stronger inducer of TRAF2 signaling. Comparison of signal transduction from the two TNF receptors, TNF-R1, a TRADD-mediated TRAF2 signaling receptor, and TNF-R2, a direct receptor-mediated TRAF2 signaling receptor, substantiated this hypothesis. TNF-induced JNK activation was used as the readout for TRAF2-mediated signal transduction because TRAF2 is the major activator of JNK (Yeh *et al.*, 1997). Indeed, in response to TNF, the TNF-R1-expressing fibroblasts had significantly higher level of JNK activation than the TNF-R2-expressing fibroblasts when normalized to receptor and cell numbers.

In addition to higher affinity, swapping mutagenesis showed a specificity of TRADD for TRAF1 and TRAF2, but not TRAF3, 4, 5, and 6 (Table II). This is in contrast to the direct TRAF recruitment by the subgroup of TNF receptors that do not contain a death domain, which exhibits a more promiscuous specificity for TRAF1, 2, 3, and 5 (Ni *et al.*, 2000; Park *et al.*, 1999; Ye *et al.*, 1999). Interestingly, TRAF1 and TRAF2 interact constitutively with the cellular caspase inhibitors cIAP1 and cIAP2 (Fig. 9D), which were originally isolated from the TNF-R2 signaling complex (Rothe *et al.*, 1995). In addition, this interaction with cIAPs requires both TRAF1 and TRAF2 (Rothe *et al.*, 1995), suggesting that TRAF1/2 hetero-oligomer interacts with cIAP1 and cIAP2. The specificity of TRADD for TRAF1 and TRAF2 ensures the recruitment of cIAPs to the signaling complex, which may be important for direct caspase-8 inhibition (Wang *et al.*, 1998) and the immediate suppression of apoptosis at the apical point of the cascade.

The high affinity and restricted specificity of TRADD for TRAF1/2 explain that although TNF-R1 and related death receptors are capable of both cell survival promotion and cell death induction, under most circumstances, they rarely induce cell death. TRAF2 signaling plays an important role in protection from apoptosis because TRAF2-deficient mice are overly sensitive to TNF-induced cell death (Yeh *et al.*, 1997). This TRAF1/2-mediated protection from apoptosis is dependent on the high affinity of

TRADD-TRAF2 interaction because TRADD mutants with even moderately decreased affinity to TRAF2, such as H65A, Y16A and S67A, showed significantly increased cell death induction. The much higher affinity of TRAF2 recruitment by TRADD versus the direct TRAF2 recruitment by TNF receptors without a death domain suggests the importance of preserving this high affinity in the signal transduction by TNF-R1 and related receptors. A modest decrease in the monomeric affinity, which may translate into a larger difference in the multimeric interaction, could lead to an imbalance in the regulation between cell survival and cell death.

There may be two ways that TRAF2 can protect cells from apoptosis and the cooperation of the two mechanisms may be necessary for efficient apoptosis suppression. The first mechanism has to do with recruitment of the cellular caspase inhibitors, cIAP1 and cIAP2, to the TNF-R1 signaling complex by TRAF1 and TRAF2 to inhibit caspase-8 activation (Rothe *et al.*, 1995; Wang *et al.*, 1998). This mechanism acts at the entry point of TNF-mediated apoptosis, is independent of gene transcription and explains the specificity of TRADD for TRAF1 and TRAF2 but not other members of the TRAF family. The second mechanism may be related to the ability of TRAF2 to activate NF-κB, which may induce the expression of anti–apoptotic genes to suppress cell death (Beg and Baltimore, 1996). Since apoptosis induction can be fast and does not require gene transcription and protein synthesis, the first mechanism is likely to be crucial in placing apoptosis under check while the second mechanism may further strengthen the anti-apoptotic function of TRAF2.

However, under certain circumstance, the TNF-R1 signaling complex can switch to its apoptotic mode. One possible mechanism may have to do with the mitochondrial release of Smac in a JNK-dependent manner (Deng *et al.*, 2003). As an IAP-interacting protein, Smac may compete with TRAF1/2 for cIAP interaction to remove cIAPs from the TNF-R1 signaling complex. Another possible mechanism is the involvement of NF-κB-inducible gene product FLIP. TNF-R1-induced apoptosis appears to involve the switch from an initial plasma membrane bound complex consisting of TNF-R1, TRADD, RIP1 and TRAF2 to a cytoplasmic complex consisting of TRADD, RIP1, FADD, and caspase-8 (Micheau and Tschopp, 2003).

VIII. TRAF Signaling Inhibitors

Because of the role of TRAFs in inflammation and tumorigenesis, down-regulation of TRAF signaling may serve potential therapeutic benefits to many diseases. A way to inhibit TRAF signaling is to identify reagents that inhibit TRAF-receptor interactions. The ability of short peptides

to interact with TRAFs provides templates for peptidomimetic drug identification approaches. In addition, small molecules may be identified that fit into the specific surface pockets of TRAFs, such as the hydrophobic P_{-2} site.

As a proof of principle that inhibition of TRAF-receptor interactions could act as potential therapeutic means, cell permeable TRAF6-interacting decoy peptides were constructed by fusing the TRAF6 binding sequences from TRANCE-R with a hydrophobic sequence of the Kaposi fibroblast growth factor signal peptide (Ye *et al.*, 2002). They were tested for their inhibitory effects on TRANCE-R-mediated signal transduction. Pre-treatment with the decoy peptides inhibited endogenous TRANCE-R mediated NF-κB activation upon TRANCE stimulation in a dose-dependent manner. Further, the decoy peptides inhibited TRANCE-induced osteoclast differentiation in a cell line model and in primary cells, without affecting cell viability. These results demonstrate that peptides containing the TRAF6 binding motif can act as decoys to inhibit TRAF6 signaling and associated biological functions.

The effectiveness of these decoy peptides to compete with endogenous oligomeric interactions raises the optimism that it is possible for monomeric interactions to compete with the endogenous oligomeric forms of the interactions. There may be two possible mechanisms by which the cell permeable decoy peptides could work. First, endogenous concentrations of receptors may be significantly low to allow competition by the decoy peptides due to concentration advantage. Second, it is possible that the hydrophobic leaders of the decoy peptides may render the peptides to associate with cellular membranes to achieve higher local concentrations of the peptides.

Several aspects of the TRAF-receptor interactions may assist the design of high affinity TRAF binding inhibitors. The low affinity interaction indicates a non-ideal steric or chemical complementarity between TRAFs and these receptor peptides, increasing the possibility for affinity improvement. Surface pockets, especially the hydrophobic P_{-2} pocket, may be targets for small molecules. In addition, as reduction of conformational entropy may contribute negatively to the interaction, an increase in affinity may be achieved by rigidifying potential TRAF2 binding moieties.

IX. DD and DD-DD Interactions

Death domains (DDs) are found in the intracellular portion of death receptors such as Fas and TNF-R1 and death receptor-interacting adapter proteins such as FADD and TRADD. They are protein-protein interaction domains (Fesik, 2000). The structure of DDs, as first revealed by the NMR

structure of the death domain of Fas, consists of six amphipathic antiparallel α-helices arranged in a unusual Greek Key topology (Huang *et al.*, 1996) (Fig. 11A). The overall fold of other death domains, such as the NGFR p75 (Liepinsh *et al.*, 1997), FADD (Jeong *et al.*, 1999), and TNFR-1 (Sukits *et al.*, 2001; Telliez *et al.*, 2000) were found to be similar to the Fas death domain with only minor differences in the length and orientation for some of the α-helices (Fesik, 2000) (Fig. 11B).

Structural studies have revealed that several other domains involved in cell death and inflammatory signaling transduction, including the death effector domain (DED) (Fig. 11C), the caspase recruitment domain (CARD) and the Pyrin domain (PYD), also possess the same six helix bundle structures of DDs (Chou *et al.*, 1998; Eberstadt *et al.*, 1998; Hiller *et al.*, 2003), forming the death domain superfamily. Interestingly, interactions have only been observed among proteins within the same subfamilies with no cross interactions between proteins from different subfamilies.

Despite the availability of a large number of structures of isolated domains in the death domain superfamily, only two complex structures have been determined, the Pelle/Tube DD/DD complex (Xiao *et al.*, 1999) and the Apaf-1/Procaspase-9 CARD/CARD complex (Qin *et al.*, 1999), revealing two samplings of modes of interactions in this superfamily (Fig. 11D, E). Regardless of the details of the interactions, the biggest surprise, in both cases, is perhaps the asymmetry of the interactions, considering what might have been expected for homophilic interactions. Pelle and Tube are DD-containing proteins involved in Toll signaling in *Drosophila* with Pelle as a Ser/Thr kinase and Tube as an adapter protein. The Pelle/Tube interaction is mediated by the insertion of the α4 helix and the following loop of Pelle into a groove of Tube formed by the α1-α2 corner, α6 and the preceding loop and by the insertion of the C-terminal tail of Tube into a cavity formed by the α4-α5 and α2-α3 hairpins of Pelle (Qin *et al.*, 1999). Apaf-1 and Procaspase-9, on the other hand, are essential components of the mitochondrial apoptotic pathway. The Apaf-1/Procaspase-9 interaction is mediated by the mutual recognition of the slightly concave surface of procaspase-9 formed by the positively charged α-1a, α-1b and α4 helices and the convex surface of Apaf-1 formed by the negatively charged α2 and α3 helices.

The structural basis of DD/DD interactions involved in death receptor signaling, such as those in the Fas-FADD complex, remains largely unresolved. Although no solid experimental evidence is available, the Fas-FADD and TNF-R1/TRADD complexes are likely to be trimeric, most likely with Fas and TNF-R1 possessing oligomerization surfaces. Therefore such complexes might comprise at least two interfaces, a self-oligomerization surface

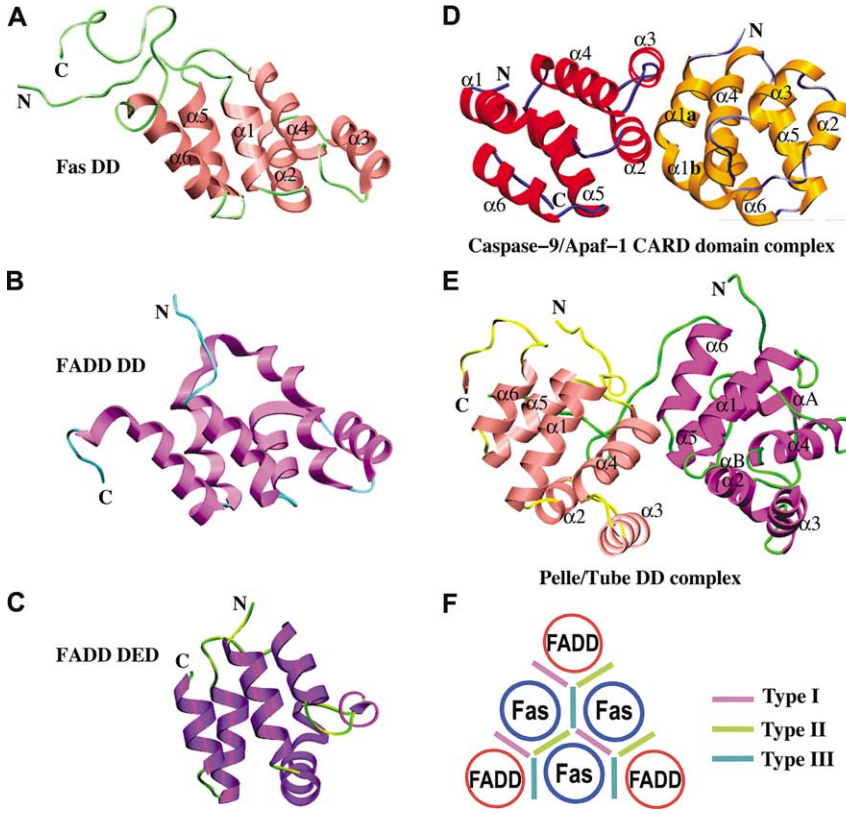


FIG. 11. Structures of DDs and DEDs. (A) DD of Fas. (B) DD of FADD. (C) DED of FADD. (D) CARD domain complex of caspase-9 (red) and Apaf-1 (orange). (E) DD complex of Pelle (pink) and Tube (purple). (F) A hypothetical model of the oligomeric Fas-FADD complex, showing the potential involvement of three types of interfaces.

and a Fas-FADD interaction surface. This conjecture is supported by mutational data that showed that residues important for binding and/or function spread throughout the surfaces of Fas (Martin *et al.*, 1999), FADD (Hill *et al.*, 2004), TRADD (Park and Baichwal, 1996) and TNF-R1 (Telliez *et al.*, 2000).

An interesting model of the Fas-FADD complex has been proposed based on the Pelle/Tube and Apaf-1/Procaspase-9 structures (Weber and Vincenz, 2001) (Fig. 11F). Although there is no particularly convincing rational for using these structures as models, assuming that Fas has

both a surface like Procaspase-9 for interaction with Apaf-1 (type I) and a surface like Pelle for interaction with Tube (type II), superposition of Pelle and Procaspase-9 in their respective complexes brought Tube and Apaf-1 into interaction vicinity to create a third interface. In addition, further propagation of alignment created a hetero-hexameric complex of Fas-FADD. Although details of the interactions are unlikely to be correct, the opposed surfaces in the complex appear to be chemically compatible and exhibit no serious steric clashes. In the model, FADD binds at the oligomerization interface of Fas and therefore only interacts with oligomerized Fas. One potential problem with this model is the lack of three-fold symmetry for Fas oligomerization. It nonetheless provided an interesting conceptual model to perceive DD-DD complexes. It remains to be shown by oligomeric DD-DD complex structures whether these interactions and models are true and whether they have any general implications for the DD superfamily.

X. Conclusion: Emerging Principles of Post-Receptor Signal Transduction

A. Ligand-Induced Receptor Activation: Re-orientation of Intracellular Domains into Closer Proximity for Signaling

TNF and related cytokine ligands form trimers or higher order oligomers in solution and on cell surface (Eck and Sprang, 1989). Upon receptor interaction, three receptor molecules interact with a TNF trimer to form 3:3 ligand-receptor complexes (Banner *et al.*, 1993). In the conventional view, this ligand-mediated receptor trimerization is the induction of receptor signal transduction across the cell membrane. However, more recent studies have demonstrated that TNFRs exist in pre-formed non-signaling trimers before ligand binding through a region of the extracellular domain named pre-ligand-binding assembly domain (PLAD), which is physically separate from the ligand binding site (Chan *et al.*, 2000; Siegel *et al.*, 2000). PLAD appears to be required for all aspects of receptor signaling, including ligand interaction, receptor activation and dominant interference of mutant receptors. The presorting of receptor chains into homotypic complexes on the cell surface makes biological sense in that it could promote rapidity and specificity of ligand interaction and prevent interference among different receptors of a same ligand.

Receptor pre-assembly has also been described for other receptor families and appears to be a fundamental principle for the signal transduction of oligomeric receptors. For example, the insulin receptor is a

constitutive disulfide-linked dimer, while both epidermal growth factor receptor (EGFR) and erythropoietin receptor (EpoR) may exist as dimers independent of ligand interaction (Constantinescu *et al.*, 2001; Grotzinger, 2002; Moriki *et al.*, 2001; Yu *et al.*, 2002). The erythropoietin receptor dimers undergo a scissors-type movement to accommodate the ligand (Livnah *et al.*, 1999; Remy *et al.*, 1999) and the extracellular domain orientations are apparently tightly coupled to transmembrane helix rotations to activate the receptors (Moriki *et al.*, 2001; Seubert *et al.*, 2003).

Whether or not transmembrane helix rotation is a crucial event that leads to receptor activation in the case of TNFR superfamily, a net effect of ligand binding and receptor activation appears to be the induced closer proximity of the intracellular domains (Fig. 12A), as shown by fluorescence energy transfer experiments (Chan *et al.*, 2000). In the structures of TRAF-receptor complexes, the distance between bound receptor peptides is approximately 50 Å. This suggests that a distance of separation between the intracellular domains of receptor chains on the order of 50 Å may be optimal for TRAF recruitment and signaling transduction.

B. Geometry of TRAF-Receptor Interactions

Structural, biochemical, biophysical and cell biological analyses of the TRAF-receptor interactions have eluted to the following structural mechanisms of the interactions (Fig. 12A). First, the intracellular domains of members of the TNFR superfamily do not appear to have well-defined tertiary structures. Rather, they may be fairly flexible and disordered in the absence of TRAF interaction. Second, short linear sequences within the intracellular domains are responsible for TRAF recruitments and the interactions are driven by favorable enthalpy. The interactions between core TRAF binding sequences and TRAFs and possibly secondary interactions mediated by residues flanking the core sequences provide conformational restraint to the intracellular domains upon TRAF interactions. This is implicated from the entropic loss in TRAF-receptor interactions. Third, the directionality of receptor peptides in TRAF-receptor complexes places the mushroom-shaped cap against the membrane, allowing the amino-terminal domains in full-length TRAFs to be exposed to the cytosol for interaction with down-stream signaling molecules (Fig. 1). 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 TRAF2 binding CD40 peptide has been shown capable of both TRAF interaction and wild type like NF-κB activation when linked immediately after the transmembrane region of CD40 (Cheng and Baltimore, 1996).

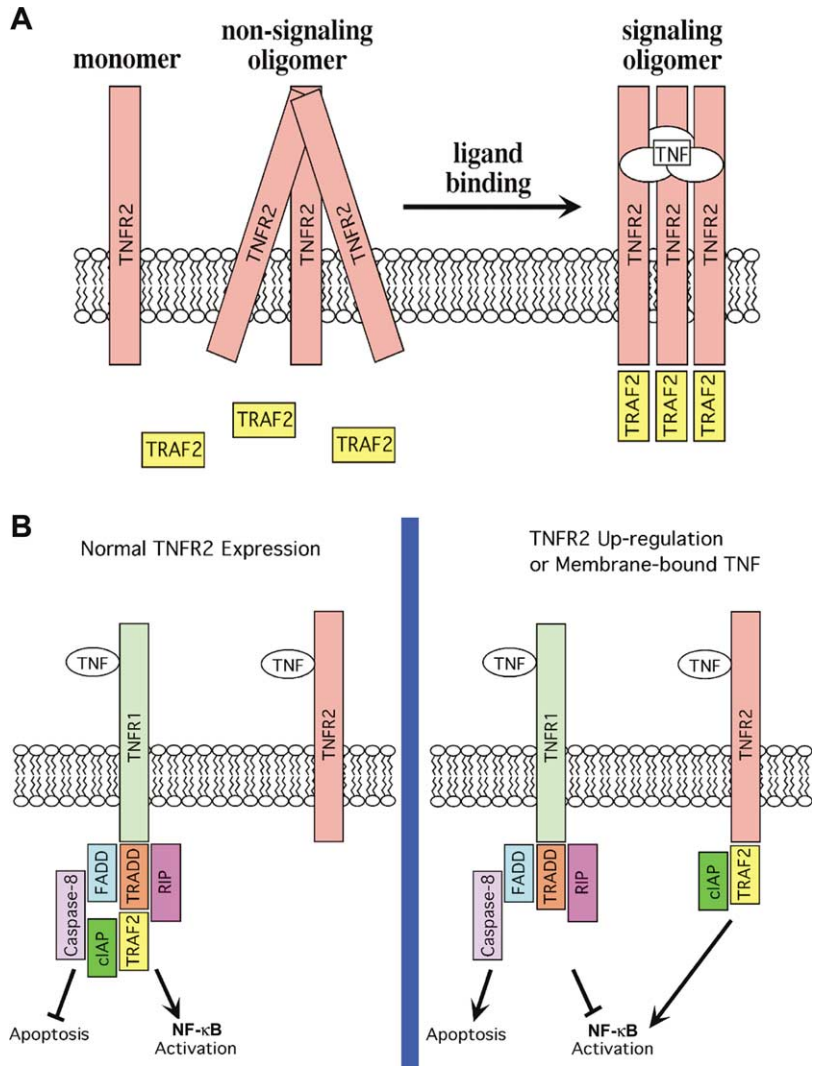


FIG. 12. Principles of post-receptor signal transduction. (A) Receptor activation and TRAF recruitment. (B) Competitive TRAF recruitments and regulation of cell survival and death.

This oligomeric TRAF-receptor interaction geometry provides avidity to increase the affinity of TRAF recruitment upon receptor activation. This is necessary because interactions between TRAFs and monomeric receptors are relatively weak (Table III), which ensures that TRAFs do not

interact with non-activated receptors. The dependence of TRAF recruitment on ligand-induced receptor activation has been shown for several TNFRs including TNF-R2, CD40 and LTβR (Kuhne *et al.*, 1997; Shu *et al.*, 1996; VanArsdale *et al.*, 1997) and is considered a common feature of the TNF receptor superfamily. Both affinity and specificity of the interaction will be greatly amplified by the avidity contribution from this oligomeric association, transforming a low affinity interaction into a tight and highly specific one.

For TRAF recruitment, since each receptor peptide contacts one TRAF domain only, avidity is the only factor that contributes to enhanced affinity of TRAFs for activated receptors. Due to the lack of structural information on DD interactions, it is not known whether ligand-induced receptor recruitment of FADD and TRADD (Hsu *et al.*, 1996b) is also purely driven by avidity or the intracellular DD of the receptors also form composite binding sites for FADD and TRADD upon receptor activation. In keeping with the latter scenario, a protein known as silencer of death domains (SODD) (Jiang *et al.*, 1999) has been shown to associate with the intracellular DD of TNF-R1 and get released upon ligand stimulation to activate the receptor.

C. Energetics: Affinity Differences of TRAF Recruitment and Different Avidity Requirements for Efficient Signaling

Quantitative characterization of monomeric TRAF-receptor and TRAF-adapter protein interactions showed a wide range of affinities from 7.8 μM to 1.9 mM (Table III), raising the question whether these receptors require different avidity contribution for efficient TRAF signaling. Many of the TNF-like cytokine ligands are membrane-bound and therefore may be capable of inducing membrane patching and higher order of receptor aggregation. Even though the minimal aggregation state of TRAFs appears to be trimeric, higher orders of aggregation may be possible as well in response to the higher order of receptor aggregation. This would increase the avidity in the TRAF-receptor interaction and the strength of the signal transduction. In keeping with this hypothesis, soluble trimeric CD40L can be fairly inefficient in inducing CD40 signaling under certain circumstances, compared to cell-bound or cross-linked hexameric CD40L (Pullen *et al.*, 1999b). In addition, TNF-R2 is mostly activated by membrane-bound form of TNF (Grell *et al.*, 1995). In some instances, multiple TRAF binding sequences in a single protein may also allow additional avidity from the interaction of TRAFs with neighboring TRAF binding sites. Therefore, TRAF-mediated signal transduction may be modulated at several levels including affinity and avidity.

The consideration on the combined effects of affinity and avidity explains why TNF-R2 is rather non-responsive to soluble TNF but mostly respond to cell-bound TNF (Grell *et al.*, 1995), while TNF-R1 can be stimulated efficiently with soluble TNF. TNF is synthesized as a transmembrane ligand and efficiently converted to soluble TNF under most but not all circumstances. The affinity of TRAF2 for TNF-R2 (K_d = 0.5 mM) is remarkably lower than the TRADD-TRAF2 interaction (K_d = 7.8 μM). The cell-bound TNF may be capable of creating higher order of receptor clustering than soluble TNF and may therefore provide enhanced avidity to TRAF2 recruitment by TNF-R2 to potentiate this signal transduction. TNF-R2 signaling can also be induced by the up-regulation of expression, which again increases the avidity contribution of TRAF2 recruitment (Fig. 12B).

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

D. Specificity and Diverse Recognition: Conserved Interaction with Key Residues

A fundamental principle derived from the structural studies of TRAF-receptor complexes is that although the interactions are of low affinity, they are highly specific as shown by the identical interactions in different crystal packing environments and with different lengths of receptor peptides. This is in contrast to low affinity non-specific protein-DNA interactions that are mediated solely by charge attractions.

In addition to the specific recognition of a particular receptor peptide by TRAFs, a diverse set of receptor sequences may be recognized in a conserved mode of interaction by TRAFs. This ability of TRAFs to recognize diverse receptor sequences forms the basis for the wide spectrum of biological effects that TRAFs mediate. The molecular basis of this recognition resides on the conserved interaction of TRAFs with a few conserved key residues of receptor sequences rather than structural plasticity.

E. Biological Interplay: Competitive TRAF Recruitments and Context-Dependent Regulation of Survival and Death

Because TRAFs can be recruited to many different receptors, including survival receptors and receptors with potential for both cell death and cell survival induction, the competitive TRAF recruitment may modulate the signaling components of these receptors to induce differential biological effects. This would relate the outcome of 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.

This competitive recruitment hypothesis explains how TNF-R2, a survival receptor, could induce apoptosis (Fig. 12B). It has been observed that mice deficient in TNF-R2 showed decreased cell death following TNF treatment (Erickson *et al.*, 1994), while overexpression of TNF-R2 could lead to increased sensitivity to TNF-induced apoptosis (Chan and Lenardo, 2000; Haridas *et al.*, 1998; Heller *et al.*, 1992; Vandenabeele *et al.*, 1995; Weiss *et al.*, 1997). Moreover, TNF-induced apoptosis of activated primary T lymphocytes has also been shown to require TNF-R2 (Sarin *et al.*, 1995; Zheng *et al.*, 1995). Functionally, this may be important for Fas-independent peripheral deletion of T lymphocytes and the regulation of mature T-cell homeostasis.

The observed cooperation of TNF-R2 in TNF-induced apoptosis could be explained by a decreased recruitment of TRAF1, TRAF2 and cIAPs in the TNF-R1 signaling complex. Overexpression or up-regulation of expression of TNF-R2 upon T-cell activation could lead to an increased association of TRAF1 and TRAF2 with TNF-R2, sequestering intracellular TRAF1, TRAF2 and the constitutively associated cIAPs away from the signaling complex of TNF-R1 and related receptors. In keeping with this proposal, the TRAF2 binding region of TNF-R2 is required for this potentiation of TNF-R1-mediated apoptosis (Weiss *et al.*, 1998).

This interplay between TNF-R2 and TNF-R1 may be extended to other members of the TNF receptor superfamily such as CD40, CD30, LTβR and CD27. These receptors have been shown to induce cell death under certain circumstances (Grell *et al.*, 1999). Similar to TNF-R2, activation of any of these receptors could lead to a sequestration, and possibly degradation (Duckett and Thompson, 1997), of TRAF1, TRAF2 and cIAPs. As these receptors have been shown to induce the expression of TNF (Grell *et al.*, 1999), the thus activated TNF-R1 would be tipped to apoptosis induction under the lack of recruitment of TRAF1, TRAF2 and cIAPs.

F. Remaining Questions

While the current structural and functional studies have shed lights on the molecular mechanisms of post-receptor signal transduction by the TNFR superfamily, many important questions remain. One such question is the structural basis for the formation of death receptor signaling complexes, involving DD-DD and DED-DED interactions. Another question is the molecular basis of TRAF downstream signaling. Does it involve oligomerization and proximity induced activation of down-stream effectors, or conformational modulations? Because of the importance of the TNFR superfamily in human disease, an ultimate question lies on the translation of structural and functional studies into therapeutic applications.

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