

Chapter 6

Expression, Purification, and Crystallization of Toll/Interleukin-1 Receptor (TIR) Domains

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Summary

Toll-like receptors (TLRs) and interleukin-1 receptor (IL-1R) play crucial roles in host innate immune response against microbial infections. These receptors share a conserved cytoplasmic domain, the Toll/interleukin-1 receptor (TIR) domain, which is required for signaling through these receptors. Structural information on the TIR domains will be essential for understanding the molecular basis for signal transduction by these receptors.

Key words: Innate immunity, Adaptive immunity, Crystallography, Protein structure and function, Signal transduction, Protein complexes.

1. Introduction

Toll-like receptors (TLRs) have crucial roles in innate immunity (1–6). They recognize various pathogen-associated molecules and initiate host defense responses. Ten TLRs have been identified in humans, and their ligands include cell-wall components from Gram-positive and Gram-negative bacteria (LPS and others), bacterial flagellin, viral dsRNA, unmethylated CpG DNA, and others.

TLRs contain leucine-rich repeat (LRR) and cysteine-rich domains in their extracellular domains. The intracellular region of the TLRs contains a conserved domain of about 150 amino acid residues, which also shares sequence homology with the intracellular region of the interleukin-1 receptor (IL-1R) superfamily. Therefore, this domain is known as the TIR (Toll/interleukin-1

receptor) domain. Upon receptor activation, the TIR domain of the receptor recruits downstream adaptor signaling molecules such as MyD88, Mal, Trif, and others (7). These adaptors also contain a TIR domain, suggesting that signal transduction by TLRs and IL-1Rs may be mediated by a homotypic receptor–adaptor TIR domain complex. Mutations in the TIR domain can abrogate this signaling process and cause serious diseases in humans (8). Understanding the molecular basis of TLR and IL-1R signaling will require structural information on the TIR domains (9–11).

This chapter describes protocols about how to express TIR domains of human TLR1 and TLR2 in bacteria, and how to purify and crystallize these TIR domains for structural studies. The protocols described here should be applicable to other TIR domains. In fact, a large number of TIR domains have been purified this way and the TIR domain from IL-1RAPL has also been crystallized (11).

2. Materials

2.1. Protein Expression of the TIR Domains of Human TLR1 and TLR2

1. Kanamycin should be made in a stock solution of 35 mg/ml in water, and filtered through a syringe filter for sterilization.
2. IPTG (0.4 M stock solution) is prepared in water, and filtered to sterilize. It can then be divided into smaller aliquots (1 ml each for example) and stored at -20°C .

2.2. Protein Purification of the TIR Domains of Human TLR1

1. PMSF (100 mM stock solution) is prepared in isopropanol, and should be added to the buffers just prior to use. PMSF is toxic and so should be handled with care.
2. β -Mercaptoethanol (β ME) and DTT should be added to the buffers just prior to use, and unused portions of these buffers should be discarded (or supplemented with fresh compounds) in a few days. The oxidized compounds will increase the baseline in the A_{280} readings.
3. Imidazole (0.5 M stock solution) is prepared in water, but make sure to adjust the pH to about 7.
4. Nickel–agarose resins (Qiagen and others) are stored in ethanol. It is good to exchange them into the lysis buffer before adding the bacterial lysate.
5. Lysis buffer: 20 mM Mops (pH 7.0), 300 mM NaCl, 5 mM imidazole (pH 7.0), 5% (v/v) glycerol, 10 mM β ME, and 0.3% (v/v) Triton X-100.

6. Nickel column wash buffer: 20 mM Mops (pH 7.0), 300 mM NaCl, 20 mM imidazole (pH 7.0), 5% (v/v) glycerol, and 10 mM β ME.
7. Nickel column elution buffer: 20 mM Mops (pH 7.0), 200 mM NaCl, 150 mM imidazole (pH 7.0), 5% (v/v) glycerol, and 10 mM β ME.
8. Gel filtration running buffer: 20 mM Mops (pH 7.0), 200 mM NaCl, and 5 mM DTT.
9. A 5 ml cation exchange column can be packed using SP Fast Flow resin (GE Healthcare) following the manufacturer's instructions.
10. The Sephacryl S-300 gel filtration column is from GE Healthcare.

2.3. Protein Purification of the TIR Domains of Human TLR2

1. Lysis buffer: 20 mM Mops (pH 7.0), 200 mM NaCl, 5% (v/v) glycerol, 2 mM DTT, and 0.3% (v/v) Triton X-100.
2. Gel filtration running buffer: 20 mM Mops (pH 7.0), 150 mM NaCl, and 3 mM DTT.

3. Methods

The TIR domains of human TLR1 (residues 625–786, about 20 residues from the transmembrane region) and human TLR2 (residues 626–784, about 16 residues from the transmembrane region) were subcloned into the pET26b vector (Novagen) using the NdeI and XhoI restriction sites, following standard protocols. The resulting protein of TLR1 contains a C-terminal His6 tag (with the sequence LEHHHHHH) while that of TLR2 does not have any tag.

3.1. Protein Expression of the TIR Domains of Human TLR1 and TLR2

1. Transform the desired expression plasmid into competent cells of expression host (BL21 (DE3)) and plate onto an LB-agar plate with 35 μ g/ml kanamycin.
2. Incubate the plate at 37°C overnight.
3. Pick a single colony from the plate and inoculate a 50 ml LB culture with 35 μ g/ml kanamycin, and shake at 37°C overnight.
4. Dilute the 50 ml overnight culture 100-fold into prewarmed LB media with 35 μ g/ml kanamycin and let the cells grow at 37°C for 3–4 h or until the OD₆₀₀ reaches around 0.6.
5. Induce the culture with 0.4 mM IPTG and continue shaking at 20°C overnight (about 16 h).

6. Harvest the bacterial cells by centrifuging at $5,000 \times g$ for 15 min.
7. Flash-freeze the cell pellet in liquid nitrogen and store at -80°C for later use, or start the protein purification right away.

**3.2. Protein
Purification of the
TIR Domain of Human
TLR1**

1. Resuspend the cell pellet in lysis buffer. Use about 30 ml lysis buffer per 700 ml bacterial culture.
2. Add PMSF to a final concentration of 0.5 mM.
3. Lyse the cells by sonicating with 30 bursts on ice, with 50% interval. Let the mixture sit on ice for 30 s to cool. Repeat three more times.
4. Centrifuge at $15,000 \times g$ for 30 min at 4°C .
5. In the meantime, prepare the Ni-agarose resin by washing them twice with ddH_2O . Then pre-equilibrate the resin with lysis buffer (*see Note 1*).
6. Add the supernatant from **step 4** into the pre-equilibrated Ni-agarose resin.
7. Gently mix the resin and the supernatant at 4°C for about 1 h for protein binding.
8. Load the resin onto a column under gravity flow.
9. Wash the resin with five bed volumes of lysis buffer, followed by ten bed volumes of wash buffer.
10. Elute the bound protein with five bed volumes of elution buffer. The protein should be more than 80% pure already after this step.
11. Dilute the eluate threefold with 20 mM Mops (pH 7.0) to reduce the salt concentration.
12. Load the diluted sample onto a cation exchange column (SP-FF, GE Healthcare) pre-equilibrated with 20 mM Mops (pH 7.0) and 50 mM NaCl (*see Note 2*).
13. Wash the column until the UV reading reaches baseline.
14. Elute the protein with a 50–500 mM linear NaCl gradient in ten column volumes.
15. Collect the peak fractions from the column, and concentrate to about 2 ml.
16. Load the concentrated protein onto a gel filtration column (Sephacryl S-300, GE Healthcare) pre-equilibrated with at least one column volume (~ 120 ml) of running buffer (*see Note 3*).
17. Collect the peak fractions from the gel filtration column (keep those fractions with UV reading more than half the maximum UV reading) and concentrate to 30 mg/ml. Exchange the protein buffer to one without salt during the

concentration. The protein concentration was determined by the Bradford (Biorad) method using bovine serum albumin (BSA) as the standard.

18. Divide the sample into small aliquots (20–50 μ l each), flash-freeze in liquid nitrogen, and store at -80°C .
19. Analyze the progress of purification by SDS-PAGE, with Coomassie staining. The final protein sample should be more than 95% pure.

3.3. Protein Purification of the TIR Domain of Human TLR2

1. Resuspend the cell pellet in lysis buffer. Use about 30 ml lysis buffer per 700 ml bacterial culture.
2. Add PMSF to a final concentration of 0.5 mM.
3. Lyse the cells by sonicating with 30 bursts on ice, with 50% interval. Let the mixture sit on ice for 30 s to cool. Repeat three more times.
4. Centrifuge at $15,000 \times g$ for 30 min at 4°C .
5. Mix the supernatant with 1% (w/v) streptomycin sulfate (Sigma) and incubate at 4°C for half an hour to precipitate DNA.
6. Centrifuge again at $15,000 \times g$ for 30 min at 4°C .
7. Load the supernatant onto a cation exchange column (SP FF, GE Healthcare) pre-equilibrated with 20 mM Mops (pH 7.0) and 100 mM NaCl (*see Note 4*).
8. Wash the column extensively with buffer containing 20 mM Mops (pH 7.0) and 250 mM NaCl.
9. Elute the protein with a 250–700 mM NaCl gradient in ten column volumes.
10. Collect the peak fractions from the column, and concentrate to about 2 ml.
11. Load the concentrated protein onto a Sephacryl S-300 gel filtration column (GE Healthcare) pre-equilibrated with at least one column volume (~ 120 ml) of the running buffer.
12. Collect the peak fractions from the gel filtration column (keep those fractions with UV reading more than half the maximum UV reading) and concentrate to 30 mg/ml.
13. Divide the sample into small aliquots (20–50 μ l each), flash-freeze in liquid nitrogen, and store at -80°C .
14. Analyze the progress of purification by SDS-PAGE, with Coomassie staining. The final protein sample should be more than 95% pure.

3.4. Crystallization of the TIR Domains of Human TLR1 and TLR2

Crystals of the TIR domain of human TLR1 were obtained at 21°C , using the hanging-drop vapor diffusion method (*see Note 5*). The reservoir solution contained 100 mM Tris (pH 8.0),

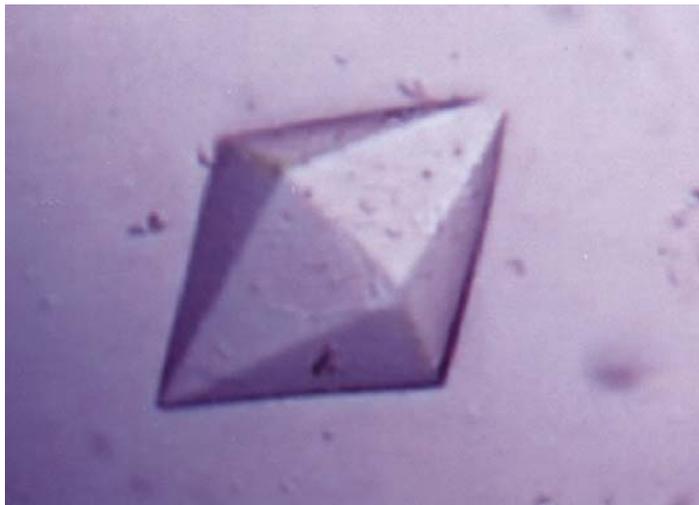


Fig. 1. A crystal of the Toll/interleukin-1 receptor (TIR) domain of human Toll-like receptor (TLR).

1.2 M $\text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, 5 mM DTT, and 20% (v/v) glycerol. Crystals in the shape of hexagonal bipyramids (Fig. 1) generally appeared in 2 weeks and took another week to grow to full size ($0.3 \times 0.3 \times 0.3 \text{ mm}^3$). For cryo-protection, the crystals were transferred to an artificial mother liquid containing 100 mM Tris (pH 8.0), 1.7 M ammonium sulfate (or phosphate), 25% (v/v) glycerol, 20 mM MgCl_2 , and 10 mM DTT, and flash-frozen in liquid nitrogen (or propane) for data collection at 100 K.

Crystals of the TIR domain of TLR2 were obtained at 4°C, using the hanging-drop vapor diffusion method. The reservoir solution contained 100 mM cacodylate (pH 6.8), 10% (w/v) PEG8000, 20% (v/v) DMSO, 200 mM MgCl_2 , and 5 mM DTT. The crystals in the same morphology as those of TIR domain of human TLR1 generally appeared overnight and grew to full size ($0.3 \times 0.3 \times 0.3 \text{ mm}^3$) in 2–3 days.

4. Notes

1. Generally the amount of nickel–agarose used for purification is just sufficient for binding all the His-tagged proteins. This should prevent the nonspecific binding of contaminant proteins to the resin. For TLR1 and TLR2 TIR domains, generally about 1 ml slurry of the resin is used for each 700 ml of bacterial culture. The nickel–agarose is supplied as a 50% slurry in ethanol.

2. The TIR domain of TLR1 has a high pI, and therefore can bind a cation exchange column at pI 7. This cation exchange step allows further purification of the protein.
3. The gel filtration column both allows further purification of the protein and gives an indication of the solution behavior (mono-disperse or aggregated) of the protein. The TIR domains of TLR1 and TLR2 consistently migrated as monomers on the gel filtration column, suggesting that TIR domains alone have weak affinity for self-association.
4. The TIR domain of TLR2 has a rather high pI value of 8.5. It binds tightly to the cation exchange column even at pH 7, and can be eluted only at higher salt (about 300 mM NaCl). Therefore, the TIR domain is mostly pure after the single cation exchange purification step.
5. In a hanging-drop vapor diffusion crystallization setup, the precipitant solution is placed in a well (typically on a 24-well plate). The protein solution is mixed (generally 1 μ l + 1 μ l) with the precipitant solution on a cover slip. The cover slip is then inverted and placed over the well, forming an airtight enclosure (grease is applied to the top edge of the well). Over time, equilibration between the drop and reservoir leads to changes in the drop and crystallization of the protein.

Acknowledgments

We thank Yingwu Xu and Javed Khan for their contributions to the TIR domain project. This research was supported in part by a grant from the National Institutes of Health (AI49475 to LT).

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