The Crystal Structure of the Human Toll-like Receptor 10 Cytoplasmic Domain Reveals a Putative Signaling Dimer*

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The Toll/interleukin-1 receptor (TIR) domain is a highly conserved signaling domain found in the intracellular regions of Toll-like receptors (TLRs), in interleukin-1 receptors, and in several cytoplasmic adaptor proteins. TIR domains mediate receptor signal transduction through recruitment of adaptor proteins and play critical roles in the innate immune response and inflammation. This work presents the 2.2 Å crystal structure of the TIR domain of human TLR10, revealing a symmetric dimer in the asymmetric unit. The dimer interaction surface contains residues from the BB-loop, DD-loop, and αC-helix, which have previously been identified as important structural motifs for signaling in homologous TLR receptors. The interaction surface is extensive, containing a central hydrophobic patch surrounded by polar residues. The BB-loop forms a tight interaction, where a range of consecutive residues binds in a pocket formed by the reciprocal BB-loop and αC-helix. This pocket appears to be well suited for binding peptide substrates, which is consistent with the notion that peptides and peptide mimetics of the BB-loop are inhibitors for TLR signaling. The TLR10 structure is in good agreement with available biochemical data on TLR receptors and is likely to provide a good model for the physiological dimer.

The Toll-like receptors (TLRs)3 are type I transmembrane receptors (1) that play an important role in innate immunity as they recognize conserved pathogen signatures and induce early gene expression responses to infections (2, 3). TLR signaling is mediated through the cytoplasmic Toll/interleukin-1 receptor (TIR) domain, the oligomerization of which initiates the recruitment of TIR domain-containing adaptor proteins. The cytoplasmic adaptor subgroup comprises five members: MyD88, MyD88-adaptor-like (MAL), TIR-domain-containing adaptor protein inducing IFNβ (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo-motif containing protein (SARM) (4), which mediate or modulate the downstream signaling. Subsequent kinase activation results in the induction of transcription factors such as interferon regulatory factor and NF-κB followed by the production of numerous regulators and effectors of the innate immune response. In addition to the TLRs and the cytoplasmic TIR adaptor subgroup, the family of TIR domain-containing proteins encompasses the interleukin-1 (IL-1) receptor family (5).

The 10 members of the human TLR family recognize a wide variety of pathogen-associated molecular patterns. As no ligand has yet been reported for TLR10, it remains the only orphan family member. Activation of TLRs is believed to involve receptor oligomerization, and the current view is that the receptors are active as either homodimers or heterodimers, depending on the receptor type. The cytoplasmic TIR domains dimerize to form a platform for the recruitment of adaptor proteins and additional signaling molecules. TLR10 has been shown to form homodimers and to interact with TLRs 1 and 2 (6). All of the TLRs, except TLR3, have been shown to signal through the common TIR adaptor MyD88 (6–9), whereas the other adaptors are more selective for specific receptors (4).

Functional analysis of TIR domains has pointed to areas and residues of specific importance for receptor function. In particular, several studies point to the BB-loop as being essential for TLR signal transduction, and it has been suggested to be the site of adaptor protein recruitment (10). Prior to this work, the structures of three other TIR domains have been determined. Analysis of the structures of TLR1 and TLR2 (10, 11) and IL-1RAPL (12) TIR domains revealed that the BB-loops are ordered but accommodate different conformations in the different structures. The structural studies have also led to different suggestions for how a biological dimer is formed (11, 13).

This work presents the structure of the human TLR10 TIR domain, revealing a symmetric dimer with an extensive and highly compatible interaction area. The BB-loop and helix αC of each monomer constitute the major part of the dimer interface. The TLR10 dimer is compatible with existing data from functional analysis of TLR10 and related receptors, and it is likely that the TLR10 structure represents the physiological active dimer.

EXPERIMENTAL PROCEDURES

Cloning and Expression—Full-length human TLR10 cDNA was achieved from mammalian gene collection (ID: BC089406). A sequence carrying codons 622–776, corresponding to the TIR domain, was subcloned into the vector pNIC-Bsa4, joining the sequence to an N-terminal hexahistidine tag with integrated tobacco etch virus protease cleavage site. The resulting plasmid was transformed into BL21(DE3) cells. Cell cultivation
and protein expression were performed as described earlier (14). Harvested cells were frozen at −80 °C until protein purification.

Extraction and Purification—Cell extract preparation, purification protocol, and buffer compositions were as described earlier (14). Purification was conducted on an AKTA Xpress system equipped with IMAC (HiTrap chelating HP 1 ml, GE Healthcare) and gel filtration (HiLoad™ 16/60 Superdex 200 prep grade, GE Healthcare) columns. Final gel filtration was performed at 4 °C, in 20 mM HEPES, 300 mM NaCl, 10% glycerol, 2 mM tris(2-carboxyethyl)phosphine, pH 7.5. The resulting gel filtration chromatogram displayed a single symmetric peak at a retention volume corresponding to the monomeric TIR domain. The peak fractions contained the TIR domain to 95% homogeneity, as judged by SDS-PAGE analysis (not shown). The protein was concentrated to 10.5 mg/ml as determined using a molar absorption coefficient of 36,900 (15), from a protein solution (10.5 mg/ml) and 2 mM of well solution (11% polyethylene glycol 3350, 0.2 mM NaSCN), incubated at 20 °C. Crystals appeared on day 2 and were harvested on day 4. A crystal was briefly soaked in cryoprotectant solution (15% polyethylene glycol 3350, 25% glycerol, 0.2 mM NaSCN, 0.2 mM NaCl) prior to freezing in liquid nitrogen. X-ray diffraction data were collected at 100 K on the Berliner Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung (BESSY) beamline 14.1 (Berlin, Germany). The data were processed using the program XDS (16).

Structure Solution and Refinement—The TLR10 TIR domain was crystallized in hanging drops containing 1 μl of protein solution (10.5 mg/ml) and 2 μl of well solution (11% polyethylene glycol 3350, 0.2 mM NaSCN), incubated at 20 °C. Crystals appeared on day 2 and were harvested on day 4. A crystal was briefly soaked in cryoprotectant solution (15% polyethylene glycol 3350, 25% glycerol, 0.2 mM NaSCN, 0.2 mM NaCl) prior to freezing in liquid nitrogen. X-ray diffraction data were collected at 100 K on the Berliner Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung (BESSY) beamline 14.1 (Berlin, Germany). The data were processed using the program XDS (16).

Structure Solution and Refinement—The structure was solved by molecular replacement using MOLREP (17) and the human TLR1 structure (PDB ID 1FYV) (10) as template. Two monomers were found within the asymmetric unit, arranged as a dimer with two-fold symmetry. The structure was refined with data between 23 and 2.2 Å using Refmac (18), and iterative model building between refinement rounds was carried out in Coot (19). Water molecules were added into difference electron density maps using ARP/wARP (20). Crystal data and refinement statistics are shown in Table 1. The coordinates and structure factors were deposited in the PDB under the accession code 2J67. Structure analysis was done with Coot and PyMOL (21). Figures were made using PyMOL.

RESULTS

Overall Structure—Several constructs of the TLR10 TIR domain were expressed, purified, and subjected to crystallization trials. The construct containing residues Lys-622–Asn-776 yielded crystals that allowed the TLR10 TIR domain structure to be determined at 2.2 Å resolution. The structure reveals a dimer in the asymmetric unit where each monomer contains a central five-stranded parallel β-sheet (βA–βE) surrounded by five α helices (αA–αE) (Fig. 1A). In line with previous work on TIR domains, the loops are named by the strands and helices that they connect. For instance, the BB-loop connects strand βB and helix αB. The TLR10 TIR domain displays a high degree of sequence conservation (25–75% identity) to the other human TLRs, and the TLR10 structure is therefore similar to the previous structures of TLR1 (10) and TLR2 (11) with r.m.s.d. of 1.4–1.7 Å. The most significant difference in Ca positions among the three TLR structures is seen in the CD-loops, which differ considerably (not shown), whereas both the αC-helix and the following βD-strand overlap with low r.m.s.d.

The TLR10 Dimer—The TLR10 dimer displays a two-fold symmetry (Fig. 1A) with a buried surface interaction area of 974

### Table 1

| Data processing and refinement statistics | Values in parentheses correspond to the highest resolution shell. |
|------------------------------------------|---------------------------------------------------------------|
| Space group                             | P2₁                                                           |
| Unit cell dimensions a, b, c (Å)         | 66.2, 43.3, 71.3                                             |
| Unit cell dimensions α, β, γ (°)         | 90, 101, 90                                                  |
| Resolution (Å)                          | 23–2.2 (2.3–2.2)                                             |
| R_sym (%)                               | 6.5 (40.2)                                                   |
| Completeness (%)                        | 97.6 (92)                                                    |
| Redundancy (%)                          | 3.8 (3.7)                                                    |
| <l/σ> (%)                               | 13.8 (3.5)                                                   |
| Refinement                              |                                                               |
| R-factor (%)                            | 21.4                                                         |
| R_free (%)                              | 26.6                                                         |
| r.m.s.d. bond length (Å)                | 0.015                                                        |
| r.m.s.d. bond angle (°)                 | 1.5                                                          |
| Ramachandran plot                       |                                                               |
| Most favored (%)                        | 93.1                                                         |
| Additionally allowed (%)                | 6.1                                                          |
| Generously allowed (%)                  | 0.8                                                          |
| Disallowed (%)                          | 0                                                            |

**FIGURE 1.** A, ribbon diagram of the TLR10 TIR dimer structure. Part of the DD-loop and the αD-helix of monomer B is disordered and not resolved in the structure. B, stereo diagram outlining the key residues involved in the dimer interface.
Å, which is in the range of typical physiological interaction surfaces. Based on the solvation energy calculated from the buried surface area and specific electrostatic interactions, the dimer interface was judged by the PISA server (22) to constitute a physiological interaction. The major contributions to the dimer interface are made by residues of the BB-loops and the αC-helices. The dimer interface can be seen as made up of a hydrophobic core surrounded by a hydrogen-bonded network (Fig. 1B). The hydrophobic core is constituted primarily by residues of the BB-loop: Tyr-668, Phe-672, Pro-674, and Ile-678. Apart from contributing to the dimer contact, these four residues are stabilizing the observed conformation of the BB-loop, consisting of residues 667–678. At the end of the central αC-helix, Phe-710 and Cys-706 also contribute hydrophobic interactions. The hydrogen-bond network surrounding the hydrophobic core of the interface consists of residues of the BB-loop (Tyr-668, Lys-676), the αB-helix (Ser-679), and the central distorted αC-helix (His-707). The Ser-679 and His-707 residues form a Ser-His-His-Ser chain of hydrogen bonds, connecting the two αC-helices at the very center of the dimer. Above this hydrogen-bond network, the monomers separate slightly, forming a cleft where a number of well defined water molecules mediate hydrogen bonding between the two subunits. At the top of the cleft in monomer A, Arg-741 of the DD-loop makes a polar interaction with Glu-709 in helix αC of monomer B. However, the main part of the DD-loop of monomer A is not involved in the dimer interface. Also, in monomer B, the DD-loop is mainly disordered, which appears to be due to the exposure of the side chains of Phe-735 and Tyr-736, making a crystal contact to the BB-loop of a symmetry-related molecule.

A striking feature of the TLR10 structure is the positions of the BB-loops. Although this element constitutes an important part of the dimer interface, a large part of the BB-loop is exposed to the surrounding solvent. This results in the formation of an extended patch of well preserved residues (Fig. 2A). Considering the reported importance of BB-loop residues in the function of TLRs, this opens for the possibility that this extended patch is involved in the recruitment of TIR domain-containing adaptor molecules.

The TIR domain dimer seen in this work represents a unique structure. In previous structural work, other TIR-dimer interaction modes have been proposed and correlated to mutagenesis data. Based on a crystal interaction in the C713S mutant of the TLR2 TIR domain, the authors argue for a physiological role of an asymmetric dimer found in this structure (11). Since TLR2 is not functional as a homodimer (23), the authors suggested that this interface is involved in heterodimer formation between TLR2 and TLR1 or TLR6. This asymmetric dimer interface involves interactions of the BB-loop and αB-helix with the αC-helix and DD-loop of the contacting monomer. In the TLR1 structure (10), a disulfide-linked symmetric dimer was seen, also with the αC-helix and the BB-loops as the central components. In the structure of the IL-1RAPL TIR domain, a dimer is seen in the asymmetric unit (12), where the interface has the αC-helix as the central component. Although these previous structure dimers are different from the one seen in TLR10, they have focused the attention of previous mutational work on the same interaction motifs as those seen in the TLR10 structure.

The TLR10 Dimer Is Consistent with Mutation Data—A wealth of data has been generated on the effects of TLR TIR domain mutations on reporter gene-based signaling assays. However, the structural interpretation of these data is complicated by the fact that it is not known which specific interactions are affected by the mutations, i.e. if it is an inter-receptor interaction, if it is a receptor-adaptor interaction, or if another, still to be revealed, interaction is affected. Furthermore, inactivating mutations have not been analyzed for misfolding defects. The lack of a good biochemical system for in vitro studies of the relevant signaling events and interactions in the TLR-signaling cascades have hampered progress toward understanding the detailed mechanism of these signaling cascades. Structural analyses, however, have the potential to reveal interaction modes of TLR domains, which can serve as working models for the physiological interactions, where the models can be validated by comparison with in vivo mutational data.

Previous cell-based mutational work has implicated the BB- and DD-loops as important for the function of TIR domains. Several studies have pointed to the important role of a conserved proline within the BB-loop for the downstream signaling. The well studied P714H mutation of TLR4 (corresponding
to Pro-674 in TLR10) abolishes the response to lipopolysaccharides of Gram-negative bacteria in the C3H/HeJ mouse and acts as a dominant-negative mutant (24). Mutations of this proline apparently had no effect on the ability of TLR4 to form a complex with MyD88 (25). Mutation of the corresponding proline within TLR2 (Pro-681) was demonstrated to result in an inability to signal through MyD88 (10). TLR10 has been shown to complex with MyD88 in cell extracts, and the P674H mutation affects both signaling capacity and the interaction with MyD88 (6). The position of Pro-674 in the TLR10 structure suggests that mutations of this residue are likely to affect dimer interaction. Pro-674 contributes to the interaction surface but also seems important in stabilizing the specific conformation of the BB-loop by restraining its main chain conformation. Moreover, Pro-674 is also exposed on to the surface of the dimer, potentially affecting the interaction with adaptor molecules (Figs. 1B and 2B).

The mutation of BB-loop residue I718A in TLR4, corresponding to Ile-678 in TLR10, was found to abolish signaling through the receptor (26). In an alanine mutation scan, it was found that Phe-679, also of the BB-loop, in TLR2, corresponding to Phe-672 in TLR10, inhibited TLR2 signaling (13). As discussed above, the BB-loop residues correspond to Phe-672, Pro-674, and Ile-678 in TLR10 are all involved in the central hydrophobic interaction seen in the TLR10 dimer (Fig. 1B). Phe-672 is conserved as a Phe or a Trp in all TLRs and plays a dual role in the TLR10 structure; it anchors the BB-loop onto the TIR domain but also makes an homotypic interaction with Phe-672 in the neighboring subunit. Ile-678 is conserved as an aliphatic residue (ILV) in all TLRs, except TLR3 (Glu) and TLR5 (Arg).

In work on TLR2 and TLR4, it has been demonstrated that the residues corresponding to Cys-706 and Phe-710 of TLR10 are important for receptor function (11, 27). These two residues fall in the central region of the dimer interaction of helix αC and make direct van der Waals interactions at the TLR10 dimer interface. Together with His-707, Cys-706 and Phe-710 constitute the bulk of the αC-helix interactions of the dimer.

The TLR2 DD-loop mutations of residues Arg-748, Phe-749, Leu-752, and Arg-753, corresponding to Arg-741, Tyr-742, Leu-745, and Lys-746 in TLR10, had significant effects on TLR2-TLR1 signaling, whereas most other alanine mutations of DD-loop residues showed no such effect (13). Only Arg-741 of these residues participates directly in the TLR10 dimer interface, where in molecule A, it sits on the tip of the DD-loop, making a salt bridge to Glu-709 of molecule B (Fig. 1B). The Tyr-742, Leu-745, and Lys-746 residues appear to be stabilizing the position of the DD-loop onto the core of the molecule. Since the DD-loop in monomer B is flexible and partially disordered, it is conceivable that mutations of these residues might destabilize the conformation of the region and therefore inhibit the possibility for Arg-741 to participate in the dimer interaction. Both the main chain and the side chain conformations of the DD-loops in the TLR1 and TLR2 structures are similar to those in TLR10, suggesting that the stabilizing function of DD-loop residues could be similar in the three proteins.

In a large scale mutational study of the TLR4 TIR domain, mutations of sets of 1–3 consecutive residues were made at a number of different places and shown to abolish or attenuate activity (26). Our interpretation of these complex data is that the effects seen from mutations of two and three consecutive residues are likely to be due to significant structural perturbations. Most single mutations in the study retained activity, and only mutations in the BB-loop and in the αβ-helix abolished activity. A number of mutations showing no or minor effects on TLR signaling have also been made in TLR TIR domains. These mutations map almost invariably to areas outside the interaction surface of the TLR10 dimer. Conversely, it is striking that nearly all of the reported mutations of TLR TIR domains affecting receptor function maps to the TLR10 dimer interface revealed in the present study, thus confirming its relevance as a model for a physiological signaling dimer for several members of the TLR family. As mentioned above, there are, however, mutations in the DD-loop region and in the αβ-helix, which affect the activity but which do not fall directly on the dimer interaction surface. The effects of these mutations could potentially be explained by secondary structural effects on the interaction surface through misalignment of the DD-loop or the αβ-helix.

In a recent report, the TLR4 TIR domain dimer was modeled using our TLR10 dimer, made available from the PDB prior to the publication of this work as template (27). These authors also concluded that the interaction mode of the TLR10 dimer is likely to be physiological and that the TLR4 TIR domain dimer modeled from the TLR10 crystallographic structures is consistent with available experimental data.

**DISCUSSION**

Over the last decade, research on Toll receptor signaling has gathered a wealth of data on the effects of point mutations in the TIR domains. Despite this, a detailed understanding of the interaction modes between TIR domains in activated signaling complex is still lacking. The TLR10 structure presented in this work adds the first direct structural data on a complex of two TLR-TIR domains known to form a signaling pair. The extent of the interaction surface indicates that it corresponds to a physiological interaction. By analyzing the reported effects of TIR domain mutations of TLR10 and other TLRs, we confirm that the TLR10-dimer interaction mode is consistent with observed effects of such mutations. This analysis therefore supports the notion that the TLR10 dimer could serve as a model for TIR domain signaling in the TLR10 system, as well as for other members of the family, most notably TLR1, TLR2, and TLR4, where extensive mutational data exist. However, due to the limitations in information content in the reporter assays and the lack of good in vitro systems for studying activated TIR domain complexes, further studies are required to provide conclusive evidence for the interaction modes of the activated TLR receptors.

The BB-loop interaction seen in the TLR10 dimer is very intriguing as it involves most residues of the consecutive sequence Tyr-668–Ser-679 (Ser-679 being the first residue of the αβ-helix). The interaction of this segment is extensive and highly complementary, with relatively few water molecules involved in mediating the interaction (Fig. 2B). The interaction is quite hydrophobic (Tyr-668, Phe-672, Pro-674, Ile-678) but
also contains polar components (Tyr-668, Lys-676, Ser-679). Peptides and peptidomimetics modeled from the BB-loops of MyD88 and other adaptor TIR domains have been shown to inhibit TLR (28) and IL-1R signaling (29–31). Likewise, peptides corresponding to the BB-loop region of several TLRs were shown to block, with some specificity, the signaling of specific TLRs (32). It is therefore possible that the binding pocket for the BB-loop revealed by the TLR10 structure also constitutes the binding site for peptide-like inhibitors.

In conclusion, the extensive interaction surface and its agreement with mutational data support the physiological relevance of dimer formation (30, 31). The conserved and exposed BB-loop surface seen on one face of the TLR10 dimer (Fig. 2A) is a potential candidate interaction surface with MyD88. If this is in fact the case, it would further underline the importance of the BB-loop in the TLR signaling since it could be involved in both homodimer and heterotetramer formation. However, future structural studies of relevant complexes will be required to resolve this issue.

In conclusion, the extensive interaction surface and its agreement with mutational data support the physiological relevance of the dimer observed in the TLR10 structure. This work provides a structural background for the critical role of the BB-loop in TLR signaling and reveals a potential peptide-binding pocket, which could be utilized for rational design of specific antagonists of selected TLR pathways.

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