Preliminary X-ray crystallographic studies of the TIR domain of human Toll-like receptor 6

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Toll-like receptor (TLR) proteins have been identified and shown to play a role in the innate immune response. TLR6 associated with TLR2 can recognize diacylated lipoprotein. In this study, the human TLR6 TIR domain corresponding to amino acids 640–796 was overexpressed in *Escherichia coli* using engineered C-terminal His tags. The TLR6 TIR domain was then purified to homogeneity and crystallized at 20°C. Finally, X-ray diffraction data were collected to a resolution of 2.2 Å from a crystal belonging to space group C2, with unit-cell parameters $a = 127.60$, $b = 44.20$, $c = 75.72$ Å, $\beta = 118.89^\circ$.

1. Introduction

Toll receptor was first identified in *Drosophila* as an essential component of the pathway that establishes the dorsal–ventral axis in developing embryos (Hashimoto *et al.*, 1988). Toll-like receptors (TLRs) have been identified in mammals and are known to be critically involved in innate immune responses (Iwasaki & Medzhitov, 2004). Innate immunity is initiated by the recognition of special patterns on pathogens known as pathogen-associated molecular patterns (PAMPs). TLRs can recognize these PAMPs and initiate the innate immune response. To date, 13 TLRs have been described in mammals (Hoebe *et al.*, 2004). The TLR protein is composed of three domains: N-terminal leucine-rich repeats (LRRs) in the extracellular portion, a transmembrane domain and a C-terminal Toll/IL-1 receptor (TIR) domain in the cytoplasm. The N-terminal LRRs domain can recognize specific components of pathogens, while the TIR domain interacts with MyD88 or Mal (TIRAP), which are TIR-domain-containing adaptor molecules. In 1999, TLR6 was identified as a product of dimerization with TLR2 (Takeuchi *et al.*, 1999). The heterodimeric complex of TLR6 can detect diacylated lipoprotein and mediates signalling to NF- κ B (Takeda & Akira, 2004). Previously reported crystal structures of the TIR domain of TLR1, TLR2 and TLR10 have shown that TIR domains contain central five-stranded parallel β -sheets surrounded by a total of five helices (Xu *et al.*, 2000; Nyman *et al.*, 2008).

As a first step towards elucidating the molecular structure of TLR6 and further understanding its molecular interaction with many downstream components, we overexpressed, purified by affinity chromatography followed by gel-filtration chromatography and crystallized the TIR domain of human TLR6 corresponding to amino acids 640–796 (hereafter referred to as TLR6^{640–796}). Structural studies of protein-interaction modules can often provide critical information regarding the interaction mode (Bae & Park, 2011; Park, 2011). After optimization, the final crystals of TLR6^{640–796} diffracted to a resolution of 2.2 Å and refinement is currently in progress. Details regarding the structure of TLR6^{640–796} should help us better understand TLR6-mediated signalling events, which are critical to the innate immune response.

Table 1

Diffraction data of the crystal of the TIR domain of TLR6.

Values in parentheses are for the highest resolution shell.

X-ray source	SB II 5C at PAL
Wavelength (Å)	1.0000
Space group	C2
Unit-cell parameters (Å, °)	$a = 127.60$, $b = 44.20$ $c = 75.72$, $\beta = 118.89$
Resolution limits (Å)	50–2.2 (2.24–2.20)
No. of observations	132143
No. of unique reflections	18245
Mean $I/\sigma(I)$	43.6 (4.0)
Completeness (%)	96.4 (97.2)
$R_{\text{merge}}^{\dagger}$ (%)	9.1 (66.0)

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

2. Materials and methods

2.1. Expression and purification

To express C-terminally His-tagged protein, human TLR6^{640–796} (GenBank BAA78631) was amplified by PCR using forward (5'-GGGCATATGCTCCAGTTTCATGCTTTTATTTCAT-3') and reverse (5'-GGGCTCGAGAGATTTACATCATTGTTTCA-GTG-3') primers. The PCR product was then digested with the *NdeI* and *XhoI* restriction enzymes (Enzymomics, Republic of Korea), after which it was inserted into pOKD5 vector which had been cut with the same restriction enzymes. The plasmid was then transformed into *Escherichia coli* BL21 (DE3) competent cells, after which its expression was induced by treating the bacteria with 0.25 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) overnight at 20°C during culture in 21 Luria–Bertani (LB) media. Cells expressing TLR6^{640–796} were pelleted by centrifugation, resuspended and lysed by sonication in 25 ml lysis buffer (20 mM Tris pH 8.0, 500 mM NaCl, 25 mM imidazole).

The lysate was then centrifuged at 16 000 rev min^{−1} for 30 min at 4°C, 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 lysis buffer (defined above). The C-terminal His-tagged TLR6^{640–796} was eluted from the column using elution buffer (20 mM Tris buffer pH 8.0, 500 mM NaCl,

250 mM imidazole). 0.5 ml peak fractions were pooled, concentrated to 2 ml and applied onto a Superdex 200 gel-filtration column (GE Healthcare) that had been pre-equilibrated with a solution consisting of 20 mM Tris pH 8.0, 150 mM NaCl. A gel-filtration standard (Bio-Rad) containing a mixture of molecular-weight markers (thyroglobulin, 670 kDa; globulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; and vitamin B₁₂, 1.35 kDa) was used for size calibration. TLR6^{640–796}-containing fractions were collected and concentrated to 4–5 mg ml^{−1} and the peak was then confirmed to contain TLR6^{640–796} by SDS-PAGE. Purified TLR6^{640–796} retained the C-terminal extra residues LEHHHHHH.

2.2. Crystallization

The crystallization conditions were initially screened at 20°C by the hanging-drop vapour-diffusion method using screening kits from Hampton Research (Crystal Screen, Crystal Screen 2, Natrix, MembFac, Index, Crystal Screen Cryo, Crystal Screen Lite, SaltRX and PEG/Ion) and from the deCODE Biostructures Group (Wizard I, II, III and IV). Initial crystals were grown on the plates by equilibrating a mixture comprising 1 μ l protein solution (4–5 mg ml^{−1} protein in 20 mM Tris–HCl pH 8.0, 150 mM NaCl) and 1 μ l reservoir solution No. 77 from the Index screen [0.2 M lithium sulfate monohydrate, 0.1 M Tris pH 8.5, 25%(w/v) polyethylene glycol 3350] against 0.4 ml reservoir solution. Crystallization was further optimized by changing the concentration of the precipitant and the pH. Crystals appeared after between three and six months and grew to maximum dimensions of 0.5 $\times$ 0.3 $\times$ 0.1 mm (Fig. 1) in the presence of 0.2 M lithium sulfate monohydrate, 0.1 M Tris pH 8.6, 25%(w/v) polyethylene glycol 3350, 4%(v/v) formamide.

2.3. Crystallographic data collection

For data collection, the crystals were transiently soaked in a solution corresponding to the reservoir solution supplemented with 30%(v/v) glycerol. The soaked crystals were then flash-cooled in

Figure 1

Crystal of the TIR domain of human TLR6. Crystals were grown for six months in the presence of 0.2 M lithium sulfate monohydrate, 0.1 M Tris pH 8.6, 25%(w/v) polyethylene glycol 3350, 4%(v/v) formamide. The approximate dimensions of the crystals were 0.5 $\times$ 0.3 $\times$ 0.1 mm.

Figure 2

Diffraction image (1° oscillation) from a crystal of the TIR domain of TLR6 with a 2.2 Å resolution limit.

liquid nitrogen. A 2.2 Å resolution native diffraction data set was collected on beamline BL-4A of the Pohang Accelerator Laboratory (PAL), Republic of Korea (Fig. 2). Overall, 360 images were collected with 1° oscillation. The data set was indexed and processed using *HKL-2000* (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 1.

3. Results and discussion

To understand the TLR6-mediated signalling pathway, we over-expressed, purified and crystallized TLR6^{640–796}, which is responsible for interaction with various downstream signalling components. TLR6^{640–796} was purified by two chromatography steps, His-tag affinity chromatography and gel-filtration chromatography, which produced 98% pure target protein. After the affinity chromatography step, the target protein was concentrated and purified by gel-filtration chromatography. TLR6^{640–796} was eluted at approximately 18 ml upon gel-filtration chromatography, which corresponds to a molecular weight of 20 kDa. Since the calculated monomeric molecular weight of TLR6^{640–796} including the C-terminal His tag was 19.74 kDa, TLR6^{640–796} appears to exist as a monomer in solution, similar to TLR1 and TLR2.

We initially attempted to obtain a crystal of the target protein using the TIR domain containing amino acids 640–782. This was easily crystallized, but the crystals never diffracted to a resolution above 6 Å. Success in obtaining a highly diffracting crystal of TLR6^{640–796} was the result of making a longer construct. The domain construct containing amino acids 640–796 diffracted to a resolution of 2.2 Å. The crystals belonged to space group *C2*, with unit-cell parameters $a = 127.60$, $b = 44.20$, $c = 75.72$ Å, $\beta = 118.89^\circ$. Assuming the presence of one dimer in the crystallographic asymmetric unit, the Matthews coefficient (V_M) was calculated to be $2.67 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of 53.92% (Matthews, 1968). Diffraction data statistics are given in Table 1. The molecular-replacement

phasing method was conducted with *Phaser* (McCoy, 2007) using the TLR1 structure (PDB entry 1fyv; Xu *et al.*, 2000), which has 78% amino-acid sequence identity to the TLR6 TIR domain, as a search model. Probable solutions with rotation-function and translation-function *Z*-scores of 7.5 and 7.9 for molecule *A*, and 6.0 and 13.5 for molecule *B*, respectively, were initially obtained. Initial refinement with *REFMAC5* (Murshudov *et al.*, 2011) using the initial *Phaser* model gave an R_{work} of 37.0% and an R_{free} of 42.9%. There was one dimer in the asymmetric unit. Further structural refinement is currently being conducted.

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References

- Bae, J. Y. & Park, H. H. (2011). *J. Biol. Chem.* **286**, 39528–39536.
- Hashimoto, C., Hudson, K. L. & Anderson, K. V. (1988). *Cell*, **52**, 269–279.
- Hoebe, K., Janssen, E. & Beutler, B. (2004). *Nature Immunol.* **5**, 971–974.
- Iwasaki, A. & Medzhitov, R. (2004). *Nature Immunol.* **5**, 987–995.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McCoy, A. J. (2007). *Acta Cryst.* **D63**, 32–41.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* **D67**, 355–367.
- Nyman, T., Stenmark, P., Flodin, S., Johansson, I., Hammarström, M. & Nordlund, P. (2008). *J. Biol. Chem.* **283**, 11861–11865.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Park, H. H. (2011). *Apoptosis*, **16**, 209–220.
- Takeda, K. & Akira, S. (2004). *Semin. Immunol.* **16**, 3–9.
- Takeuchi, O., Kawai, T., Sanjo, H., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Takeda, K. & Akira, S. (1999). *Gene*, **231**, 59–65.
- Xu, Y., Tao, X., Shen, B., Horng, T., Medzhitov, R., Manley, J. L. & Tong, L. (2000). *Nature (London)*, **408**, 111–115.