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The use of construct variation and diffraction data analysis in the crystallization of the TRAF domain of human tumor necrosis factor receptor associated factor 6

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Obtaining soluble proteins and diffraction-quality crystals often represents the bottleneck in macromolecular crystallography. Here, it is shown that construct variation can be an efficient strategy in expressing soluble proteins from bacteria and that clues from poor crystals may be used to improve crystal packing and to optimize crystal quality.

1. Introduction

The tumor necrosis factor (TNF) receptor associated factors (TRAF1–6) are major intracellular signal transducers for the TNF receptor (TNFR) superfamily (Chung *et al.*, 2002). They are genetically conserved across multicellular organisms including *Drosophila* (Liu *et al.*, 1999), *Caenorhabditis elegans* (Wajant *et al.*, 1998) and *Dictyostelium discoideum* (Regnier *et al.*, 1995). The downstream effectors of TRAF signaling are transcription factors in the NF-κB and AP-1 family (Baud *et al.*, 1999; Malinin *et al.*, 1997; Nishitoh *et al.*, 1998), which can turn on numerous genes involved in various aspects of cellular and immune functions.

The TRAF proteins are characterized by the presence of a novel TRAF domain at the C-terminus, which in turn consists of a coiled-coil domain followed by a conserved TRAF-C domain (Rothe *et al.*, 1994). The TRAF domain plays an important role in TRAF function by mediating self-association and upstream interactions with receptors and other signaling proteins (Takeuchi *et al.*, 1996). The N-terminal portion of most of the TRAF proteins contains a RING finger and several zinc-finger motifs, which are important for downstream signaling events (Rothe *et al.*, 1995; Takeuchi *et al.*, 1996). Crystal structures of the TRAF domain of TRAF2 in complex with various receptor peptides (McWhirter *et al.*, 1999; Park *et al.*, 1999; Ye *et al.*, 1999) and with the adapter protein TRADD (Park *et al.*, 2000) and of the TRAF domain of TRAF3 in complex with CD40 peptide (Ni *et al.*, 2000) and TANK peptide (Li *et al.*, 2002) have been reported.

TRAF6 is a unique TRAF family member in that it also participates in the signal transduction of the IL-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily (Cao, Henzel *et al.*, 1996; Cao, Xiong *et al.*, 1996) and therefore plays a crucial role in both adaptive and innate immunity. Targeted deletion of TRAF6 results in perinatal lethality and severe defects in signal transduction by both the TNFR and IL-1R/TLR superfamilies (Lomaga *et al.*, 1999; Naito *et al.*, 1999). Within the TNFR superfamily, TRAF6 is important for CD40 and TRANCE-R (also known as RANK) signaling. In the absence of TRAF6, B cells failed to proliferate and activate NF-κB in response to CD40 ligation and osteoclasts exhibited deficient bone-resorption owing to defective TRANCE-R signaling, leading to severe osteopetrosis. For IL-1R/TLRs, TRAF6 is indispensable for NF-κB activation by the proinflammatory cytokine IL-1 and for cellular responsiveness to bacterial endotoxin lipopolysaccharide (LPS), the activator of TLR4 (Poltorak *et al.*, 1998).

The unique biological function of TRAF6 is determined by its unique specificity towards receptor sequences. To obtain the structural basis of this specificity, we have determined the crystal structures of the TRAF domain of TRAF6 alone and in complexes with CD40 and TRANCE-R peptides (Ye *et al.*, 2002). Here, we report the protein expression and crystallization of the TRAF domain of TRAF6.

2. Materials and methods

2.1. Protein expression, purification and crystallization

Various TRAF6 constructs were subcloned into the pET24D vector (Novagen) with C-terminal His tags. They were induced by 0.5 m*M* IPTG overnight at 293 K. The proteins were purified by Ni-affinity chromatography (Qiagen) and gel filtration (Pharmacia). An additional ion-exchange step was added when necessary. Soluble TRAF6 proteins were crystallized by the vapor-diffusion method using 5–25% PEG 8000 in 100 m*M* Tris at pH 7.5.

Table 1
Various TRAF6 constructs.

Constructs	Solubility	Proteolysis	Crystallization
G351–V522†	**Soluble**	**C-term at 508**	Aggregation, no crystals
A274–G512‡	Insoluble		
S290–G512§	Insoluble		
Q309–A347§	Insoluble		
Q309–G512§	Insoluble		
D310–G512§	Insoluble		
E315–S507§	Insoluble		
M320–L508§	Insoluble		
S324–L508§	Insoluble		
M325–507§	Insoluble		
Y326–G512§	Insoluble		
V327–L508§	Insoluble		
E329–L508§	Insoluble		
T333–L508§	**Partially soluble**		**Needle-shaped crystals**
T333–G512§	**Partially soluble**		No crystals
E343–504¶	**∼10% soluble**		No crystals
A346–504¶	**∼10% soluble**		**Good crystals**
C349–504¶	**∼10% soluble**		**Poor crystals**

† Based on sequence definition of the TRAF-C domain. ‡ Based on sequence definition of the TRAF domain (TRAF-N + TRAF-C). § Based on alignment with two crystallizable constructs of TRAF2 (310–501, 327–501). ¶ Based on the crystal packing of the earlier crystal.

Table 2
The first TRAF6 crystal form, with unit-cell parameters a = 47.1, b = 55.2, c = 94.2 Å.

Values in parentheses are for the highest resolution shell.

Protein construct	Space group	Resolution (Å)	R_{sym}	Completeness
333–508	$P2_12_12$	2.9	8.7 (32.0)	93.1 (91.3)

2.2. Data collection and structure determination

Diffraction data were collected at the X4A beamline of NSLS and the A1 beamline of CHESS and processed with the *HKL* package (Otwinowski & Minor, 1997). The structures were determined by molecular-replacement calculations using the program *REPLACE* (Tong, 1993) with the TRAF-domain structure of TRAF2 (Ye *et al.*, 1999) after the removal of non-conserved side chains as a search model.

3. Results

3.1. The use of construct variation for identifying soluble TRAF6 constructs

Since the TRAF-C domain of TRAF6 (residues 351–522) is responsible for receptor interaction (Cao, Xiong *et al.*, 1996; Takeuchi *et al.*, 1996), we attempted to produce soluble TRAF6 constructs containing this domain. We generated 18 different constructs within the TRAF domain of TRAF6, which contains the coiled-coil TRAF-N domain (residues 274–350) and the TRAF-C domain (Table 1). Initial constructs were based on domain definitions suggested by sequence alignments (Cao, Xiong *et al.*, 1996; Ishida *et al.*, 1996; Rothe *et al.*, 1994). These were either insoluble or had a tendency to aggregate. Limited proteolysis of the soluble construct (G351–V522) helped to define the C-terminus of the domain at residue L508. This is close to the corresponding residue (D504) aligned with the ordered C-terminus in the TRAF2 structure (Park *et al.*, 1999).

Success in obtaining well diffracting crystals of the TRAF domain of TRAF2 (Park *et al.*, 1999) prompted us to make similar constructs in TRAF6 that contain short coiled-coil segments in the TRAF-N domain. Of 13 such constructs, two produced proteins that were partially soluble. These two constructs (residues 333–508 and residues 333–512) contain short coiled coils, even shorter than the corresponding TRAF2 construct (327–501). Both constructs were purified to homogeneity and shown to be trimers by gel filtration at the high protein concentrations used in this purification step. The trimeric nature of the TRAF6 constructs is consistent with our earlier prediction from the TRAF2 structure (Park *et al.*, 1999) that all TRAF proteins should be able to form similar trimers.

3.2. Initial crystallization and structure determination

Initial crystallization screenings for the soluble TRAF6 constructs were performed using a protein concentration of around 5–10 mg ml^{-1}, a concentration most frequently used in protein crystallization. This failed to produce any TRAF6 crystals (either alone or with receptor peptides), even though the precipitation patterns suggested this protein concentration to be appropriate. Surprisingly, one of the proteins (residues 333–508) started to crystallize consistently when we lowered the protein concentration to around 1–2 mg ml^{-1}. The crystals obtained under low TRAF6 concentrations were largely thin needles in clusters.

From the many crystallization trials, one TRAF6 crystal suitable for data collection was obtained. Although the crystal was not single, one lattice set could be isolated to permit processing of the diffraction data. The crystals were shown to be orthorhombic (space group $P2_12_12$ or $P2_122$) and we were able to collect a 2.9 Å data set using the X4A beamline at NSLS (Table 2).

To our surprise, a calculation of the possible solvent content in the crystal suggested that the crystal contained a monomer of TRAF6 per crystallographic asymmetric unit. This is contradictory to the trimeric nature of the TRAF6 protein in solution. In addition, mass-spectrometry analysis on a dissolved crystal indicated that the protein had not undergone any proteolysis and that the coiled-coil region of the protein, which is important for trimer formation, was intact.

Structure determination by molecular replacement using the monomer structure of TRAF2 as a model confirmed the space group to be $P2_12_12$. Interestingly, in this molecular-replacement solution the coiled-coil region of the TRAF2 model was in a steric clash with a symmetry-related molecule (Fig. 1), indicating that this region has assumed a different conformation or is disordered in the TRAF6 crystal. The different conformation of the coiled-coil region of TRAF6 in the crystal explained why TRAF6 crystallized in the monomeric form. It also explained the preference for crystallization at low protein concentrations, under which the equilibrium of trimerization is pushed toward the monomeric state. Even though the TRAF6 protein we expressed was predominately trimeric at high protein concentrations, it only contains a short coiled coil and may not be a stable trimer.

3.3. New TRAF6 constructs that give well diffracting crystals

The approximate region of the coiled coil that clashes with a symmetry-related molecule was determined by crystal packing and new constructs for crystallization were designed by removing this region. Three different constructs starting at residues 343, 346 and 349 were made (Table 1). The approximate amino-terminal positions are 10–20 residues shorter than the trimeric TRAF6 constructs. Since these three constructs contain essentially no coiled-coil region, it was expected that they would be monomeric in solution. Overnight induction of these constructs at 293 K produced partially soluble proteins. Even though their yields were low (∼0.5–1.0 mg per litre of culture), these proteins were easily purified to homogeneity. One protein construct (residues 346–504) crystallized readily as single crystals and native data set to 2.4 Å resolution was collected at a synchrotron. The structure has now been solved and refined to a 20.4% R factor with good

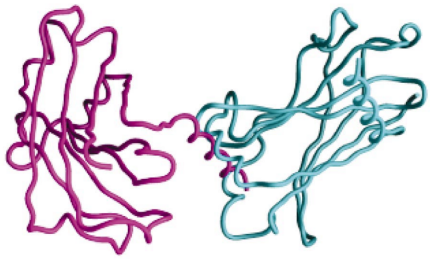

Figure 1
Molecular-replacement solution of the first TRAF6 crystal form ($P2_12_12$), showing the overlap between the coiled-coil region of the molecule (shown in magenta) with the neighboring molecule (shown in cyan). This steric overlap suggests that the coiled-coil region of TRAF6 was either disordered or assumed a different conformation in the crystal.

stereochemistry in the model (Ye *et al.*, 2002). Subsequently, crystals of this construct in complex with CD40 and TRNACE-R peptides were obtained (Ye *et al.*, 2002).

4. Conclusions

Construct variation can significantly influence protein production and crystallization. However, care should be taken to assure that the length differences do not influence the biological activity of the protein. The different constructs generated for TRAF6 all contain the full-length TRAF-C domain essential for receptor and adapter protein interaction. In addition, measurements using isothermal titration calorimetry showed that the long and the short constructs of TRAF6 (residues 333–508 and residues 346–504, respectively) possessed similar affinity to the CD40 peptide. These data therefore further support construct variation being an effective method for obtaining suitable protein for crystallization while preserving the biological function of the protein.

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