Molecular Basis for the Unique Specificity of TRAF6

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Abstract

Tumor necrosis factor (TNF) receptor (TNFR) associated factor 6 (TRAF6) is a unique member of the TRAF family of adaptor proteins that is involved in both the TNF receptor superfamily and the interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily signal transduction pathways. The ability to mediate signals from both families of receptors implicates TRAF6 as an important regulator of a diverse range of physiological processes such as innate and adaptive immunity, bone metabolism, and the development of lymph nodes, mammary glands, skin, and the central nervous system. This chapter will highlight the structural and biochemical studies of TRAF6 in receptor interactions and discuss the potential for peptidomimetic drug application based on TRAF6 receptor binding motif.

Introduction

TRAF6 was first identified in the signal transduction pathways of CD40 and IL-1R,[1,2] which makes it the only member of the TRAF family of adaptor proteins to mediate signals from both the TNFR and the IL-1R/TLR superfamily. Gene deletion studies of TRAF6 confirmed the role of TRAF6 in innate and adaptive immunity, bone metabolism, and the development of lymph nodes, mammary glands, skin, and the central nervous system.[3-7]

TRAF6 has a unique sequence specificity for receptor interaction that does not overlap with other TRAF family members.[8,9] Although TRAF6 interacts directly with TNFR family members, CD40 and TRANCE-R (also known as RANK), TRAF6 does not directly bind to IL-1R/TLR superfamily members. TRAF6 is coupled to IL-1R/TLR activation by interacting with Ser/Thr kinases IRAK1, IRAK2, IRAK-M and possibly IRAK-4.[2,10-12] IRAKs are recruited to activated receptors through interaction with adaptor proteins containing the Toll and IL-1R (TIR) domain such as MyD88, Mal/TIRAP, TRIF, TRAM, Tollip, and SARM,[13,14] which then interacts with TIR-domain of the receptors.

The downstream signaling events of TRAF proteins converge on the activation of transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1),[15,16] that transcribe genes involved in numerous cellular and immune regulation. Interestingly, the activation pathways for NF-κB and AP-1 by TRAF2 and TRAF6 may both utilize a nondegradative lysine-63 linked polyubiquitin chains for downstream signaling. In vitro reconstitution assay has shown that the RING domain of TRAF6 functions as a ubiquitin ligase to synthesize lysine-63 linked polyubiquitin in the presence of the ubiquitin conjugation enzyme system, Ubc13 and Uev1A.[17,18] These nondegradative polyubiquitin chains have been shown to be important in the activation of protein kinase complex called IκB kinase (IKK), which directly activates NF-κB.[17] Similarly, the inhibition of NF-κB

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TRAFs, edited by Hao Wu.

activation was observed by the deubiquitination of TRAF2 and RIP (receptor interacting protein) by the nondegradative deubiquitinating enzymes, CYLD[19-21] and A20,[22] respectively.

Expression and Crystallization of TRAF6

The domain organization of TRAF6 is consistent with other TRAF family members. The N-terminal domain is comprised of a RING and five zinc finger regions followed by a coiled-coil TRAF-N domain and a conserved TRAF-C domain.[23] The N-terminus of TRAF6 mediates downstream signaling, whereas the C-terminus is involved in self-association and receptor interaction.[24]

Structural studies on the TRAF domain of TRAF6 were initiated to determine the receptor interaction specificity by TRAF6. Extensive TRAF6 construct variations were utilized to produce soluble protein that led to the successful crystallization of TRAF6.[25] Mapping studies of the TRAF-C domain defined residues 351-522 to be the region responsible for receptor interaction.[2,26] Initial construct designs were based on the above domain definitions and sequence alignments.[2,24,26] These early constructs were mostly insoluble or had a tendency to aggregate. Based on the successful TRAF2 TRAF-domain crystallization,[27] similar constructs were made for TRAF6 (residues 333-508 and residues 333-512). These TRAF2 based constructs contained a small portion of the coiled-coil TRAF-N domain along with the TRAF-C domain. These new constructs were partially soluble.

At high TRAF6 protein concentrations, TRAF6 exists in trimer form, which is consistent with the structure of TRAF2.[27] However, TRAF6 333-508 construct was only able to crystallize at low protein concentrations of 1-2 mg/ml. The X-ray diffraction of this TRAF6 construct was weak, nevertheless, a dataset was collected and the structure was solved by molecular replacement.

Analysis of the structure showed one TRAF6 monomer per crystallographic asymmetric unit. Interestingly, the coiled-coil region of the TRAF6 was situated in a position that would clash sterically with another symmetry related molecule. The location of the coiled-coil region therefore explains why TRAF6 was only able to crystallize at low protein concentrations and as a monomer. Based on this information, further constructs starting at 343, 346, and 349 were made, which deleted the short coiled-coil region. These proteins were partially soluble and the construct with residues 346-504 was readily crystallized both alone and in complex with CD40 and TRANCE-R peptides.[28]

Molecular Basis for the Distinct Specificity of TRAF6

The TRAF-C domain of TRAF6 shows the highest degree of difference compared to other TRAF protein structures, when compared to TRAF2 TRAF-C domain (Fig. 1). The TRAF2 TRAF-C domain is comprised of 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[27] (Fig. 1A). Although the overall architecture is the same, superposition of TRAF6 with TRAF2 shows an r.m.s.d of 1.1-1.2 Å for 127 aligned Cα positions within 3.0 Å (Fig. 1C). This TRAF6-TRAF2 structural difference is larger than for TRAF3 TRAF-C domain (Fig. 1B).

There are numerous residue insertions or deletions within the loop regions of TRAF6 structure. Specifically, β3-β4 loop contains one residue insertion, β5-β6 loop contains three residue insertions, and β7-β8 loop contains one residue deletion. The β3-β4 loop of TRAF6 exhibit a movement of up to 12 Å in Cα positions, relative to TRAF2. Therefore, TRAF6 no longer interacts with receptor peptides in this region. The remaining loop regions show on average 2-5 Å Cα movement in comparison to TRAF2. In the absence of a receptor peptide the β6-β7 loop is disordered. Although TRAF6 crystallized as a monomer, the TRAF trimerization loops, β2-β3 and β4-β5, are conserved in TRAF6. This shows that on a structural level TRAF6 can form trimers.

In agreement with the distinct receptor specificity and function of TRAF6, crystals of TRAF6 in complex with CD40 or TRANCE-R peptides revealed novel binding modes.[28] The receptor chain binds across the TRAF domain of TRAF6 that exhibits a trajectory which is 40° away from the receptor peptide position on TRAF2 (Fig. 2A,B). This mode of receptor peptide association on TRAF6 results in a completely different receptor side-chain interactions compared to TRAF2 (Fig. 2C).

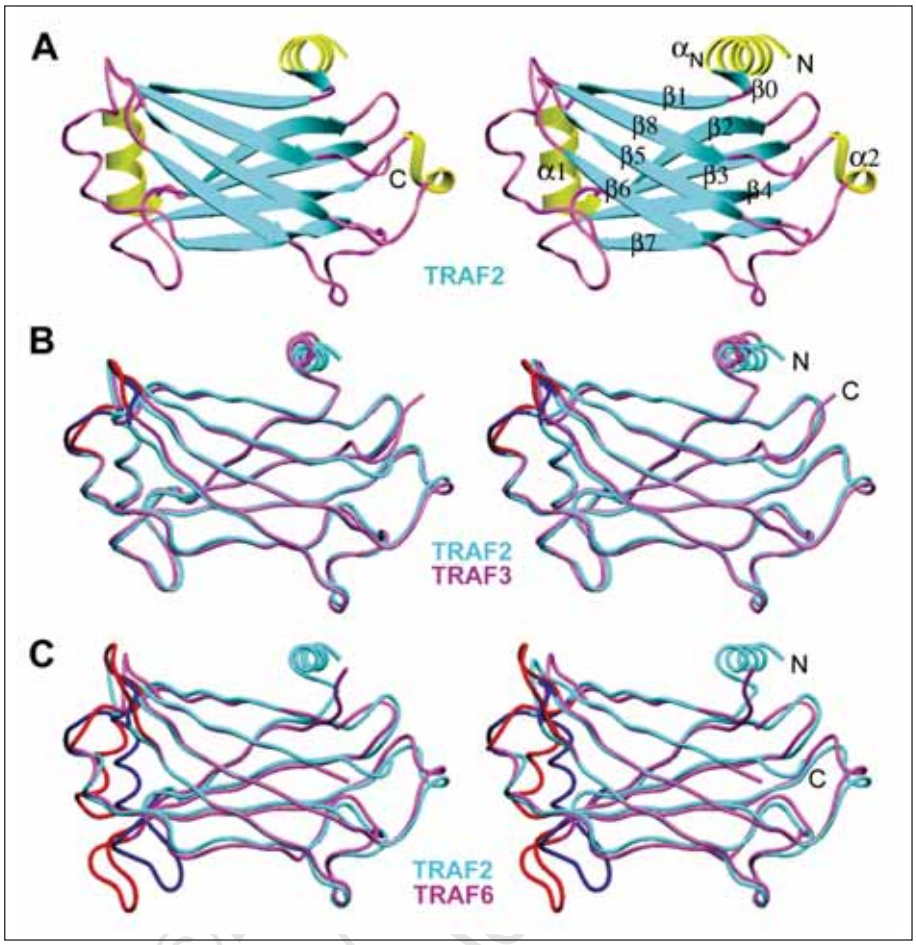

Figure 1. TRAF domain structures. A) Stereo drawing of the TRAF domain of TRAF2 with labeled secondary structures. B) Superposition of the TRAF domain of TRAF2 (cyan) and TRAF3 (magenta). Regions with large differences between the two structures are shown in blue for TRAF2 and red for TRAF3. C) Superposition 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. Modified from Wu.[29] A color version of this figure is available online at www.Eurekah.com.

One of the major structural differences between TRAF6 and TRAF2 is the insertion of a proline residue in the β-bulge of the β7 strand (P468). The P468 insertion allows a more extensive main chain hydrogen bond formations to occur between the receptor peptides and the TRAF-C domain (residues 234-238 of CD40 and 344-349 of TRANCE-R with residues P468-G472 of TRAF6 TRAF-C domain) (Fig. 2D). The CD40 and TRANCE-R peptides assume a typical β conformation rather than a highly twisted polyproline II helix-type conformation observed in TRAF2 binding peptides.

A similar nomenclature of peptide positions as TRAF2 is used for TRAF6 binding peptides. The residues E235 of CD40 and E346 of TRANCE-R were designated as the P_0 position of TRAF6 binding peptides. These residues occupy a similar, although not an identical position as P_0 residue (Q/E) in the TRAF2 binding motif. The peptide residues corresponding to P_{-4} to P_3 of both CD40

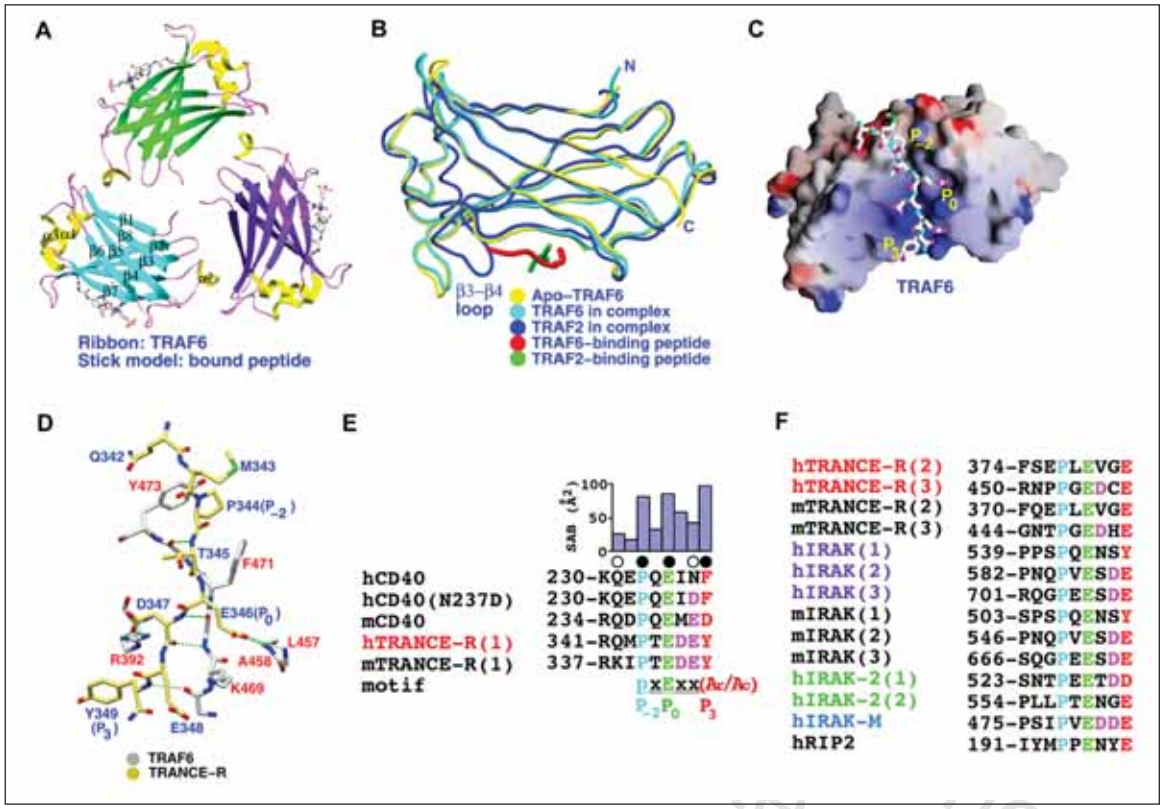

Figure 2. TRAF6-receptor interactions. A) TRAF6 trimeric model. B) Superposition of TRAF6 with TRAF2, with bound receptor peptides. C) Electrostatic surface of TRAF6 in complex with the TRANCE-R peptide. D) Detailed interactions between TRAF6 and TRANCE-R. E) Sequence alignment of CD40 and TRANCE-R and 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.[28]

and TRANCE-R directly interact with TRAF6. Based on the surface area burial and specific side chain interactions of residues at P_{-2}, P_0 and P_3, these residues contribute the most to the interactions (Fig. 2D).

The Pro at P_{-2} position interacts with the hydrophobic pocket created by the residues F471 and Y473 of TRAF6. The carboxylate of the P_0 Glu residue forms hydrogen bonds with the main chain amide nitrogen atoms of L457 and A458, while the aliphatic portion of the side chain aligns nicely along the TRAF6 surface. In addition, the carboxylate of the P_0 Glu may form a favorable charge-charge interaction with the side chain of K469. The P_3 residue in CD40 (F238) and TRANCE-R (Y349) is among several aromatic and basic residues of TRAF6, including H376, R392, H394, and R466. There is an amino-aromatic interaction observed between Y349 of TRANCE-R and R392 of TRAF6. A similar amino-aromatic interaction is possible for F238 of CD40.

Despite the differences between TRAF6 and TRAF2, the peptide interaction sites on TRAF6 are quite analogous to those of TRAF2. The residues forming the P_{-2} pocket of TRAF6 function similarly to the Ser467 and Cys469 of TRAF2. The corresponding TRAF6 P_{-2} pocket is about 3 Å away and consists of Phe471 and Tyr473. The residues forming the pocket for P_0, Leu457 and Ala458, are analogous to Ser454 and Ser455 of TRAF2. In addition, the residues R392 and H394 of TRAF6 are the structural correspondents of R393 and Y395 of TRAF2, which are two critical residues forming the P_1 pocket of TRAF2. Similarities between TRAF6 and TRAF2 indicate an evolutionary mechanism in which the same mutations result in the formation of new interaction specificity for TRAF2 while at the same time abolish interactions for TRAF6.

A consensus sequence for TRAF6 binding motif was derived from the structure-based sequence alignment of TRAF6 binding sites in human and mouse CD40 and TRANCE-R. The motif representing the positions P_{-2} to P_3 consists of pxExx(Ar/Ac), where p is written in lowercase to represent tolerance for other small to medium sized residues, x can by any residues, Ar represents any aromatic residues, and Ac represents any acidic residues (Fig. 2E,F). Mutational studies have shown that similar to what is observed in TRAF2 binding peptides, the proline at P_{-2} can accommodate changes to small residues such as Ala without loss of binding affinity to TRAF6 (Table 1). The P_0 position can also accommodate a Gln substitution from Glu, but not to Ala. Also, the side chain at P_3

Table 1. Structure-based mutational studies

TRAF6	Receptor/Adapter, Motif Position	Effects[a]	Method	Ref.
WT TRAF6	CD40 (P237A) P_{-2}	+	GST-pulldown and NF-κB activation	28
	CD40 (P237Q) P_{-2}	--		
	CD40 (E239Q) P_0	--		
	CD40 (D242A) P_3	-		
	CD40 (Q235A) P_{-4}	+		
	TRANCE-R (E342A, E375A, E449A) $P_0/P_0/P_0$	--	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) TRAF6 (F471A) TRAF6 (Y473A)	IRAK	-- --- ---	TRAF6 dominant negative effect on NF-κB activation	

[a] +: no effect; -: decreased; --: greatly decreased; ---: drastically decreased.

Table 2. Characterizations of TRAF6-receptor interactions using isothermal titration calorimetry

TRAF6	Receptor/Adapter and Sequence [a]	K_d [b]	Ref.
TRAF6 (333-508)	CD40 (216-245) KKVAKKPTNKAPHPKQEPQEINFPDDLPGS	59.9 μM	28
	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.

position is necessary for proper receptor peptide interaction with TRAF6. Furthermore, the residues at P_1 and P_2 may have a preference for acidic residues to compliment the basic TRAF6 surface formed by the side chains of R392 and K469. Isothermal titration calorimetry (ITC) measurements confirmed this hypothesis by showing much higher binding affinities to TRAF6 by peptides with acidic residues at P_1 and P_2 (Table 2).

Inhibitors of TRAF6 Signaling

TRAF proteins are known to play a critical role in regulating inflammatory responses as well as cell survival and proliferation. The down-regulation of TRAFs may be therapeutically beneficial since it has been implicated in many disease processes involving inflammation and tumorigenesis. One method of inhibiting TRAF signaling is to block TRAF-receptor interaction with short peptides or small molecules.

Based on the crystal structure of TRAF6-TRANCE-R complex, cell permeable TRAF6-interacting decoy peptides were constructed by fusing the TRAF6 interacting sequences from TRANCE-R with the hydrophobic signal peptide of the Kaposi fibroblast growth factor.[12] The effectiveness of the decoy peptides in blocking TRANCE-R mediated signaling was examined by measuring NF-κB activation in RAW264.7 cells. Pretreatment of RAW264.7 cells with the decoy peptides led to a dose-dependent inhibition of NF-κB activation (Fig. 3A). In addition, TRANCE-induced osteoclast differentiation in RAW264.7 and primary mouse monocytes was blocked by cotreatment with the decoy peptides (Fig. 3B,C). These cell-based assays demonstrate the potential of TRAF6-binding motif peptides to inhibit specifically TRAF6 mediated signal transduction.

The success of the TRAF6 binding motif decoy peptides in cell culture studies indicates two possible modes of action. The first mechanism may rely on the low level of endogenous receptors that may be competed out by the higher decoy peptide concentration. The second mechanism may involve the hydrophobic signal peptide sequence of the decoy peptide which can allow association with cellular membranes, thereby achieving high local concentrations of the decoy peptides to compete out the receptor TRAF6 interaction. These mechanisms describe how it may be possible to compete with oligomeric endogenous interactions.

Structural and thermodynamic studies indicate several features of TRAF-receptor interactions that can be manipulated to design high affinity TRAF binding inhibitors. The first feature is the inherent low affinity interaction between the receptor and TRAFs, which indicates a nonideal steric

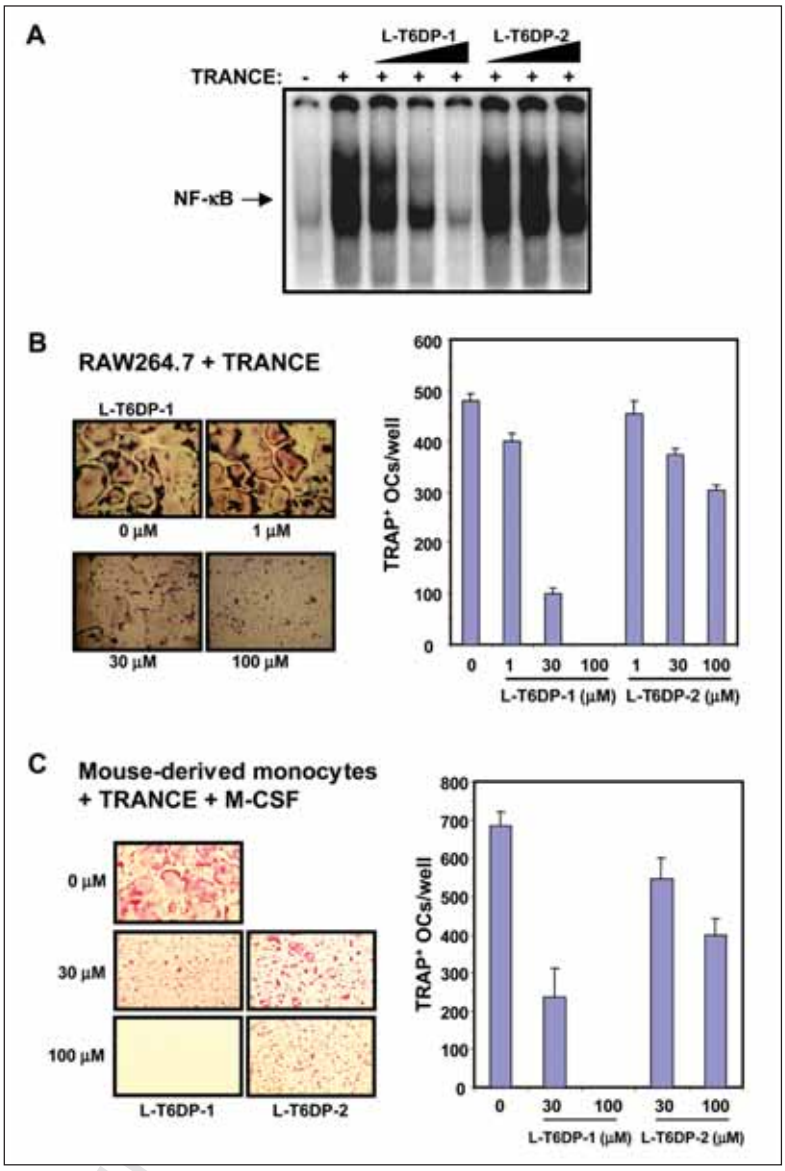

Figure 3. Inhibitory effects of TRAF6 decoy peptides (L-T6DP-1 and L-T6DP-2) in TRANCE-mediated signal transduction and osteoclast differentiation. A) Inhibition of TRANCE-mediated NF-κB activation by TRAF6 decoy peptides, as shown by EMSA. B,C) Inhibition of TRANCE-mediated osteoclast differentiation in RAW264.7 cells (B) and primary monocytes (C) by TRAF6 decoy peptides. Cells were stained for TRAP. Modified from Ye et al.[28]

or chemical complementation. Secondly, surface pockets such as the hydrophobic P_{-2} pocket, can be ideal targets for small molecule inhibitors. Finally, an increase in decoy peptide affinity for TRAFs may be achieved by rigidifying the TRAF binding moieties, since reduction of conformational entropy can lead to a negative contribution to the interaction.

Remaining Questions

The structural and functional studies of TRAF6 have revealed both the similarities as well as the differences between TRAF6- and TRAF2-receptor signaling. There are still many more studies to be conducted to elucidate specific TRAF6 activation mechanisms. For example, we still do not know whether TRAF6 is monomeric before recruitment to the receptors and whether oligomerization per se or oligomerization-induced conformational changes govern TRAF6 activation. These questions and others such as the role of ubiquitination in TRAF6 activation remain to be answered.

Acknowledgment

This work was supported by the National Institute of Health (AI45937), the Pew Charitable Trust and the Rita Allen Foundation.

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