Crystallization and preliminary X-ray crystallographic study of the extracellular domain of the 4-1BB ligand, a member of the TNF family

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Received 12 October 2005

Accepted 25 November 2005

Online 16 December 2005

The 4-1BB ligand, a member of the tumour necrosis factor (TNF) family, is an important co-stimulatory molecule that plays a key role in the clonal expansion and survival of CD8⁺ T cells. Signalling through binding of the 4-1BB ligand and 4-1BB has been reported to enhance CD8⁺ T-cell expansion and protect activated CD8⁺ T cells from death. The 4-1BB ligand is an integral protein expressed on activated antigen-presenting cells. The extracellular domain of the 4-1BB ligand fused with glutathione-*S*-transferase was expressed in *Escherichia coli* (Origami) and purified by using affinity and ion-exchange column chromatographic methods. Crystals of the 4-1BB ligand were obtained at 290 K by the hanging-drop vapour-diffusion method. X-ray diffraction data were collected from these crystals to 2.8 Å resolution and the crystals belong to space group C2, with unit-cell parameters $a = 114.6$, $b = 73.8$, $c = 118.50$ Å, $\beta = 115.5^\circ$.

1. Introduction

T cells require two signals for activation in immune responses. The primary signal is an antigen-specific MHC-restricted signal through the T-cell receptor and the second signal is a co-stimulatory signal (Plas *et al.*, 2002). The interaction of CD28 and B7 molecules is one of the best-studied co-stimulatory pathways and is considered to be the main mechanism for primary T-cell stimulation (Carreno & Collins, 2002). However, additional molecules have been identified which play crucial roles in the T-cell responses following initial T-cell activation. These molecules are divided into two main groups: the immunoglobulin superfamily and TNF and TNF receptor (TNFR) superfamily (Croft, 2003).

The 4-1BB ligand (4-1BBL) is a member of the TNF family. TNF ligands and their receptors function in a variety of biological processes, such as development of lymphoid organs, host defence, inflammation, apoptosis, autoimmunity and regulation of various immune-system cell types (Lotz *et al.*, 1996). To date, at least 18 TNF family ligands and 29 receptors have been identified in humans (Locksley *et al.*, 2001). Some ligands interact with multiple receptors and some receptors also interact with multiple ligands. Several of these ligands are involved in co-stimulation of T-cell activation and they include OX40–OX40 ligand, CD27–CD70, CD30–CD30 ligand, HVEM (herpesvirus entry mediator)–LIGHT and 4-1BB–4-1BBL (Croft, 2003). Co-stimulation is essential for induction of T-lymphocyte proliferation and inhibition of activation-induced cell death (AICD; Daniel *et al.*, 1997; Scholz *et al.*, 2002). 4-1BBL is generally expressed in antigen-presenting cells (APCs) and interacts with 4-1BB expressed on the T cells. 4-1BBL and 4-1BB-mediated signals expand primary CD8⁺ T-cell responses and suppress CD4⁺ T-cell responses (Mittler *et al.*, 1999). In particular, these signals promote the survival of activated CD8⁺ T cells and enhance the memory pool of antigen-specific CD8⁺ T cells (Bertram *et al.*, 2002).

The fact that 4-1BBL promotes expansion of CD8⁺ T cells that are stimulated by anti-CD3/CD28 (Maus *et al.*, 2002) suggests that signals through the 4-1BBL and 4-1BB complex will play important roles in immunotherapy against cancer (Vonderheide & June, 2003). In fact, TNF ligands have now been used to expand human T cells *in vitro*, which might explain their usefulness in adoptive immunotherapy

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strategies (Maus *et al.*, 2002). Targeting TNF and TNFR molecules might also prove to be of benefit in preventing transplant rejection and graft versus host disease. In animal models, interruption of TNF–TNFR interactions promotes graft survival and suppress graft versus host disease (Tamada *et al.*, 2000; Scheu *et al.*, 2002; Blazar *et al.*, 2001; Tsukada *et al.*, 2000). In addition, blockage or acceleration of these interactions might be useful in treating autoimmune diseases such as rheumatoid arthritis (Seo *et al.*, 2004), multiple sclerosis (Mouzaki *et al.*, 2004) and type 1 diabetes (Kodama *et al.*, 2005) in which T-cell tolerance breaks down.

The overall structure of TNF ligands is known as a ‘ β -jelly roll’ in which eight antiparallel β -strands form a three-dimensional sandwich structure (Idriss & Naismith, 2000; Gruss, 1996). Molecular structures have been reported for several TNF ligands, including TNF α , TNF β , CD40 ligand, TRAIL (TNF-related apoptosis-inducing ligand), APRIL (a proliferation-inducing ligand) and TALL-1 (Jones *et al.*, 1989; Eck *et al.*, 1992; Karpusas *et al.*, 1995; Cha *et al.*, 1999; Hymowitz *et al.*, 1999; Liu *et al.*, 2002). However, no structures are known for the co-stimulatory members of the TNF family.

2. Materials and methods

2.1. Protein expression and purification

DNA fragments of 564 base pairs covering the extracellular domain of 4-1BBL (positions 175–738; e4-1BBL) were inserted into pGEX-6p-1 (Amersham Biosciences) vector and transformed into *Escherichia coli* strain Origami competent cells. Transformants were selected and cultured on Luria–Bertani (LB) broth agar plates containing 100 $\mu\text{g ml}^{-1}$ ampicillin. A single clone of the Origami cells was inoculated into TP (tryptone phosphate) media (2% bacto-tryptone, 0.2% Na_2HPO_4 , 0.1% KH_2PO_4 , 0.8% NaCl, 1.2% yeast extract, 0.2% glucose) with 100 $\mu\text{g ml}^{-1}$ ampicillin and cultured at 310 K until OD_{600} reached 0.5. e4-1BBL fused with glutathione-S-transferase (GST) was expressed at a temperature of 293 K by addition of 0.1 mM isopropyl β -D-thiogalactopyranoside (IPTG) for 4 h. The cells were harvested by centrifugation at 3500 rev min^{-1} for 10 min at 277 K, resuspended and sonicated in phosphate-buffered saline (PBS) buffer. After centrifugation of the cell extracts, the supernatant was applied onto Glutathione Excellose (Takara) resin pre-equilibrated with PBS buffer. The column was washed with PBS buffer to remove contaminative proteins. GST-tagged e4-1BBL was eluted with elution buffer containing 20 mM reduced L-glutathione, 50 mM Tris–HCl pH 8.0, 50 mM NaCl and then cleaved using

Figure 1
Crystals of e4-1BBL grown by the hanging-drop vapour-diffusion method. The approximate dimensions of the crystal are 0.25 $\times$ 0.07 $\times$ 0.02 mm.

Table 1

X-ray data-collection statistics.

Wavelength ($\text{\AA}$)	0.97960
Resolution ($\text{\AA}$)	2.8
Space group	<i>C</i> 2
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = 114.6$, $b = 73.8$, $c = 60.9$, $\alpha = \gamma = 90$, $\beta = 115.5$
Total/unique reflections	92862/11437
Completeness (%)	94.0 (88.8)
$R_{\text{sym}}^\dagger$ (%)	6.8 (21.4)
Average $I/\sigma(I)$	22.6 (3.0)

$^\dagger R_{\text{sym}} = \sum_h \sum_i |I(h)_i - I(h)| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the mean intensity after rejection of reflections with greater than 90% chance of being outliers based on Bayesian statistics.

PreScission protease (Amersham Bioscience). The e4-1BBL was separated from GST using an ion-exchange column (Resource Q, Amersham Bioscience) chromatographic method.

2.2. Crystallization and data collection

Initial crystallization experiments of e4-1BBL were conducted by the microbatch method using commercially available screening solutions (Hampton Research). Crystals of e4-1BBL were obtained under various conditions and optimization of crystallization conditions was performed by the hanging-drop vapour-diffusion method at 290 K. The crystals were grown in drops consisting of a mixture of 1.5 μl protein sample and 1.5 μl reservoir solution [0.1 M Bis-Tris pH 5.5, 0.2 M ammonium acetate, 25% (w/v) PEG 3350] and equilibrated against 1000 μl reservoir solution over 7–10 d (Fig. 1). X-ray diffraction data were collected at beamline 6B of Pohang Light Source (PLS) in Korea. Prior to data collection, an e4-1BBL crystal was soaked in mother liquor with 20% (v/v) glycerol added as a cryoprotectant. Collected data were processed using *DENZO* and scaled using *SCALEPACK* from the *HKL2000* program suite.

3. Results and discussion

The crystal of e4-1BBL diffracted to 2.8 $\text{\AA}$ resolution and belongs to space group *C*2, with unit-cell parameters $a = 114.6$, $b = 73.8$, $c = 118.50$ $\text{\AA}$, $\beta = 115.5^\circ$. The presence of three molecules in the asymmetric unit gives a calculated Matthews coefficient V_M of 1.9 $\text{\AA}^3 \text{Da}^{-1}$, which corresponds to a solvent content of 36.6%. Analysis of the Patterson self-rotation function suggests that the crystal contains one further protein molecule in the asymmetric unit. The crystallographic parameters and data-collection statistics are summarized in Table 1. Structure determination by the molecular-replacement method is in progress.

Experiments at PLS were supported in part by MOST and POSTECH. We thank Sun-Sin Cha and Kyung-Jin Kim for assistance at beamline 6B of PLS. This work was supported in part by the SRC fund to the IRC at the University of Ulsan from KOSEF and MOST and by funds from the Korea Research Foundation Grant (KRF-2004-005-C00129) and the Yonsei University Faculty Research Project to H-SC.

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