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Received 28 May 2013

Accepted 10 July 2013

Preliminary X-ray crystallographic studies of the short form of the human TRAF4 TRAF domain

TNF (tumour necrosis factor) receptor-associated factor 4 (TRAF4) is a unique TRAF protein that participates in morphogenetic and developmental function and cell migration. TRAF-family proteins contain a TRAF domain for target interaction. In this study, the short form of the human TRAF4 TRAF domain, corresponding to amino acids 290–462, was overexpressed in *Escherichia coli* using engineered C-terminal His tags. The short form of the TRAF4 TRAF domain was purified to homogeneity and crystallized at 293 K. Finally, X-ray diffraction data were collected to a resolution of 4.2 Å from a crystal belonging to the hexagonal space group $P3_2$, with unit-cell parameters $a = b = 147.17$, $c = 202.69$ Å.

1. Introduction

Tumour necrosis factors (TNFs) and their receptors (TNFRs) are the main inflammatory mediators possessing a capacity to trigger apoptosis. In response to the binding of secreted TNF to TNFR located on the membrane, many signalling molecules, including TNF receptor type 1-associated death domain protein (TRADD), TNF receptor-associated factor (TRAF), receptor-interacting protein (RIP) and Fas-associated protein with death domain (FADD), can be recruited to the intracellular part of the TNFR directly or indirectly, leading to apoptosis or NF- κ B (nuclear factor kappa-light-chain-enhancer of activate B cells)-mediated cell survival.

TRAF proteins are key intracellular signalling components of TNF signalling pathways that perform scaffold functions that link signalling receptors to downstream signalling molecules (Arch *et al.*, 1998). Seven TRAF proteins have been identified in mammals: TRAF1–TRAF7 (Chung *et al.*, 2002). All of the TRAF proteins except for TRAF7 contain the TRAF domain, which mediates protein interactions (Rothe *et al.*, 1994).

Even though TRAF4 is a canonical TRAF protein, it is unique in that it functions differently in the cell (Kedinger & Rio, 2007). Although TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6 function in the inflammatory and apoptotic signalling pathway and are tightly linked to the immune response, TRAF4 is involved in morphogenetic and developmental processes and cell migration (Kedinger & Rio, 2007). The most recent studies have also shown that TRAF4 plays a role in immune-system function by interacting with nucleotide-binding oligomerization domain (NOD)-like receptors (Marinis *et al.*, 2011).

Crystal structures of the TRAF domains of TRAF2, TRAF3, TRAF5 and TRAF6 have shown that TRAF domains are composed of seven to eight antiparallel β -sheet folds followed by a coiled-coil region (Park *et al.*, 1999; Ye *et al.*, 2002). Several TRAF-domain structures showed that they exist as mushroom-like trimeric structures in solution (Fig. 1a; Zhang *et al.*, 2012). Despite the important roles of TRAF4 in human disease states, including cancer, neuronal diseases and immune disease, limited structural information is available (Izban *et al.*, 2000; Aston *et al.*, 2004).

In order to elucidate the molecular structure of TRAF4 and to further understand its molecular interaction with many downstream components, we recently solved the crystal structure of a longer form of the TRAF4 TRAF domain with the coiled-coil domain corresponding to amino acids 290–470 at 2.3 Å resolution (unpublished

work). Structural study of protein-interaction domains can provide critical information on the protein-interaction mode (Bae & Park, 2011; Park, 2011). In this study, we overexpressed, purified and crystallized a shorter form of the TRAF4 TRAF domain corresponding to amino acids 290–462. The short-form crystal was grown under a totally different condition and had a different crystal shape, space group and unit-cell parameters. The final crystals of the TRAF4 domain of TRAF4 were obtained in a solution consisting of 1.2 M lithium sulfate monohydrate, 0.01 M nickel chloride hexahydrate, 0.1 M Tris-HCl pH 8.2. The crystals belonged to space group $P3_2$, with unit-cell parameters $a = b = 147.17$, $c = 202.69$ Å. The crystals were obtained at 293 K and diffracted to a resolution of 4.2 Å. Details of the structure of the short form of the TRAF4 TRAF domain might be helpful for a better understanding of TRAF4-mediated signalling events. A comparative study with the long form of the TRAF4 TRAF

domain will be performed. This structural comparison might be helpful for understanding the trimerization mechanism and the importance of trimerization-based signalling events.

2. Materials and methods

2.1. Expression and purification

To express C-terminally His-tagged protein, the short form of the human TRAF4 (GenBank ID NM_004295) TRAF domain corresponding to amino acids 290–462 was amplified by PCR. We used the forward primer GGG CAT ATG CAG GAG CTG CAG GAG CTT and the reverse primer GGG CTC GAG AAC AGC ACG GAT GAA G for PCR. The PCR product was then digested with the *Nde*I and *Xho*I restriction enzymes (Enzynomics, Korea), after which it was inserted into pET-24a vector which had been cut with the same restriction enzymes. The plasmid was then transformed into *Escherichia coli* BL21 (DE3) competent cells and its expression was induced by treating the bacteria with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) overnight at 293 K.

Cells expressing the short form of the TRAF4 TRAF domain were pelleted by centrifugation, resuspended and lysed by sonication in 25 ml lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 25 mM imidazole). The lysate was centrifuged at 16 000 rev min⁻¹ using a Hanil Supra 22K centrifuge (No. 7 rotor) for 30 min at 277 K, after which the supernatant fractions were applied onto a gravity-flow column (Bio-Rad) packed with Ni-NTA affinity resin (Qiagen). The nonspecifically bound bacterial proteins were subsequently removed from the column using wash buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 60 mM imidazole). The C-terminally His-tagged short form of the TRAF4 TRAF domain was eluted from the column using elution buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 250 mM imidazole). The elution fractions were collected on a 0.5 ml scale to 4 ml. The eluted protein was applied onto a Superdex 200 gel-filtration column (GE Healthcare) that had been pre-equilibrated with a solution consisting of 20 mM Tris-HCl pH 8.0, 150 mM NaCl. A gel-filtration standard (Bio-Rad) consisting of a mixture of molecular-weight markers (thyroglobulin, 670 000 Da; globulin, 158 000 Da; ovalbumin, 44 000 Da; myoglobin, 17 000 Da; vitamin B₁₂, 1350 Da) was used for size calibration. The short form of the TRAF4 TRAF domain (molecular weight 19.890 Da), which eluted at around 15 ml, was collected and concentrated to 6–8 mg ml⁻¹. The protein concentration was measured using a protein-assay kit (Bio-Rad) and was determined using the Bradford method (Bradford, 1976). The peak was confirmed to contain the target protein by SDS-PAGE. The purified short form of the TRAF4 TRAF domain contained the extra C-terminal residues LEHHHHHH, which were not removed. 2 l of culture produced 8 mg of protein with >95% purity and the purified protein was kept at 193 K for subsequent use.

2.2. Crystallization

The crystallization conditions were initially screened at 293 K by the hanging-drop vapour-diffusion method using screening kits from Hampton Research (Crystal Screen, Crystal Screen 2, Natrix, MembFac, Index, Crystal Screen Cryo and Crystal Screen Lite) and Emerald BioStructures (Wizard I, II, III and IV). Initial crystals were grown on plates by equilibrating a mixture consisting of 1 μ l protein solution (6–8 mg ml⁻¹ protein in 20 mM Tris-HCl pH 8.0, 150 mM NaCl) and 1 μ l reservoir solution (condition No. 41 of Crystal Screen 2 from Hampton Research; 1 M lithium sulfate monohydrate, 0.01 M nickel chloride hexahydrate, 0.1 M Tris-HCl pH 8.5) against 0.4 ml reservoir solution. Crystallization was further optimized by searching

Figure 1
Purification of a short form of the TRAF4 TRAF domain. (a) The typical trimeric mushroom-like structure of the TRAF domain. The TRAF5 TRAF domain is shown as a representative structure (PDB entry 4gjh; Zhang *et al.*, 2012). (b) Gel-filtration chromatography profile. An SDS-PAGE of the peak fractions is shown.

over a range of concentrations of lithium sulfate monohydrate and nickel chloride hexahydrate and a range of pH values. Crystals appeared within 3 d and grew to maximum dimensions of $0.5 \times 0.2 \times 0.1$ mm in the presence of 1.2 M lithium sulfate monohydrate, 0.01 M nickel chloride hexahydrate, 0.1 M Tris-HCl pH 8.2. The crystals obtained diffracted to a resolution of 4.2 Å.

2.3. Crystallographic data collection

For data collection at 110 K, the crystals were transiently soaked in a solution corresponding to the reservoir solution supplemented with 30% (v/v) glycerol. The soaked crystals were then cryocooled in liquid nitrogen. A 4.2 Å resolution native diffraction data set was collected on beamline BL-4A of the Pohang Accelerator Laboratory (PAL), Republic of Korea (Fig. 2). The data sets were indexed and processed using *HKL-2000* (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 1.

Figure 2

Crystals of a short form of the human TRAF4 TRAF domain. Crystals were grown in 7 d in the presence of 1.2 M lithium sulfate monohydrate, 0.01 M nickel chloride hexahydrate, 0.1 M Tris-HCl pH 8.2. The approximate dimensions of the crystals were $0.5 \times 0.2 \times 0.1$ mm.

Figure 3

Diffraction image (1° oscillation) of a crystal of a short form of the TRAF4 TRAF domain with a 4.2 Å resolution limit.

Table 1

Diffraction data for the crystal of the short form of the TRAF4 TRAF domain.

Values in parentheses are for the outermost resolution shell.

X-ray source	BL-4A, PAL
Wavelength (Å)	0.97950
Space group	$P3_2$
Unit-cell parameters (Å)	$a = b = 147.17$, $c = 202.69$
Resolution limits (Å)	50–4.2 (4.25–4.20)
Mosaicity (°)	0.9
No. of observations	173946
No. of unique reflections	34891
Mean $I/\sigma(I)$	12.1 (4.7)
Completeness (%)	99.9 (100)
$R_{\text{merge}}^\dagger$ (%)	19.2 (51.5)

$^\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the i th observation of the intensity of reflection hkl and $\langle I(hkl) \rangle$ is the weighted average intensity of all observations i of reflection hkl .

3. Results and discussion

To understand the TRAF4-mediated signalling pathway and its involvement in cell polarity, we overexpressed, purified and crystallized the short form of the TRAF4 TRAF domain, which is responsible for interaction with various downstream signalling components. The short form of the human TRAF4 TRAF domain, corresponding to amino acids 290–462, was purified by two chromatographic steps (His-tag affinity chromatography and gel-filtration chromatography), which produced greater than 95% pure target protein (Fig. 1b). After the affinity-chromatography step, the target protein was concentrated and applied to gel-filtration chromatography. The short form of the TRAF4 TRAF domain eluted at approximately 70 000 Da upon gel-filtration chromatography. Since the calculated monomeric molecular weight of the TRAF domain of TRAF4 including the C-terminal His tag was 22 316 Da, the target protein may exist as a trimer in solution, similar to TRAF2 and TRAF5.

We initially tried to obtain a crystal of the target protein using the TRAF-C domain consisting of amino acids 310–470. This was easily crystallized, but the crystals never diffracted to a resolution better than 6 Å. Interestingly, however, a TRAF domain including the coiled-coil domain at the N-terminus crystallized in a totally different form that diffracted to better resolution (Fig. 2). The domain construct containing amino acids 290–462 diffracted to 4.2 Å resolution (Fig. 3). The crystals belonged to space group $P3_2$, with unit-cell parameters $a = b = 147.17$, $c = 202.69$ Å. Assuming the presence of four trimers in the crystallographic asymmetric unit, the Matthews coefficient (V_M) was calculated to be $2.35 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of 47.72% (Matthews, 1968). Diffraction data statistics are given in Table 1. The data set was indexed and processed using *HKL-2000* (Otwinowski & Minor, 1997). The molecular-replacement phasing method was conducted with *Phaser* (McCoy, 2007) using the long form of the trimeric TRAF4 structure (PDB entry 4k8u; H. H. Park & Y. H. Yoon, unpublished work), which contains amino acids 290–470, as a search model. Probable solutions with rotation-function and translation-function Z -scores of 6.8 and 10.7, respectively, for the first trimer, of 6.8 and 17.8, respectively, for the second trimer, of 6.9 and 22.4, respectively, for the third trimer, and of 7.2 and 24.4, respectively, for the fourth trimer were initially obtained. Initial refinement with *REFMAC5* (Murshudov *et al.*, 2011) using the initial *Phaser* model gave an R_{work} of 36.7% and an R_{free} of 41.2%. Further structural refinement is currently being conducted.

We are grateful to Dr Yeon Gil Kim of BL-4A at the Pohang Accelerator Laboratory. This research was supported by the Basic Science Research Program through the National Research Founda-

tion of Korea (NRF) funded by the Ministry of Education, Science and Technology (2013009083).

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