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Expression, purification and crystallization of recombinant human TRAIL

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TRAIL (also known as Apo-2L) belongs to the tumour necrosis factor (TNF) cytokine family and induces rapid apoptosis in a wide variety of tumour cell lines upon binding to the death-signalling receptors on the cell membrane. Normal cells are resistant to TRAIL, owing to the expression of decoy receptors which lack functional death domains and antagonize TRAIL-induced apoptosis. Soluble and functional human TRAIL, expressed in *Escherichia coli* and refolded into a functional form, has been crystallized. The crystals belong to space group $P6_3$ with unit-cell dimensions $a = b = 65.61$, $c = 131.70$ Å. The asymmetric unit contains two molecules of TRAIL, with a crystal volume per protein mass (V_m) of 2.41 $Å^3\,Da^{-1}$ and a solvent content of about 42% by volume. A native and a platinum-derivative data set to 2.8 and 3.5 Å resolution, respectively, were obtained from frozen crystals. Structure determination by a combined molecular replacement and isomorphous replacement method is in progress.

1. Introduction

TRAIL is a newly identified member of the tumour necrosis factor (TNF) family of cytokines, which play important roles in regulating many biological functions, especially as prominent mediators of immune regulation and inflammatory responses (Lotz *et al.*, 1996). Nine members of the TNF family are known: TNF-α, TNF-β (known as lymphotoxin or LT-α), lymphotoxin-β (LT-β), 41BBL, OX40L, CD27L, CD30L, CD40L and FasL (also known as Apo-1L) (Cosman, 1994). With the exception of LT-β, all these ligands are type II membrane proteins which are processed proteolytically to form a soluble homotrimeric structure, as suggested by the structures of the mature proteins TNF-α (Eck & Sprang, 1989; Jones *et al.*, 1989), TNF-β (Eck *et al.*, 1992) and CD40L (Karpusas *et al.*, 1995). Four of the ligands, TNF-α, TNF-β, FasL and TRAIL, induce apoptosis (programed cell death) of susceptible cells such as chronically activated T cells and B cells (Daniel & Krammer, 1994; Alderson *et al.*, 1995). These cytotoxic ligands trigger the suicide response by binding to and aggregating their cognate transmembrane receptors, which contain a cytoplasmic death domain. The death domain is a protein–protein interaction motif which ultimately activates a protease cascade leading to apoptosis (Yuan, 1997). The death domains of TNFR-1 (TNF receptor 1) and Fas interact with adapter proteins FADD (Fas-associated death domain, also known as MORT1) directly or indirectly through TRADD (TNF receptor-associated death domain; Hsu *et al.*, 1996) and activate pro-apoptotic proteases (caspases) related to the gene product of *Caenorhabditis elegans ced*3 (Yuan *et al.*, 1993; Chinnaiyan *et al.*, 1995; Cleveland & Ihle, 1995; Boldin *et al.*, 1995, 1996; Baker & Reddy, 1996; Chinnaiyan & Dixit, 1996; Fraser & Evan, 1996; Muzio *et al.*, 1996; Duan & Dixit, 1997). Transmembrane receptors for the TNF ligand family constitute the TNF/NGF (nerve-growth factor) receptor superfamily characterized by the pseudo-repeats of extracellular cysteine-rich domains (Bazan, 1993). The crystal structure of TNF-β in complex with the extracellular portion of TNFR-1 (Banner *et al.*, 1993) revealed that the receptors bind to TNF as a trimer. Thus, it is reasonable to assume that trimerization of the TNF/NGF-receptor family members constitutes an initiation of the transmembrane signalling.

TRAIL is expressed in a variety of human tissues and is a potent inducer of apoptosis in many transformed cell lines (Wiley *et al.*, 1995; Pitti *et al.*, 1996). Surprisingly, normal cells are resistant to TRAIL-induced apoptosis. Two cognate membrane-bound receptors for TRAIL, DR4 (death receptor 4; Pan, O'Rourke *et al.*, 1997) and DR5 (Pan, Ni *et al.*, 1997; Sheridan *et al.*, 1997) have been identified, both of which contain a cytoplasmic death domain. Two recently identified receptors, TRID (TRAIL receptor without intracellular domain, also known as DcR1 or LIT; Pan, Ni *et al.*, 1997; Sheridan *et al.*, 1997; Mongkolsapaya

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et al., 1998) and TRUNDD (TRAIL receptor with a truncated death domain, also known as DcR2; Marsters *et al.*, 1997; Pan *et al.*, 1998) are non-signalling decoy receptors which do not contain or contain an incomplete cytoplasmic domain. The death-domain-containing DR4 and DR5 are expressed both in transformed cell lines and multiple normal tissues. Remarkably, the decoy receptors are expressed almost exclusively in normal cells and provide a cell-surface mechanism for the regulation of cellular responsiveness to TRAIL (Sheridan *et al.*, 1997). Given the fascinating interplay of TRAIL and its receptors, it is of considerable biomedical interest to test the efficacy and safety of the anti-tumoural activity of TRAIL in animal models. Human malignant gliomas, which are resistant to current therapeutic approaches, are known to be sensitive to TRAIL-induced apoptosis, while the injection of human TRAIL in mice was well tolerated (Rieger *et al.*, 1998). Human melanoma cells were killed by TRAIL, whereas all melanoma cell lines were resistant to the other TNF-family cytokines tested (FasL, TNF-α and CD40L; Griffith *et al.*, 1998). All these studies suggest that targeting TRAIL may be a promising and safe alternative for cancer therapies. Here, we report the expression, purification and crystallization of human TRAIL (residues 114–281 of the mature protein) and preliminary X-ray crystallographic analysis of the crystals.

2. Preparation of active TRAIL

The full-length human *TRAIL* gene codes for a polypeptide of 281 amino acids. Based on the alignment of the TRAIL sequence with that of TNF, whose extracellular processing site is known, truncated recombinant TRAIL (amino acids 114–281) with a His_{10} tag and a enterokinase cleavage site was expressed in SF9 cells (Pitti *et al.*, 1996). Accordingly, a segment of *TRAIL* gene coding for amino acids 114–281 was amplified by the polymerase chain reaction using the λ gt11 human placenta cDNA library as a template. The gene was inserted downstream of the T7 promoter of the expression plasmid pET-3a (Studier *et al.*, 1990). The human *TRAIL* gene was transformed and overexpressed in *E. coli* strain BL21 (DE3). Cells were grown to an OD_{600} of approximately 0.9 in Luria–Bertani medium containing 0.1 mg ml^{-1} ampicillin at 310 K, and the expression of TRAIL was induced by 1 m*M* isopropyl-β-D-thiogalactoside. After 4 h induction, cells were harvested and disrupted by sonication. The inclusion bodies containing TRAIL as insoluble aggregates were isolated by centrifugation and solubilized in a buffer containing 20 m*M* sodium phosphate (pH 7.6), 6 *M* guanidine–HCl and 1 m*M* dithiothreitol (DTT). The denatured proteins were refolded by a rapid tenfold dilution with a buffer solution containing 20 m*M* sodium phosphate (pH 7.6) and 1 m*M* DTT, followed by overnight dialysis in the same buffer at 277 K. After removing aggregates generated during dialysis, the supernatant solution was loaded on a SP Sepharose Fast Flow column (Pharmacia) and eluted with a 0–1.0 *M* NaCl gradient in the same buffer. Fractions eluted at 0.8–1.0 *M* NaCl gradient contained TRAIL almost exclusively as judged by SDS–PAGE. The unusual high p*I* of TRAIL (p*I* 8.9) appears favourable in the anionic column chromatographic separation. The purified TRAIL was dialysed against a buffer containing 10 m*M* HEPES (pH 7.6), 1 m*M* DTT and 0.3 *M* NaCl and concentrated to 12 mg ml^{-1} for crystallization.

The cytotoxic activity of the refolded TRAIL was measured on murine L929 cells (ATCC CCL-929) treated with actinomycin D. Briefly, L929 cells were seeded at 1×10^4 cells per well into a 96-well microtitre plate in Dulbecco's modified medium containing 2% foetal calf serum. 18 h later, medium containing 2 µg ml^{-1} actinomycin D was added to the cells, together with various concentrations of the recombinant TRAIL (0–10 µg ml^{-1}). The cells were incubated for an additional 18 h at 310 K. Cell viability was determined by measuring the cellular metabolic activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (Mosmann, 1983). The specific activity of the recombinant TRAIL was 7.76×10^2 units mg^{-1}, where a unit is defined as the amount of the sample required to kill half the cells in the assay system. To our knowledge, this is the first report of successful production of active unmodified TRAIL in *E. coli*, which is required for an unbiased estimation of the biological activities of TRAIL in animal model studies.

3. Crystallization and X-ray analysis

Crystals of TRAIL were obtained by the hanging-drop vapour-diffusion method using 24-well Linbro plates at 277 K. The first crystallization screening was performed with Crystal Screen 2, the sparse-matrix screening kit (Hampton Research, USA). Small hexagonal plate crystals were produced in 1 d from 30% polyethylene glycol monomethyl ether 550 (PEG MME 550), 0.1 *M* Bicine (pH 9.0) and 0.1 *M* NaCl. Subsequently, the initial crystallization conditions were optimized to produce larger single crystals (0.2 × 0.2 × 0.1 mm) from droplets containing 2 µl protein sample (12 mg ml^{-1}) and an equal volume of precipitant solution containing 25% PEG MME 550, 0.05 *M* Bicine (pH 9.0) and 10 m*M* $CdCl_2$. The droplets were equilibrated against 1 ml of the same precipitant solution at 277 K, and the crystals grew to maximum size in two weeks. For data collection, crystals were frozen at 110 K using an Oxford Cryosystems Cryostream (Oxford Cryosystems, UK) after being briefly immersed in a cryoprotectant solution containing 10% MPD in the same precipitant solution. A native data set (92.9% completeness, R_{sym} = 6.3% in the resolution range 20–2.8 Å; 86.1% completeness, R_{sym} = 17.4% in the resolution range 3.3–2.8 Å) was obtained using Cu $K\alpha$ radiation on a MacScience DIP2020 imaging-plate system mounted on a M18XHF X-ray generator operated at 50 kV and 90 mA. Using an autoindexing program provided with the program *HKL* (Otwinowski & Minor, 1997) and examining the diffraction data set, we found that the crystals belong to space group $P6_3$ with unit-cell dimensions $a = b = 65.61$ and $c = 131.70$ Å. Two molecules of TRAIL were contained in the asymmetric unit, corresponding to a crystal volume per unit molecular weight (V_m) of 2.41 Å^3 Da^{-1} and a solvent content of 42% (Matthews, 1968). Owing to the crystal geometry of TRAIL, aligning the *c* axis of the unit cell along the spindle is difficult. In addition, the TRAIL crystals show an anisotropic mosaicity, which results in much broader line widths of diffraction spots along the c^* axis and which worsens at higher resolution and on heavy-atom soaking. These two problems, in addition to the size of the crystal, limit the resolution of reflections which can be used.

4. Discussion

In an attempt to determine the structure of TRAIL by molecular replacement (MR) using as a search model the 1.8 Å resolution structure of a TNF-α mutant determined in our laboratory (Cha *et al.*, 1998), promising positions of the two molecules in the asymmetric unit were found using *AMoRe* (Navaza, 1994). The translation functions were calculated with the highest peak, which had a correlation of 17.7% in a rotation search. The search yielded a solution with a correlation of 20.0% and an *R* factor of 50.5%. The position of this solution was

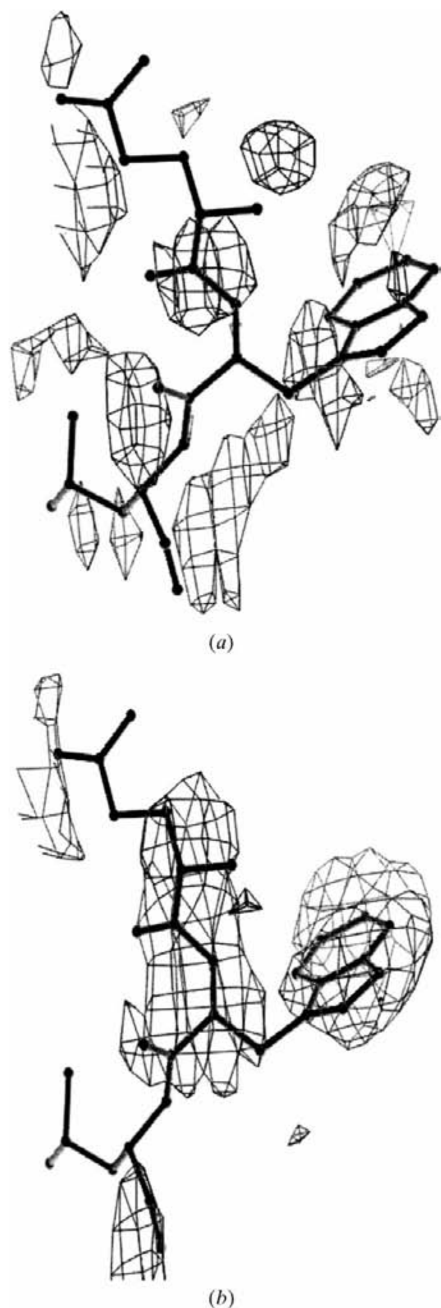

Figure 1
Electron-density maps at 3.5 Å and 1.0σ contour showing a loop region containing Trp154 invariant in TNF-α family members. The TNF-α model was positioned by molecular replacement and a rigid-body refinement is shown as a ball-and-stick model. (*a*) The $2F_o - F_c$ map was calculated with the TNF-α model containing only the core β-sheets. (*b*) The F_o map was calculated after phase combination, solvent flattening and non-crystallographic twofold density averaging in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The SIR phases from the platinum-derivative crystals were combined with phases calculated with the same model in (*a*). The improvement of the map quality in this region is visible. Consistently, the correlation coefficient for one of the two core β-sheet regions (core 1) between the experimental electron density and the calculated model density (the two core β-sheets only) improved from 0.3830 (MR) to 0.6809 (phase combination plus density modification). For the purpose of this calculation using the program *OVERLAPMAP* (Brändén & Jones, 1990), core 1 is omitted for the phase combination. The phase combination, non-crystallographic symmetry matrix calculation and molecular envelope were carried out with the programs *SIGMAA*, *LSQKAB* and *NCSMASK* from the *CCP*4 suite, respectively.

fixed in a subsequent search for the other solution, yielding a promising translation-function peak with a correlation of 27.8% and an *R* factor of 49.2%. The two solutions were then subjected to rigid-body refinement, resulting in a correlation of 43.9% and an *R* factor of 45.8%. When examined on a graphics computer, the two molecules were found to belong to each of the two trimeric organizations of TRAIL molecules in the unit cell with favourable crystal packings. The molecular threefold axes of the two trimers coincide with the crystallographic sixfold screw axis and threefold axis, respectively.

Even in the early stages of refinement, the electron densities for the β-strands were strong and interpretable and the fitting of β-strands was easily accomplished. However, further model building and refinement were hampered by low sequence homology (19% sequence homology between TNF-α and TRAIL) and a high loop-structure content (46% in the structure of TNF-α). Some loop regions of TNF family cytokines have been known to interact directly with their receptors (Van Ostade *et al.*, 1991, 1994; Banner *et al.*, 1993; Cha *et al.*, 1998). Residues 129–161, 215–218 and 264–274 of TRAIL are especially likely to interact with receptors, as the corresponding loops of TNF-β have been revealed to interact with its receptor in the structure of TNF-β in complex with TNF-R1. Many TNF-α mutants exhibiting altered receptor-binding activity have been constructed which contain substitutions on these loops (Zhang *et al.*, 1992; Banner *et al.*, 1993; Loetscher *et al.*, 1993; Van Ostade *et al.*, 1993). Thus, the correct loop structure of TRAIL is critical for the understanding and manipulation of its biological properties. In order to define the loop structure correctly, efforts towards obtaining additional phasing information is in progress. Using synchrotron radiation, we have obtained a 3.5 Å data set from isomorphic platinum (K_2PtCl_6) derivative crystals, which provide a moderate improvement of the map quality (Fig. 1). Structure determination by combined molecular replacement and isomorphous replacement methods appears promising. So far, our results indicate that the active form of TRAIL is a homotrimer and that each monomer consists of two antiparallel β-pleated sheets with a jelly-roll topology similar to other TNF-related cytokines.

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