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Toll-like receptor 5 forms asymmetric dimers in the absence of flagellin

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ABSTRACT

The structure of full-length human TLR5 determined by electron microscopy single-particle image reconstruction at 26 Å resolution shows that TLR5 forms an asymmetric homodimer via ectodomain interactions. The structure shows that like TLR9, TLR5 dimerizes in the absence of ligand. The asymmetry of the dimer suggests that TLR5 may recognize two flagellin molecules cooperatively to establish an optimal flagellin response threshold. A TLR5 homology model was generated and fitted into the electron microscopy structure. All seven predicted *N*-linked glycosylation sites are exposed on the molecular surface, away from the dimer interface. Glycosylation at the first five sites was confirmed by tandem mass spectrometry. Two aspartate residues proposed to interact with flagellin (Asp294 and Asp366) are sterically occluded by a glycan at position 342. In contrast, the central region of the ectodomains near the dimer interface is unobstructed by glycans. Ligand binding in this region would be consistent with the ligand binding sites of other TLRs.

1. Introduction

Vertebrates rely on their innate immune system to detect microorganisms. Toll-like receptors (TLRs) are the principal family of molecular sentries for the innate immune recognition of microbial patterns outside the cytoplasm. TLRs recognize broadly conserved structures such as bacterial cell wall components, viral nucleic acid signatures and certain highly conserved proteins (Kawai and Akira, 2010). Binding of the ectodomains of TLRs 1, 2, 3 and 4 to their respective ligands induces homo- or hetero-dimerization of the receptors (Botos et al., 2011; Jin et al., 2007; Liu et al., 2008; Park et al., 2009). TLR5 can detect femtomolar concentrations of monomeric flagellin from flagellar filaments that have been depolymerized by the acidic environment of the phagosome (Hayashi et al., 2001; Smith et al., 2003). TLR5 recognition of flagellin is dependent on a region of flagellin that has been mapped by alanine-scanning mutagenesis to a highly conserved surface responsible for protofilament assembly (Smith et al., 2003). This recognition event induces the secretion of antimicrobial and inflammatory cytokines by dendritic cells within minutes of infection via activation of the NF-κB signaling pathway (Hayashi et al., 2001; Kawai and Akira, 2010). α and ε *Proteobacteria* such as *Campylobacter jejuni* and *Helicobacter pylori* evade TLR5 recognition due to divergent sequences in their flagellins (Andersen-Nissen et al., 2005).

While a direct interaction between TLR5 and flagellin has not been demonstrated biochemically using purified components, TLR5 is thought to associate directly with flagellin based on the following observations. Purified flagellins from various bacterial species bind to TLR5 in detergent lysates from COS-1 cells transiently transfected with a human TLR5 expression plasmid (Mizel et al., 2003). Moreover, cell lysates from bacteria expressing flagellin variants with insertions in the D1 domain induce an NF-κB luciferase reporter in CHO cells transiently transfected with mouse TLR5 but not human TLR5 (Smith et al., 2003). This species-specific difference in TLR5 recognition implies a direct interaction between flagellin and TLR5, as any additional factors were common in experiment (Smith et al., 2003). Additionally, a naturally occurring amino acid variation in TLR5 residue 268 is responsible for the differential affinities of human and mouse TLR5 for flagellins from different bacterial species (Andersen-Nissen et al., 2007). Mutations in mouse TLR5 residues D295 or D367 weakened flagellin recognition but did so to different extents for different variants of flagellin, an effect which has been interpreted as further evidence of a direct interaction between flagellin and TLR5 (Andersen-Nissen et al., 2007).

We report here the first structural analysis of TLR5. The electron microscopy (EM) structure of full-length human TLR5 reveals an asymmetric homodimer via ectodomain interactions in the

Abbreviations: TLR, toll-like receptor; ECD, ectodomain; TM, transmembrane; LRR, leucine-rich repeat; EM, electron microscopy; TEA, triethanolamine.

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absence of flagellin. The asymmetry of the dimer suggests that TLR5 may recognize two flagellin molecules cooperatively. A TLR5 homology model fitted into the electron microscopy structure shows that all seven predicted *N*-linked glycosylation sites are exposed on the molecular surface. Two aspartate residues proposed to interact with flagellin (Andersen-Nissen et al., 2007) are sterically occluded by a glycan. In contrast, the central region of the ectodomains near the dimer interface, where other TLRs 1–4 bind their ligands (Botos et al., 2011), is unobstructed by glycans.

2. Materials and methods

2.1. Expression and purification of human toll-like receptor 5

A gene encoding the mature chain (residues 22–858) of full-length human TLR5 (FL-hTLR5) followed by a Gly–Ser linker and a C-terminal six-histidine purification tag was cloned into the pAcGP67-A vector (BD Biosciences) in frame with the baculovirus gp67 signal sequence using the Bam HI and Not I restriction sites. Sf9 insect cells (Invitrogen) were infected with a recombinant baculovirus containing the FL-hTLR5 gene. After culture in suspension for 72 h at 27 °C the cell pellet was lysed in Buffer A (10 mM triethanolamine (TEA) pH 7.5, 0.5 M NaCl, 3 mM β-mercaptoethanol (β-ME), 0.5 mM PMSF) plus 1 mM EDTA, 2 mM benzamidine, 2 μg/ml pepstatin A and 2 μg/ml leupeptin. The insoluble fraction was separated by centrifugation at 100 kg for 1 h at 4 °C. The supernatant was discarded and FL-hTLR5 was extracted by resuspending the pellet and incubating 16 h at 4 °C with gentle stirring in Buffer A plus 5 mM imidazole, 1% (w/v) Fos-Choline-14 (FC-14, Anatrace) and protease inhibitors (Roche). The suspension was diluted 1:1 with Buffer A to reduce the FC-14 concentration to 0.5% (w/v), incubated for 1 h at 4 °C, and then centrifuged at 100 kg for 1 h at 4 °C. The supernatant was loaded onto a HisTrap HP nickel-affinity column (GE Healthcare) pre-equilibrated in Buffer A. The column was washed with Buffer A containing 20 mM imidazole and eluted with a linear gradient of imidazole from 20–200 mM (4 mM/min). Protease inhibitors and 1 mM EDTA were added to the eluate. Optionally, the glycans were trimmed from FL-TLR5 by adding 5 μl of endoglycosidase F1 or H, or *N*-glycosidase F (PNGase F) (New England Biolabs) per liter of cell culture and incubating 16 h at 4 °C. The protein was transferred to Buffer Q (50 mM Tris/HCl pH 8.5, 25 mM NaCl, 1 mM β-ME, 0.3 mM FC-14, Roche protease inhibitors, 1 mM EDTA), loaded on a MonoQ anion exchange column (GE Healthcare) pre-equilibrated with Buffer Q containing 50 mM NaCl, and eluted with a linear gradient of 50–400 mM NaCl (5 mM NaCl/min). The protein was then subjected to a second round of nickel-affinity chromatography as described above except with lower concentrations of FC-14 and β-ME (0.3 and 1 mM, respectively) in the wash and elution buffers. FL-hTLR5 was purified to homogeneity by size-exclusion chromatography with a Superdex 200 10/300 GL column (GE Healthcare) in 10 mM TEA pH 7.5, 0.15 M NaCl, 3 mM FC-12 (or 0.3 mM FC-14). This procedure typically yielded 4 mg of pure FL-hTLR5 per liter of insect cell culture. A typical size-exclusion chromatogram with the corresponding SDS–PAGE gel is shown in Fig. 1.

2.2. Expression and purification of human toll-like receptor 5 ectodomain

A gene encoding residues 22–636 of human TLR5 (hTLR5-ECD) followed by a Gly–Ser linker and a C-terminal eight-histidine purification tag was cloned into the pAcGP67-A vector in frame with the baculovirus gp67 signal sequence using the Xma I and Bgl II restriction sites. High Five cells (Invitrogen) were infected with recombinant baculovirus expressing hTLR5-ECD and cultured in suspension for 96 h at 20 °C. hTLR5-ECD protein was extracted from the cell lysate pellet and purified as described above for FL-hTLR5 except for the following modifications: Buffer A contained 10 mM imidazole; the nickel-affinity column was washed with Buffer A containing 25 mM imidazole and eluted with a linear gradient of 25–250 mM imidazole; Buffer Q contained 20 mM NaCl; the MonoQ column was pre-equilibrated with 40 mM NaCl and eluted with a linear gradient of 40–400 mM NaCl; the second round of nickel-affinity chromatography was omitted; and the buffer for size-exclusion chromatography contained 0.1 M NaCl. This procedure typically yielded 0.6–1.0 mg of pure hTLR5-ECD per liter of insect cell culture. A typical size-exclusion chromatogram is shown with the corresponding SDS–PAGE gel in Fig. S1A.

2.3. Electron microscopy sample preparation and data collection

Freshly purified FL-hTLR5 or hTLR5-ECD treated with endoglycosidase H or F1 was diluted 20-fold into 10 mM TEA pH 7.5, 100 mM NaCl to a concentration of 50 nM immediately before preparing the EM specimen. 4 μl of the solution was applied onto a thin-carbon-covered holey carbon copper grid and the protein was stained with 1% uranyl-formate as described (Liu and Wang, 2011). We captured micrographs on a FEI Tecnai-12 electron microscope at 120 kV acceleration voltage with a Gatan Ultrascan4000 CCD camera at a nominal magnification of 49,000 with a defocus value of approximately −1.0 μm. For the random conical tilt reconstruction, about 50 tilt pairs of micrographs were collected at 0° and +55°.

2.4. Single-particle image reconstruction

Individual particles were boxed with EMAN BOXER (Ludtke et al., 1999). A total of about 2000 tilt pairs of particles were picked

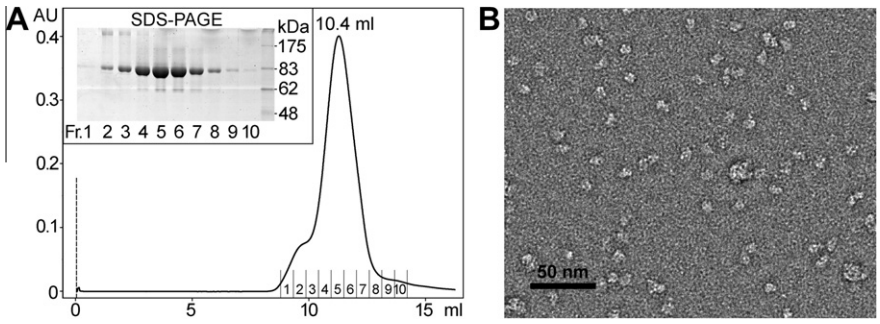

Fig. 1. Purification of full-length human TLR5 (FL-hTLR5). (A) Size-exclusion chromatogram of FL-hTLR5 from a Superdex 200 10/300 GL column after treatment with endoglycosidase H. The elution volume of the main peak corresponds to a hydrodynamic radius of ~7.4 nm (Young et al., 1980). Inset: Coomassie-stained SDS–PAGE gel showing pure FL-hTLR5 in the peak fractions. (B) Representative electron micrograph of FL-hTLR5. Most particles are approximately spherical with a 10 nm diameter. A few larger aggregates are visible, consistent with the presence of the 9.5-ml shoulder in (A).