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Crystallization and preliminary X-ray analysis of the TRAF domain of TRAF3

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Tumor necrosis factor receptors (TNFR) signal events in immune responses, Ig class switching, activation of NF-κB or regulation of apoptosis. TNFR-associated factors (TRAFs) are adaptor proteins that connect TNFRs to downstream signaling pathways, including the NF-κB and c-JUN N-terminal kinase (JNK) pathways. Members of the TRAF family exist as trimers and share a conserved TRAF domain that mediates binding to the cytoplasmic domains of TNFRs. The TRAF domain from TRAF3 has been crystallized. In addition, an N-terminally truncated form of the domain has been crystallized in space group *P*321 with a shortened *c* axis and markedly improved diffraction (2.5 Å resolution).

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1. Introduction

The question of how TNF-receptor signaling affects events such as immune responses or regulation of apoptosis is central to understanding immune disorders and cancer (Bazzoni & Beutler, 1996; Naismith & Sprang, 1998; Smith *et al.*, 1994). TNF-receptor-associated factors (TRAFs) are proteins that bind to the cytoplasmic domains of TNF receptors and are co-inducers of downstream signaling. Importantly, TRAFs bind selectively to different TNF receptors. As adaptor proteins, TRAFs can activate intracellular signaling pathways including the NF-κB and c-JUN N-terminal kinase (JNK) pathways (Natoli *et al.*, 1997; Liu *et al.*, 1996; Grammer *et al.*, 1998; Leo, Welsh *et al.*, 1999; Leo, Zapata et al., 1999; for a review, see Gravestein & Borst, 1998). TRAFs are also part of an interacting web of molecules that promote either cell death or survival depending on the cellular context (reviewed in Dragovich *et al.*, 1998).

Signals from the TNF receptor CD40 are critical for B-lymphocyte proliferation, growth and differentiation (Vogel & Noelle, 2000; Foy *et al.*, 1996; Banchereau *et al.*, 1994; Clark & Ledbetter, 1994). Thus, the role of CD40 in the humoral response and regulation of apoptosis is relevant to a number of human diseases, such as autoimmune disorders, allergy and asthma. CD40 also contributes to neoplastic cell regulation, partially through its effects on apoptosis. For example, the CD40 trigger has been shown to promote spontaneous and chemotherapeutic drug-induced apoptosis in epithelial cancer cell lines, including ovarian, bladder, colorectal and breast carcinomas (Eliopoulos *et al.*, 1996, 1997). Alternatively, CD40 generally provides signals that enhance survival and proliferation of malignant B cells, including follicular B-cell non-Hodgkin's lymphoma (the most common lymphoma) and chronic lymphocytic leukemia (CLL; the most common form of leukemia). Thus, the CD40 receptor pathway and the TRAF molecules that propagate CD40 signals represent a new focus for targets that may have utility in cancer treatment (Eliopoulos *et al.*, 2000).

Six TRAF proteins have been identified and are numbered sequentially in order of their discovery. From amino-acid sequence homology, it was determined that some features are conserved throughout the family. TRAFs 2–6 have ring fingers and zinc-finger motifs (Freemont, 1993; Schwabe & Klug, 1994), followed by the TRAF domain that is conserved in all TRAFs. This domain mediates binding to the cytoplasmic domains of TNFRs. TRAF proteins play critical roles in signal transduction pathways stimulated by TNFRs (Lomaga *et al.*, 1999; Nakano *et al.*, 1999; Xu *et al.*, 1996; Yeh *et al.*, 1997). The interactions of TRAFs with these receptors have been defined by mutational analyses, demonstrating that the interactions are required for many TNF-induced signaling events (Leo, Welsh *et al.*, 1999; Leo, Zapata et al., 1999; Akiba *et al.*, 1998; Cheng *et al.*, 1995; Hostager & Bishop, 1999; Hu *et al.*, 1994; Lee *et al.*, 1999).

To provide a molecular framework for understanding the role of TRAFs in TNF-induced signaling, we have crystallized the TRAF domain of TRAF3 containing the contact regions for the cytoplasmic domain of CD40 and other TNF receptors. Here, we report the crystallization conditions used to produce high-quality crystals for use in studies of the domain. Proteolytic trimming was used to promote the production of crystals with

increased resolution, facilitating structural studies of the protein alone or in complex with peptides from the interacting regions of TNF receptors.

2. Experimental

2.1. Purification

A fragment of TRAF3 encoding the TRAF3 NC region (residues 341–568) was expressed as a His_6-fusion protein from plasmids inserted into a pET21b vector and transformed into *E. coli* BL21 DE3 cells. Bacterial cultures were grown in LB/amp media at 310 K to a cell density approximating to A_{600} = 0.8–0.9. Protein expression was induced by the addition of 0.5 m*M* IPTG and the cultures were incubated for 4 h at 299 K. Cells were harvested by centrifugation, flash-frozen and stored at 193 K. Frozen cells were thawed and lysed by douncing in denaturing lysis buffer (6 *M* urea, 25 m*M* Tris pH 7.5, 5 m*M* imidazole, 5 m*M* β-mercaptoethanol, 50 µg ml^{-1} lysozyme). The lysate was clarified by centrifugation and the soluble fraction was applied to Ni^{2+}–NTA agarose resin (Qiagen, Inc.) which had been equilibrated with denaturing lysis buffer. The loaded column was washed overnight at 277 K with denaturing buffer. TRAF3 was then renatured while bound to the resin using a linear gradient from 4 to 0 *M* urea in 25 m*M* Tris pH 7.5, 5 m*M* β-mercaptoethanol. Finally, TRAF3 was eluted using a linear gradient of 0 to 0.4 *M* imidazole in 25 m*M* Tris pH 7.5, 150 m*M* NaCl, 5 m*M* β-mercaptoethanol.

To produce a shortened molecule for crystallization trials, purified TRAF3 was subjected to limited proteolysis. TRAF3 was digested with trypsin in 25 m*M* Tris buffer pH 7.5, 150 m*M* NaCl, 5 m*M* β-mercaptoethanol using a 1:750 enzyme:protein molar ratio and the reaction was allowed to proceed for 4 h at 277 K. The reaction was terminated by the addition of 1 m*M* AEBSF. 'Short' TRAF3 was separated from the cleaved peptide product by gel-filtration chromatography using Sephacryl S-100 (Pharmacia, Inc.). Mass spectrometry was used to confirm that 36 residues were removed from the N-terminus of TRAF3 by tryptic cleavage.

2.2. Crystallization

TRAF3 and the proteolytically shortened TRAF3 fragment were each tested for crystallization. The proteins were first dialyzed into crystallization buffer: 10 m*M* Tris pH 7.5, 150 m*M* NaCl, 1 m*M* DTT, 1 m*M* EDTA. The samples were concentrated to 3.5 mg ml^{-1} and screened using the Crystal Screen I and II reservoir solutions (Hampton Research, Inc., CA, USA) in vapor-diffusion format with hanging drops at room temperature. Trials were prepared by mixing 3 µl protein with 3 µl reservoir solution. From the initial screen, several conditions produced thin needle crystals. Crystallization conditions were screened further by varying the pH and precipitant concentrations and conducting an additive search. Crystals suitable for diffraction were obtained from crystallization buffer plus 10–15% methanol as reservoir solution. Hexagonal prisms (or half-hexagonal trapezoids) formed in 2–3 d at room temperature. The crystals grew in two morphologically indistinguishable space groups and exhibited hemihedral twinning. The first crystals formed in space group *R*3, with unit-cell parameters *a* = *b* = 84.5, *c* = 319.6 Å, and the second crystal form belonged to space group *P*321, with unit-cell parameters *a* = *b* = 83.8, *c* = 212.6 Å. Both crystals had a high solvent content of 70% and diffracted to 2.8 and 3.5 Å, respectively (see Table 1).

The proteolytically truncated TRAF3 protein crystallized from reservoir solutions containing 15% PEG 4000 in 0.1 m*M* MES pH 6.5. Hexagonal crystals grew at room temperature in 2–3 d. The space group for these crystals was *P*321, with unit-cell parameters *a* = *b* = 83.1, *c* = 77.8 Å. These crystals with modified TRAF3 had a lower solvent content (64%), were significantly improved with respect to stability and reproducibility and diffracted beyond 2.5 Å resolution (see Table 1).

2.3. Data collection

Crystals were transferred to cryoprotectant solution containing 35% glycerol in crystallization buffer. X-ray diffraction data were collected at 108 K at the Stanford Synchrotron Research Laboratory (SSRL; beamlines 9-1 or 7-1) using a MAR345 image-plate detector. Data were indexed and processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). In each of the crystal forms TRAF3 is a trimer with crystallographic threefold symmetry relating the subunits within the complete TRAF3 molecule. The data-collection statistics are presented in Table 1.

Table 1
Crystallographic data and data collection statistics.

Crystals	Form I	Form II	Truncated
Space group	*R*3	*P*321	*P*321
Unit-cell parameters (Å, °)	*a* = *b* = 84.5, *c* = 319.5, γ = 120	*a* = *b* = 83.8, *c* = 212.6, γ = 120	*a* = *b* = 83.1, *c* = 77.8, γ = 120
Data collection			
Resolution (Å)	2.8	3.5	2.5
No. of observed reflections, $I \geq 0$	59690	34625	50911
No. of unique reflections, $I \geq 0$	20792	16569	9905
$R_{\rm merge}$† (%)	5.1	7.8	5.7
Completeness (%)	98.5	78.7	87.8
Average $I/\sigma(I)$	21.7	11.0	24.8
Monomers per asymmetric unit	2	2	1
$V_{\rm M}$ (Å^3 Da^{-1})	4.1	4.0	3.4
Solvent content (%)	70	69	64

† $R_{\rm merge} = \sum |I - \langle I \rangle| / \sum I$, where I is the observed intensity and $\langle I \rangle$ is the mean intensity of all symmetry-related reflections.

3. Results and discussion

The improved crystals that have been obtained with the proteolytically trimmed TRAF3 fragment diffract to high resolution and are suitable for binding studies with peptides or short fragments derived from the cytoplasmic domain of TNFRs. TRAF3 is a mushroom-shaped molecule (Ni *et al.*, 2000) composed of an N-terminal α-helical segment (TRAF N-domain) and a C-terminal β-sandwich (TRAF C-domain). The TRAF3 trimer is stabilized mainly by formation of a coiled coil between intertwined N-terminal helical segments. In the crystal the intermolecular packing interactions between TRAF3 trimers in the crystal lattice are exclusively between the TRAF C-domains. Truncation of the N-terminus by tryptic digestion shortened the helical segment by 36 residues. The effect of this truncation on crystal packing is seen in the dramatic decrease in the *c* cell dimension from 212.6 to 78.3 Å. Consequently, the diffraction limit of crystals produced with the shorter TRAF3 molecule is markedly improved. It is interesting to note in other studies that the N-terminus of TRAF2 was also cleaved with trypsin prior to crystallization (Park *et al.*, 1999; McWhirter *et al.*, 1999).

The crystal lattice of the TRAF3 crystals contains large solvent channels, primarily around the extended coiled-coil region of the trimer (Ni *et al.*, 2000). Peptides of at least 20 residues in length can be soaked into these crystals. We have already demonstrated that peptides derived from CD40, bearing the P*x*Q*x*T recognition motif that is shared among TNFRs that bind to TRAFs, can be targeted to the binding crevice on

TRAF3 (Ni *et al.*, 2000) after soaking. In previous work, the CD40 contacts were defined in the TRAF3 crevice and compared with contacts of the same motif with TRAF2 (Ye *et al.*, 1999; McWhirter *et al.*, 1999). The comparison suggested that CD40 may assume different conformations when bound to different TRAF family members. With the new TRAF3 crystals reported here, it is possible to pursue binding studies of TRAF3 and TNFRs at high resolution. The complex structures determined from such experiments can guide the design of inhibitors and potentially provide the basis for small-molecule development based on the peptide structures.

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