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RESEARCH ARTICLE

Structural biology study of human TNF receptor associated factor 4 TRAF domain

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ABSTRACT

TRAF4 is a unique member of TRAF family, which is essential for innate immune response, nervous system and other systems. In addition to being an adaptor protein, TRAF4 was identified as a regulator protein in recent studies. We have determined the crystal structure of TRAF domain of TRAF4 (residues 292–466) at 2.60 Å resolution by X-ray crystallography method. The trimericly assembled TRAF4 resembles a mushroom shape, containing a super helical "stalk" which is made of three right-handed intertwined α helixes and a C-terminal "cap", which is divided at residue L302 as a boundary. Similar to other TRAFs, both intermolecular hydrophobic interaction in super helical "stalk" and hydrogen bonds in "cap" regions contribute directly to the formation of TRAF4 trimer. However, differing from other TRAFs, there is an additional flexible loop (residues 421–426), which contains a previously identified phosphorylated site S426 exposing on the surface. This S426 was reported to be phosphorylated by IKKα which is the pre-requisite for TRAF4-NOD2 complex formation and thus to inhibit NOD2-induced NF-κB activation. Therefore, the crystal structure of TRAF4-TRAF is valuable for understanding its molecular basis for its special function and provides structural information for further studies.

KEYWORDS TRAF4, TRAF domain, crystal structure, additional loop, phosphorylation site

INTRODUCTION

The tumor necrosis factor (TNF) receptor-associated factors (TRAFs) are essential signaling adaptor proteins, which are initially identified and named due to their interaction with TNF receptor superfamily (TNFR) directly in the cytoplasm (Inoue et al., 2000; Xie, 2013). TRAF family contains six members named from TRAF1 to TRAF6, respectively, which share a homologous TRAF domain at C-terminal region (Bradley and Pober, 2001; Chung et al., 2002). Recently, an additional protein, TRAF7, was found as a new member of TRAF family but named controversially, because it lacks the characteristic TRAF domain at the C-terminus, instead contains a WD40 repeats domain (Bouwmeester et al., 2004; Zotti and Vito, 2012). TRAF domain of this family is functional as a scaffolding region to interact with upstream and downstream effector proteins, as well as to mediate TRAF-TRAF homo/hetero-oligomerization (Bradley and Pober, 2001). Except for TRAF1, other TRAFs contain a RING domain at N-terminal region, which is involved in the process of ubiquitin-dependent protein degradation and functions as an E3-like ubiquitin ligase. Besides, all TRAFs (including TRAF1 and TRAF7) possess different number of Zinc finger motifs following the RING domain in the middle, but their functions are still unclear (Bradley and Pober, 2001; Wajant et al., 2001; Xie, 2013).

In the past twenty years, it was reported that TRAFs play important roles in many cellular processes, such as survival, proliferation, differentiation, cytokine production and so on. They can be employed by numerous receptor families, including TNFR, Toll-like receptors (TLRs), RIG-I like receptors (RIRs), NOD-like receptors (NLRs) and cytokine receptors (Xie, 2013). Thus, many domain structures and complex structures from TRAF family were successfully solved to explain how TRAFs interact with receptor proteins and transduce signals from upstream to downstream, such as the TRAF domains of TRAF2, 3, 6, the RING domains of TRAF2, 6 and complexes of TRAF2-TRADD/CD40/CD30, TRAF3-CD40/TANK, TRAF6-RANK/CD40 (McWhirter et al., 1999; Park et al., 1999; Ye et al., 1999a; Ni et al., 2000; Park et al., 2000; Ye et al., 2002; Yin

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et al., 2009; Zheng et al., 2010). Interestingly, Zhang et al. reported the crystal structures of TRAF5 and TRAF3-Cardif complex and revealed that single mutation can swap their functions so that they could block or activate the binding with Cardif (aliases as MAVS, VISA), a mitochondrial antiviral signaling protein that activates NF-κB and IRF3 (Zhang et al., 2012).

TRAF4, a unique member of TRAF family, was identified from breast cancer-derived metastatic lymph node cells and originally named as CART1 in 1995 (Régnier et al., 1995). As a common feature of domain organization from TRAF family, TRAF4 contains a RING domain, six or seven (the seventh was not confirmed) Zinc finger motifs and a homologous TRAF domain from N-terminus to C-terminus (Kedinger and Rio, 2007). Comparing with other TRAFs, TRAF4 is a unique member in its primary sequence and biology function. On one hand, TRAF4 has two additional nuclear localization signal motifs (NLS) (residues 11–15 and 123–140) in the N-terminal region which result its predominant localization to the nucleus, although it can also be detected in the cytoplasm and cell membrane when overexpressed (Krajewska et al., 1998). Therefore, it may transduce signals to the nucleus directly due to the two special NLS regions. On the other hand, although TRAF4 possesses a highly conserved TRAF domain as other TRAFs, it was identified to interact with two receptors p75NTR (a member of TNFR present in the nervous system) and glucocorticoid-induced TNFR (GITR, a receptor expressed in T cells, B cells and macrophages), not the common receptors for other TRAFs, like TNFR1, TNFR2, CD40 and MyD88. Sequence alignment analysis showed the three residues S, F and F of TRAF4 are not conserved as R, Y and S in TRAF1, 2, 3 and 5, respectively. Those residues are responsible to recognize the TRAF-interacting motifs (TIMs) of TNFR superfamily (Ye et al., 1999b; Kedinger and Rio, 2007).

In addition, TRAF4 was reported to have multi-functions as "regulator" protein other than adaptor protein in the past several years. Li et al. found that TRAF4 binds to p47phox, a subunit of NADPH complex, which is essential for NADPH oxidase activation and ROS production (Li et al., 2005). Another group revealed that TRAF4 could compete with TRAF6 to bind Act1, an E3-ligase NF-κB activator, through the same TRAF binding sites and play the regulation role in IL-17-mediated pathology and signaling pathway (Zepp et al., 2012). Especially, TRAF4 is the only member in TRAF family to function as a negative regulator in NOD2 signaling pathway. It was phosphorylated at the site of S426 by IKKα and then bound with NOD2 directly to inhibit NOD2-induced NF-κB activation (Marinis et al., 2011, 2012). Thus, TRAF4 may play multi-functional roles in innate immune system, even in nervous and other systems. Although two structures containing Zinc finger regions of TRAF4 (residues 102–164 and 190–248, PDB code: 2YUC and 2EOD, respectively) had been determined by NMR method, it is still difficult to understand its distinctive functions clearly and many questions left to be addressed. So we determined the crystal structure of TRAF domain of TRAF4 by X-ray crystallography and revealed structural basis for its phosphorylation and other functions.

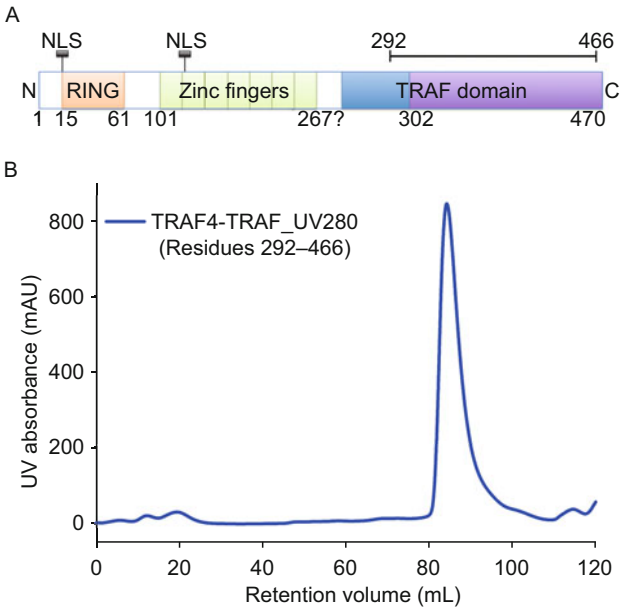

Figure 1. Production of TRAF4-TRAF. (A) Linear domain arrangement of TRAF4, composing of a RING domain, 7 Zinc fingers and a typical TRAF domain from N- to C-terminus. The solved structure contains residues 292–466 and residue L302 is a boundary between N- and C-terminal regions of TRAF4-TRAF domain. (B) Size-exclusion chromatography (SEC) result of TRAF4-TRAF (residues 292–466).

RESULTS

Protein production

To study the structure of human TRAF4, a large number of constructs were designed based on its domain organization and homologues comparison with other TRAF proteins (Kedinger and Rio, 2007) (Fig. 1A). After initial screening, the region (residues 267–466) with 6× His tag in the C-terminus could be overexpressed abundantly in the *E. coli* cells with pET-22b (+) vector, although it was not stable in the process of purification due to non-homogeneity. After degradation experiment in the room temperature for two days, a stable fragment was detected and then we determined its fragment boundary by N-terminal amino acid sequencing assay (the sequence was ^{292}L-Q-E-L^{295} in the N-terminus). Then we re-constructed and obtained the high-level expressed fragment (residues 292–466) (Fig. 1A), which contained the TRAF domain of human TRAF4 (TRAF4-TRAF). Fortunately, this stable fragment was homogeneous and eluted as a single peak during size-exclusion chromatography (SEC) (Fig. 1B), which was assembled as a trimer judging from the retention volume compared with the standard profile of protein markers.

Overall structure of human TRAF4-TRAF

The crystals of TRAF4-TRAF (residues 292–466) were obtained by hanging drop vapor diffusion method at 16°C and we

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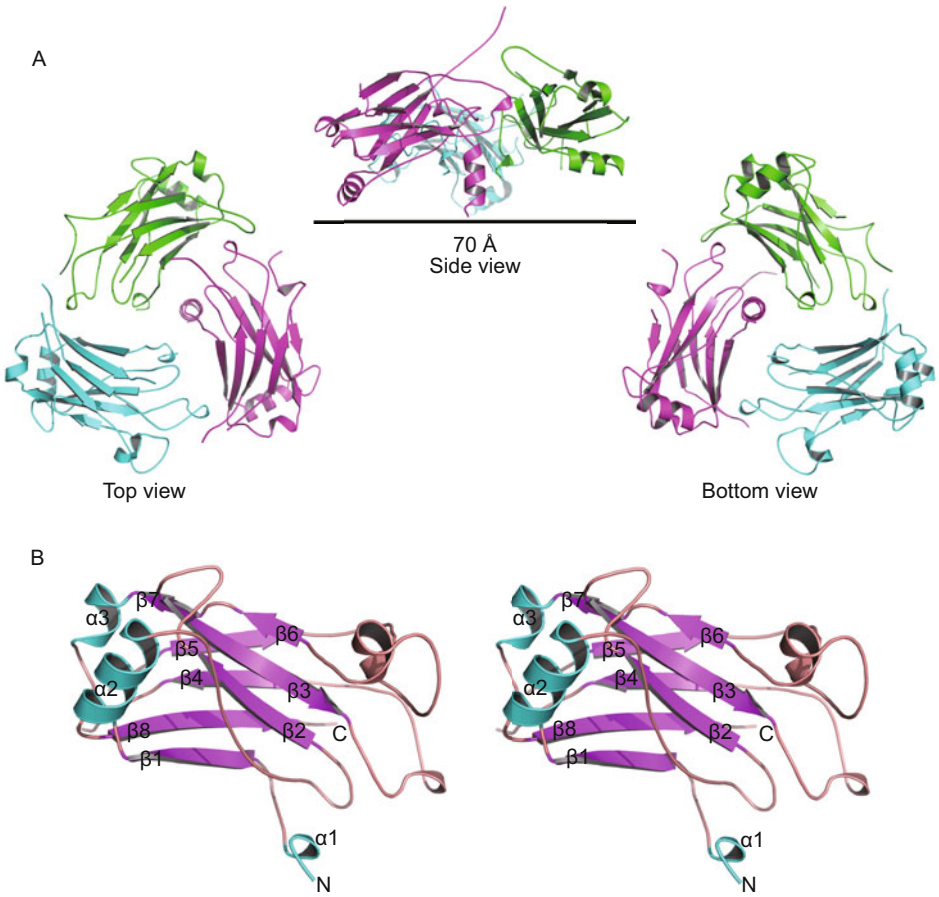

Figure 2. Overall structure of TRAF4-TRAF. (A) Canton representation of TRAF4-TRAF assembly as a trimer, like a mushroom containing an N-terminal super helical "stalk" and a C-terminal "cap", viewed from top, side, bottom. The chains A, B, C were colored in green, cyan and magenta, respectively. (B) Stereo view of TRAF4-TRAF protomer (chain B). The helix, sheet and loop were colored in cyan, magentas and salmon, respectively.

solved its structure by molecular replacement method (McCoy et al., 2007) using TRAF2-TRAF domain (PDB code: 1CA4) as the search model. Finally this model was refined to 2.60 Å resolution with an R factor of 20.11 (R_{free} = 27.57). More detailed crystallography information is listed in Table 1.

In our TRAF4-TRAF structure, three protomers are constructed as a trimer assembly in an asymmetry unit (ASU), which is consistent with the assembly state in solution examined in SEC. It seems like an equilateral triangle shape from top view with a side length of 70 Å (Fig. 2A). Interestingly, the trimer resembles a mushroom shape from the side view, the residues 303–466 in the C-terminus constitute its cap while the residues 292–302 in the N-terminus form its stalk (Fig. 2A). It clearly indicates that the residue L302 is the end of super helical "stalk" region and defines the boundary between the two parts within TRAF4-TRAF domain (Fig. 1A). For each protomer, the "cap" region contains eight β-sheet strands packing as an eight-stranded antiparallel β-sandwich structure in the middle and two helixes and several loops on both sides, while the "stalk" region is short and just composed of a helix (Fig. 2B). The three helixes (α1) from three protomers in a trimer intertwine and stabilize together as a right-handed super helical structure. Except that several loop regions are invisible (A chain: 423–431; B chain: 398–399; C chain: 398–399 and 423–431), the three chains are highly identical with a RMSD of 0.67 Å.

The formation of trimer assembly

As other TRAFs, TRAF4-TRAF is also assembled as a trimer both in solution and in crystals. Trimer probably is the smallest functional unit for TRAF family, hence it is essential to understand how the trimer is formed and stabilized. Generally, in stalk region, three helixes (α1) form a right-handed super helical structure via hydrophobic interactions (Fig. 3A). In addition to stalk region, more hydrogen bonds are identified between the cap areas of each TRAF4-TRAF. PDBePISA software (Krissinel and Henrick, 2007) found several pairs of hydrogen bonds at this region. For example, there are seven, six and five pairs of hydrogen bonds between chains A–B, B–C and C–A, respectively. The side chains of residues K341 and Y342

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Table 1. Data collection and refinement statistics

Data collection	
Crystal	TRAF4-TRAF domain
PDB code	4M4E
X-ray source	SSRF
Crystal to detector distance (mm)	270
Number of images	180
Oscillation width (°)	1.0
Wavelength (Å)	0.98
Space group	$P2_12_12_1$
a, b, c (Å)	57.30, 88.21, 118.43
α, β, γ (°)	90.00, 90.00, 90.00
Mosaicity (°)	0.69
No. Molecules/ASU	3
Resolution range (Å)	50.00–2.60 (2.69–2.60)
Rsym (%)	5.9 (31.7)
Mean I/σ (I)	33.41 (5.68)
Completeness (%)	96.5 (99.0)
Redundancy	6.7 (6.6)
Refinement	
Resolution (Å)	36.03–2.60
No. Reflections	18451
R_{work}/R_{free} (%)	20.11(27.57)
No. Atoms	4088
No. Protein atoms	3998
No. Water	90
Mean B ($Å^2$)	45.84
R.m.s deviations	
Bond lengths (Å)	0.008
Bond angles (°)	1.279
Ramachandran analysis	
Favored region (%)	96.88
Allowed region (%)	3.12
Outliers (%)	0

The numbers in parentheses represent values for the highest resolution shell.

from chain A interact with the residues V304, V309 and I311 of chain B. In addition, residues A373, N376 and W380 of chain A, in an extended loop, insert into chain B and contact with the residues K313, Q393, D395 and D453 of chain B. Similar interaction pattern exists between chains B–C and chains C–A, respectively. Generally, when viewing from stalk to cap direction, the strand β1, β8 and their two neighboring loops (residues 392–404, 447–453) form a shallow groove, which captures the loop (residues 370–385) from the adjacent chain (Fig. 3B). However, the average interface area between the three protomers at their cap region is just only 558 $Å^2$. Thus, the trimeric assembly of TRAF4-TRAF is mainly achieved by the two kinds of contacts, hydrophobic interaction at stalk region and the hydrogen bonds between loops and grooves among neighboring chains.

An additional loop containing a phosphorylation site (S426)

Not surprisingly, TRAF4-TRAF shared a highly conserved three-dimensional structure of TRAF domain with TRAF family members (TRAF2-TRAF6). Actually, Dali analysis (Holm and Rosenström, 2010) also calculated an RMSD of 1.7 Å (Z-score = 22.4) with TRAF domain of TRAF2 (PDB code: 1CA4). We also made structural comparison between TRAF4-TRAF and other TRAFs and found that an additional loop region (residues 421–431), between strand β5 and strand β6, exists only in TRAF4-TRAF structure. All the similar loops in other TRAFs are shorter. Coincidently, the alignment of primary amino acid sequence among TRAF domains of all TRAF proteins obviously showed that the extra six-residue region (residues ^{421}G-T-W-R-G-S^{426}) only exists in TRAF4. In addition, S426, a phosphorylation site identified previously (Dephoure et al., 2008; Olsen et al., 2010), locates in this extra loop. This loop is located on the surface and the exposed S426 can be easily phosphorylated by other kinases (Fig. 4A and 4C). Meanwhile, we examined the other two identical loops in other chains and we found that only loop in chain B has visible electron density, while the same loops in chain A and chain C are missing (Fig. 4B). This indicates that this loop is quite mobile and its flexibility may have some functional indications.

Coincidentally, Derek W. Abbott's group had reported in 2012 that an atypical IKK family member, IKKα, could phosphorylate the unique site S426 of TRAF4 (Marinis et al., 2012).

DISCUSSION

TRAF4 is a unique member of TRAF family and plays multifunctional roles in innate immune system, nervous system and others. It was reported recently to be a key negative regulator protein in NOD-like receptor pathway, which is not a common feature within TRAF family (Marinis et al., 2012). It is essential to investigate the molecular basis of TRAF4's new function. We have screened a large number of constructs for protein expression and eventually solved the crystal structure of TRAF4-TRAF (residues 292–466). Our structure showed that the TRAF4-TRAF is composed with a "stalk" region (residues 292–302) and a "cap" region (residues 303–470). For tertiary structure, TRAF4-TRAF is very similar to TRAF domain of other TRAFs, due to their highly conserved amino acid sequence (44% identity to TRAF domain of TRAF2). It shares a typical trimeric assembly as other TRAFs, which is stabilized by both super helical structure and hydrogen bonds in cap region.

However, differing from other TRAFs, an additional loop (421–426) is identified, this loop contains a pre-identified phosphorylation site S426. On the other hand, this loop has been tested by functional assays in 2012 (Marinis et al., 2012). As a result, the phosphorylation of S426 conferred the ability of TRAF4 to interact with receptor protein NOD2 and prompted them to form a complex. Eventually, this interaction would block the signal transduction from upstream to downstream. Thus, as a key negative regulator protein, TRAF4, when phosphorylated at S426 by IKKα, bound to NOD2 directly and then

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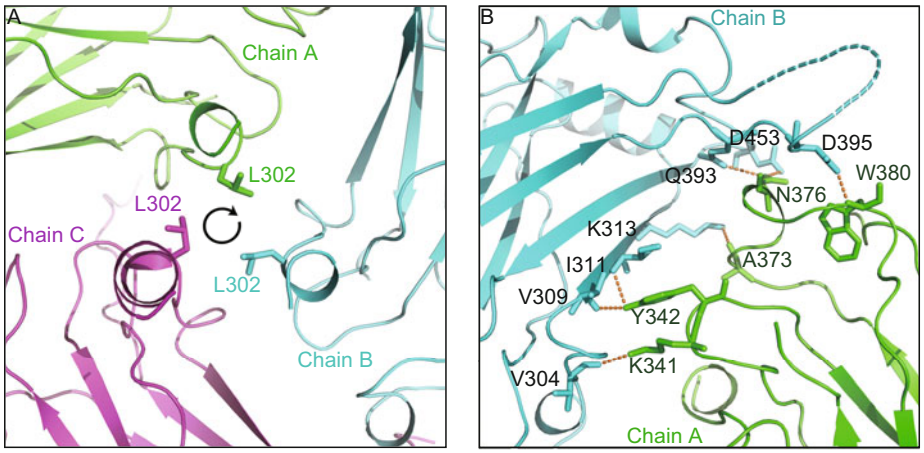

Figure 3. The formation of trimeric assembly. (A) The super helical structure formed by three N-terminal helixes from three protomers stabilized the trimeric oligomerization via hydrophobic interaction. The side chains of three residues L302 indicated three helixes intertwined following a right-handed direction, clearly. (B) A shallow "groove" was built in chain B to capture chain A and twelve residues from two chains donated seven hydrogen bonds in their interface. The chains A, B, C of two figures were colored in green, cyan and magenta, respectively.

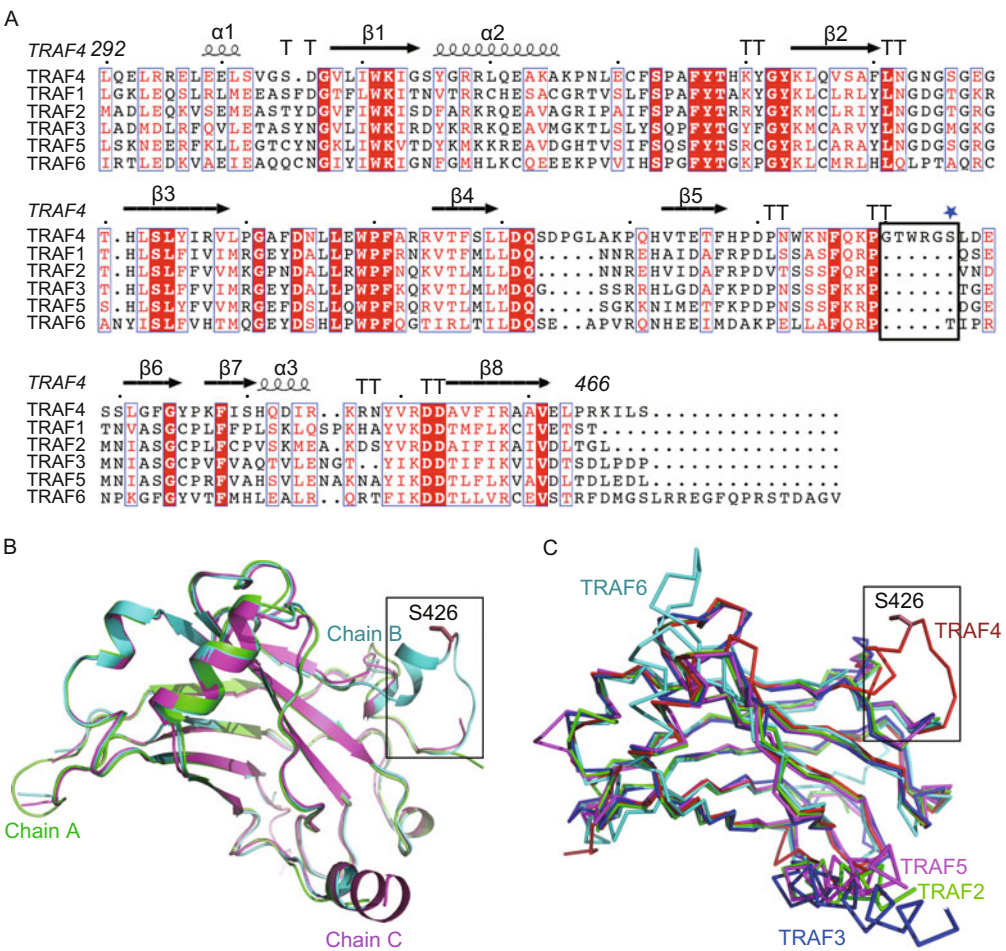

Figure 4. An additional loop containing a phosphorylated site S426. (A) TRAF4-TRAF and its homologs' sequence alignment with secondary structure elements indicated. The phosphorylated site S426 was marked with a blue star. (B) Three chains belonging to TRAF4-TRAF were structurally aligned and indicated the additional loop was just visible in chain B (cyan), not chain A (green) and chain C (magenta). (C) Structural alignment of TRAF domain of TRAF2, 3, 4, 5, 6 (colored in green, blue, red, magenta, cyan, respectively) showed the additional loop only existed in TRAF4. The region of additional loop, containing a phosphorylated site S426, was labeled with a black box in all three figures.

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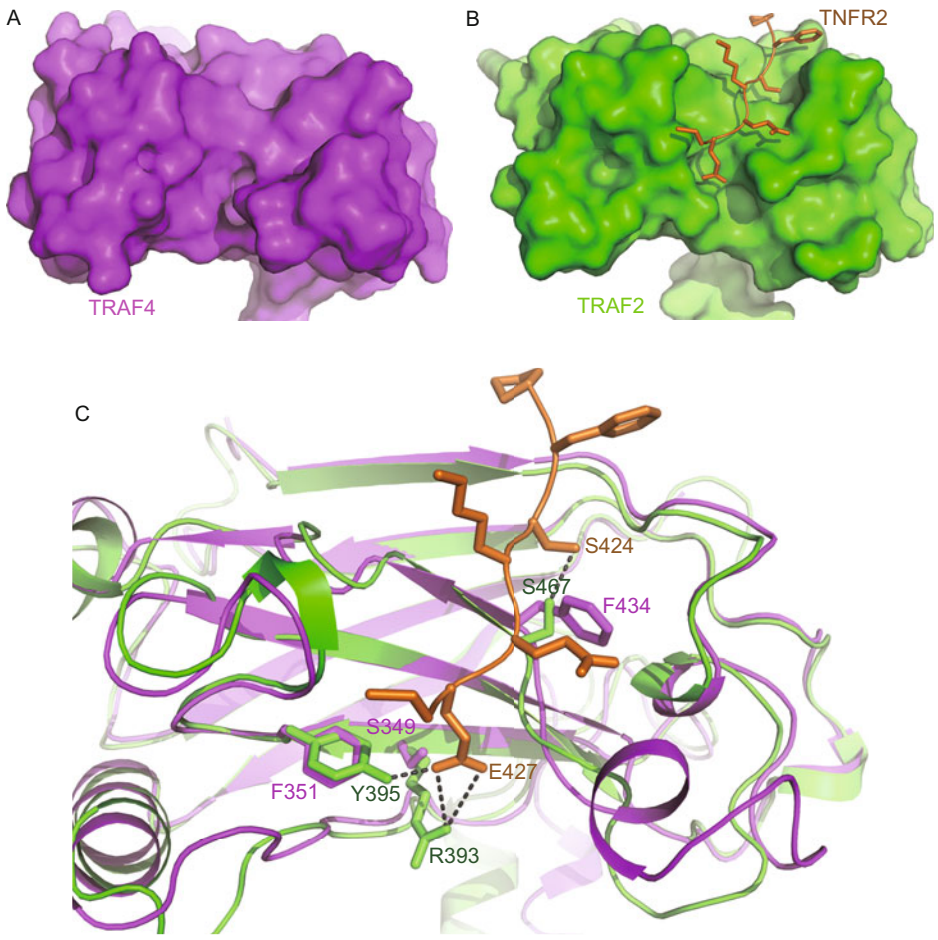

Figure 5. The surface of mediating with TIMs remains exposed in TRAF4. The surfaces of TRAF4-TRAF (A) and TRAF2-TRAF-TNFR2 complex (B) (PDB code: 1CA9), viewed from the same direction, indicate that the binding sites for TIMs remain exposed completely. (C) Structural alignment clearly shows the key residues recognizing the TIM peptide of TNFR2 vary between TRAF4 and TRAF2. TRAF4, TRAF2 and TNFR2 peptide in all three figures are colored in magenta, green and orange, respectively.

inhibited NOD2-induced NF-κB activation, which was originally activated by NOD2-RIP2 complex. Furthermore, based on sequence alignment and homology modeling using structure of TRAF2, they proposed that, in TRAF4, an outstretched loop would exist between strand β6 and strand β7 of TRAF2-TRAF (corresponding to strand β5 and strand β6 of our solved TRAF4-TRAF) and the phosphorylated residue S426 should locate there. The prediction is very much consistent with our solved structure of TRAF4-TRAF.

However, it is still hard to understand why TRAF4 cannot recognize the identical TIM motif (P/S/A/T) X (Q/E) E, which locates in the receptors like TNFR2, CD40 and interacts with TRAF1, 2, 3, 5 to transduce signals to downstream (Wu, 2007). Structural alignment between TRAF2 and TRAF4 clearly showed the surface mediating with TIMs exposed completely, even two main chains Cα atoms are nearly overlapped (Figs. 4C, 5A and 5B). Maybe some side chains of residues S349, F351 of TRAF4 are too short comparing with the corresponding residues R393, Y395 of TRAF2, which interacted with the last amino acid E of TIMs. However, the side chain of another residue F434 is longer than the corresponding residue S467 of TRAF2, which also participates in recognizing TIMs (Fig. 5C). Thus, this question remains open and further study and analysis are needed.

In summary, we determined the crystal structure of TRAF4-TRAF, a unique member of TRAF family. It possesses the same trimeric assembly as other TRAFs. However, an additional flexible loop region was found clearly differing from other TRAFs, which explained structurally why TRAF4 could be phosphorylated at S426 to inhibit signal transduction of NOD2 pathway. This structure also supplied structural basis for further studies.

MATERIALS AND METHODS

Protein expression and purification

The TRAF domain gene (amino acids 292–466) of human TNF receptor associated factor 4 (TRAF4-TRAF), as well as the full length and

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different length ones from TRAF4, was cloned into the vector pET-22b (+) using *Nde* I and *Xho* I restriction enzyme recognition sites. The recombinant TRAF4-TRAF protein with a 6× His tag in the C-terminal region was over-expressed by BL21 (DE3) cells in *E. coli* system. The cells were grown in Luria-Bertani medium containing ampicillin (100 μg/mL) at 37°C until OD_{600nm} reached 0.8 and then induced with 0.4 mmol/L isopropyl-β-D-thiogalactoside (IPTG) for 16 h at 20°C. Cells were harvested by centrifugation (4,670 *g*, 30 min) and re-suspended in lysis buffer containing PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 50 mmol/L Na_2HPO_4, and 10 mmol/L KH_2PO_4, pH 7.4). After the cells were broken by ultrasonication and separated by centrifugation (38,900 *g*, 30 min), the supernatant was loading into a nickel-nitrilotriacetic acid (Ni-NTA) resin gravity column (Qiagen) that had been previously equilibrated with the lysis buffer. The column was firstly washed with 20 mL PBS, followed by washing with 50 mL PBS containing 20 mmol/L and 50 mmol/L imidazole, and finally eluted with PBS containing 300 mmol/L imidazole. The eluted protein was further purified by S-cation ion exchange chromatography followed by Superdex-200 gel filtration chromatography (GE Healthcare). Finally the purified protein was obtained in the buffer containing 20 mmol/L Tris-HCl, pH 7.4, 300 mmol/L NaCl, 2 mmol/L DTT and 5% glycerol and stored at –80°C for further using.

Crystallization and data collection

The purified TRAF4-TRAF protein was concentrated to approximately 6.5 mg/mL for initial crystal screening by Mosquito Robot (TTP LabTech). Crystals were optimized by hands with mixing 1 μL protein with 1 μL reservoir solution by hanging drop vapor diffusion methods at 16°C. The best diffracting crystals were obtained in the condition containing 0.2 mol/L Ammonium fluoride, 20% *w/v* Polyethylene glycol 3350, pH 6.2 and the crystals were harvested and cryo-protected in the well solution containing an additional 40% (*v/v*) glycerol and then flash cooled in a dry nitrogen stream. Finally the diffraction data for TRAF4-TRAF was collected at 100 K using an ADSC Q315 CCD detector on beamline BL17U1 of Shanghai Synchrotron Radiation Facility (SSRF).

Data processing and structure determination

The data set was indexed, integrated, and scaled using the HKL2000 software package (Otwinowski and Minor, 1997). The TRAF4-TRAF was crystallized in $P2_12_12_1$ space group with unit cell parameters of a = 57.30, b = 88.21, c = 118.43 Å. The structure was solved by molecular replacement method (McCoy et al., 2007) using the TRAF domain of TRAF2 structure (PDB code: 1CA4) as a search model. The TRAF4-TRAF structure model was manually improved in Coot (Emsley et al., 2010). Refinement was carried out using Refmac (Murshudov et al., 1997) and PHENIX Refine (Adams et al., 2010), alternately. Finally the model could be refined to 2.60 Å resolution with an R factor of 20.11 (R_{free} = 27.57), containing three protein chains in one asymmetric unit (ASU). Data scaling, refinement and validation statistics are shown in Table 1. The atomic coordinates and diffraction data were deposited in the Protein Data Bank (www.pdb.org) and the PDB code is 4M4E. Structural figures were prepared using PyMOL (www.pymol.org).

N-terminal amino acid sequencing

The protein was firstly fractionated by SDS-PAGE (12%) and then transferred electrophoretically to polyvinylidene difluoride membrane (PVDF) in the ice bath using BioRad machine for 1 h (300 mA). The stable band after degradation was visualized by staining with Coomassie brilliant blue (0.1% Coomassie brilliant blue R-250 in 1.0% acetic acid and 40% methanol) and cut out to dry manually. The PVDF membrane containing target band was digested and extracted. Finally the protein was sequenced on an automated protein sequencer (ABI Procise 491) to recognize the first four or five amino acids in the N-terminus.

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ABBREVIATIONS

GITR, glucocorticoid-induced TNFR; IKKα, I-kappa-B kinase α; IRF3, Interferon regulatory factor 3; MAVS, Mitochondrial antiviral signaling protein; MyD88, Myeloid differentiation primary response 88; NLR, NOD-like receptor; NLS, nuclear localization signal; NOD2, Nucleotide-binding oligomerization domain containing 2; p75NTR, nerve growth factor receptor; RIP2, receptor-interacting serine/threonine-protein kinase 2; RIR, RIG-I like receptor; RNAK, receptor activator of NF-κB; ROS, reactive oxygen species; TANK, TRAF family member-associated NF-Kappa-B activator; TIM, TRAF-interacting motif; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFR, TNF receptor; TRADD, TNFR1-associated DEATH domain protein; TRAF, TNF receptor-associated factor; TRAF4-TRAF, TRAF domain of TRAF4; VISA, Virus-induced-signaling adapter

COMPLIANCE WITH ETHICS GUIDELINES

Fengfeng Niu, Heng Ru, Wei Ding, Songying Ouyang, Zhi-Jie Liu declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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