Structural Insights into the Assembly of Large Oligomeric Signalosomes in the Toll-Like Receptor–Interleukin-1 Receptor Superfamily

Ryan Ferrao, Jixi Li, Elisa Bergamin, Hao Wu

The Toll-like receptor (TLR)–interleukin 1 receptor (IL-1R) superfamily plays fundamentally important roles in innate immune and inflammatory responses. Structural studies have begun to show that upon ligand stimulation, TLRs and IL-1Rs assemble large oligomeric intracellular signaling complexes, or “signalosomes,” to initiate the activation of kinases and ubiquitin ligases, leading eventually to the activation of the transcription factors that are responsible for the expression of genes whose products mediate immune and inflammatory responses. The different scaffolds identified by these structural studies provide a molecular foundation for understanding the formation of microscopically visible signaling clusters that have long been known to cell biologists. Here, we illustrate the potential mechanistic steps of step-by-step assembly from the membrane-proximal interactions to the more downstream events. Formation of large oligomeric signalosomes may help to establish a threshold response in TLR and IL-1R signaling.

Introduction

Toll-like receptors (TLRs) and interleukin-1 (IL-1) receptors (IL-1Rs) belong to a superfamily of evolutionarily conserved transmembrane receptors that are involved in innate and inflammatory immune responses. To date, a total of 10 human TLRs (TLR1 to TLR10) have been identified. They are characterized by an extracellular leucine-rich repeat (LRR) motif that is responsible for the identification of distinct microbial structures, referred to as pathogen-associated molecular patterns (PAMPs), enabling the innate immune system to recognize invading microorganisms and initiate protective immune responses (1–4). Members of the IL-1R family are distinct from TLRs in their extracellular region, which contains three immunoglobulin-like domains. IL-1R family members interact with accessory proteins to form heterodimeric receptor signaling complexes (1).

Members of the TLR–IL-1R superfamily share a common cytoplasmic domain, the Toll–IL-1R homology (TIR) domain, which is essential for initiating intracellular signaling. Recognition of cognate ligand triggers a signaling cascade by the recruitment of TIR-containing adaptor proteins through homotypic TIR-TIR interactions (1–4). Myeloid differentiation protein 88 (MyD88), a TIR-containing adaptor protein that is essential for IL-1 and IL-18 signaling (5), was the first to be identified as being involved in TLR signaling (6). Subsequently, a number of other TIR-containing adaptors were shown to participate in signaling from a subset of TLRs, including MyD88 adaptor-like (Mal, also known as TIRAP) (7), TIR domain–containing adaptor inducing interferon-β (IFN-β) (TRIF, also known as TICAM1), and TRIF-related adaptor molecule (TRAM, also known as TICAM2) (8, 9). TIR-containing receptors and TIR-containing adaptors interact to form membrane-proximal signaling complexes in the TLR and IL-1R pathways.

MyD88 is the major adaptor protein shared by almost all TLRs, except TLR3 (10). In addition to its TIR domain, MyD88 possesses an N-terminal death domain (DD) (Fig. 1A). Upon stimulation, MyD88-dependent signaling propagates through the recruitment of IL-1R–associated kinase (IRAK) family members. IRAKs are multi-domain proteins containing both a DD and a serine and threonine kinase domain (KD). Targeted deletions in mice have identified IRAK1, IRAK2, and IRAK4 as stimulators of signaling and IRAK-M as an inhibitor of signaling (11–13). After MyD88 is recruited to receptor TIR domains, homotypic DD-DD interactions between MyD88 and IRAK family members lead to the formation of an oligomeric DD signaling scaffold. Formation of this signaling scaffold promotes IRAK activation, which is followed by the recruitment and activation of tumor necrosis factor (TNF) receptor–associated factor 6 (TRAF6).

TRAF6 is a RING domain–containing E3 ubiquitin ligase (14). Together with the E2 ubiquitin-conjugating enzyme Ubc13 and a ubiquitin-conjugating enzyme variant, Uev1A, TRAF6 catalyzes the synthesis of Lys63–linked polyubiquitin chains. Formation of K63-linked polyubiquitin enables the recruitment of two downstream kinase complexes. The first is the transforming growth factor–β (TGF-β)–activated kinase 1 (TAK1) complex, which is composed of TAK1 and the TAK1-binding proteins TAB1 and either TAB2 or TAB3 (15–17). The second is the inhibitor of NF-kB (IκB) kinase (IKK) complex, which consists of the catalytic subunits IκKα or IκKβ (or both) and the regulatory subunit NEMO (NF-κB essential modulator; also known as IκKγ). The recruitment of these complexes to polyubiquitin triggers the phosphorylation and activation of their kinase activities. IKK phosphorylates IκB proteins, which are ubiquitinated by the E3 ubiquitin ligase complex Skp1–Cullin–F-box β-transducin repeat-containing protein (SCFβcr) and subsequently degraded by the proteasome. Degradation of IκB enables the entry of NF-kB into the nucleus, where, together with two additional transcription factors, AP-1 and an IRF (interferon regulatory factor), it induces the expression of target genes whose products mediate inflammatory and immune responses (18, 19).

Engagement of the TLR–IL-1R pathway in an appropriate physiological environment initiates the development of protective immune responses. However, the complexity of this pathway renders itself susceptible to interruption and deregulation, leading to its association with many human diseases (20, 21). For example, inherited mutations or polymorphisms in the genes encoding TIR-containing adaptors and IRAKs may cause either extreme sensitivity to or protection from infection (22). Other types of deregulation in the pathway contribute to diseases of the immune system, such as inflammatory disorders (22), autoimmune diseases (23), and allergy (24), as well as other diseases such as cancer (25), insulin
resistance (26), atherosclerosis (27), and painful neuropathy (28). Given that the major components of the TLR–IL-1R pathway have been identified, obtaining the relevant structural information about the core signaling complexes involved could enhance our understanding of the molecular mechanism of signaling and assist the targeting of the pathway by therapeutics. Here, we illustrate the structural features of the TLR–IL-1R signaling pathway and discuss recent advances in the field.

Receptor Clustering and TIR Domain Interactions

In humans, the 10-member TLR family recognizes various PAMPs (29). Each TLR recognizes and binds to its cognate PAMP through an extracellular LRR domain. Agonistic ligand binding triggers signaling by promoting the dimerization of TLRs (30) or by altering the conformation of existing dimers (31). In addition, TLRs partition into lipid raft microdomains upon ligand binding; thus, higher-order oligomers of TLRs may be critical for signal initiation (32, 33). Structural information is available on several dimeric TLR ectodomain-ligand complexes, including TLR4 bound to its co-receptor MD-2 and its ligand lipopolysaccharide (LPS) (34), a TLR3 homodimer bound to double-stranded RNA (dsRNA) (35), heterodimers of TLR2 and TLR1 (36) and TLR2 and TLR6 bound to lipopeptides (37), and TLR5 bound to Salmonella flagellin (38). In each structure, the TLRs adopt similar M-shaped dimers, which brings the C-terminal juxtamembrane ends in close proximity to each other (Fig. 1B, top right). Although the overall conformation of the TLR dimers is similar, each ligand is bound to a distinct surface of the LRR.

Cellular responses to the cytokine IL-1β require a receptor complex containing both IL-1RI and IL-1RACP (29). Initial binding of IL-1β to IL-1RI requires the subsequent association of IL-1RI with IL-1RACP to form an active signaling complex (39). The IL-1β–binding extracellular regions of both receptors contain three immunoglobulin-like domains. The crystal structure of the ternary complex of IL-1β and the extracellular domains of IL-1RI and IL-1RACP has been solved (Fig. 1B, top left) (40). This structure superimposes well with both the structure of the IL-1RI–IL-1β heterodimer (41) and the ternary complex structure containing the TIR-deficient decoy receptor IL-1RII bound to IL-1β and IL-1RACP (42). In the IL-1β–IL-1RI–IL-1RACP ternary complex, the juxtamembrane immunoglobulin-like domains of both receptors are brought in close proximity to each other. All of the TLR and IL-1R family members share a cytoplasmic TIR domain. The TIR domain is small and globular, consisting of a parallel β sheet surrounded by α helices (43). Binding of an agonistic ligand to its receptor is thought to promote homotypic interactions of these domains, creating a nucleation platform to promote oligomerization with TIR-containing adaptor proteins. Several structures of TIR domains are available, including those of TLR1, TLR2 (43), TLR10 (44), the IL-1R family member IL-1RAPL (45), and the TIR-containing adaptors MyD88 (46) and Mal (47). Despite the abundance of structural information about the TIR domain, the nature of TIR domain oligomerization remains elusive. This is in part attributable to the difficulty of reconstituting stable TIR domain multimers in solution. Nonetheless, several models of TIR oligomerization have been proposed, which are based on crystal structures of TLR10 (44) and IL-1RAPL (45), mutagenesis experiments, and molecular docking studies (46, 47).
Oligomeric DD Interactions and IRAK Activation

MyD88, the primary signaling adaptor for TLR and IL-1R signaling, also contains an N-terminal DD, a member of the death domain superfamily, whose additional members include caspase recruitment domains, pyrin domains, and death effector domains. Superfamily members share a common fold that resembles a Greek key bundle of six antiparallel α helices (48). DD-containing proteins are critical components of apoptotic and inflammatory signaling. Through a conserved set of homotypic interactions, DDs form oligomeric molecular scaffolds that are responsible for the activation of proapoptotic caspases (49–51), proinflammatory caspases (52), and kinases.

In the TLR and IL-1R pathways, the DDs of MyD88 associate with the DDs of the IRAK family of serine and threonine kinases (53). The DDs of MyD88 and IRAKs assemble into oligomeric signaling platforms known as the “Myddosomes” (32) (Fig. 1B). The structure of a ternary Myddosome containing the DDs of MyD88, IRAK4, and IRAK2 has been solved (54). The DDs are arranged in a four-layered tower, with six MyD88 DDs forming the top two layers, below which is a layer of four IRAK4 DDs, and finally a layer of four IRAK2 DDs (Fig. 1B). The DDs form a single, left-handed helical scaffold held together by three conserved DD interaction types (55). Shape and charge complementarity between the top and bottom surfaces of each layer confer a sequential assembly order to the Myddosome. This structure explains the observation that the recruitment of IRAK1 to the IL-1R complex is markedly reduced in IRAK4-deficient cells (56, 57). The DD of IRAK4 is monomeric in solution, whereas that of MyD88 is prone to oligomerization at high concentrations (54). These data suggest a strict hierarchical assembly mechanism that begins with ligand binding and receptor clustering. Juxtaposition of receptor TIR domains leads to MyD88 recruitment and DD oligomerization. The MyD88 DD oligomer then serves as a nucleation platform for the recruitment of IRAK4. Only after four IRAK4 molecules are present in the Myddosome can IRAK1 or IRAK2 join the complex.

The DD interactions provide an oligomeric platform for the activation of the IRAKs. In vitro kinase assays have shown that IRAK4 can autophosphorylate itself in addition to phosphorylating the activation loop of IRAK1 (58). MyD88 promotes the phosphorylation of IRAK1 by IRAK4 (59). These data suggest a mechanism for signal propagation in which the initial recruitment of IRAK4 results in its autophosphorylation and activation. Activated IRAK4 is then able to phosphorylate and activate downstream IRAK1 and IRAK2. Phosphorylated IRAK1 or IRAK2 (60) then recruits TRAF6 to the membrane (61) through TRAF6 interaction motifs (62).

**TRAF6 and Polyubiquitin as Key Scaffolds**

The E3 ubiquitin ligase TRAF6 is essential for TLR- and IL-1R–mediated activation of the NF-κB and AP-1 signaling pathways (63). Similar to other TRAFs, TRAF6 contains N-terminal RING and zinc finger (ZF) domains, followed by a C-terminal TRAF domain (Fig. 2A). Crystal structures of the C-terminal TRAF domain of TRAF6 and TRAF2 in complex with TRAF interaction motif peptides revealed trimeric complexes (Fig. 2B) (62, 64), similar to the oligomerization state of TNFR1 bound to its ligand (65). In contrast, the N-terminal region of TRAF6 is unexpectedly dimeric, both in the crystal lattice and in solution, with the RING and ZF domains arranged linearly in a rigid, golf club–like conformation (66, 67) (Fig. 2C). The RING and ZF domains mediate K63-linked polyubiquitination that is essential for NF-κB activation. Dimerization of TRAF6 is critical for its E3 ligase activity, because mutants defective in dimerization are compromised in the assembly of polyubiquitin and the ability to promote IκB phosphorylation (67).

The recruitment of TRAF6 to TLR or IL-1R signalosomes through IRAK1 or IRAK2 promotes the oligomerization of TRAF6 and its E3 ubiquitin ligase activity (68). TRAF6, in conjunction with the E2 enzyme Ubc13 and the E2 variant Uev1A (Fig. 2C), catalyzes K63-linked polyubiquitination of target proteins, including IRAK1, NEMO, and TRAF6 itself. Lys124 of TRAF6 is the major site of auto-polyubiquitination (69), and a point mutation in this residue abolishes...
the ability of TRAF6 to activate TAK1 and IKK. The structures of a complex of Ubc13 and Uev1A with and without ubiquitin have been reported (70–72) (Fig. 2C). The ubiquitin moiety is covalently linked to the active site residue of Ubc13. In addition, K63 of ubiquitin is bound to the acceptor-binding site of an adjacent complex, revealing the mechanism by which Uev1A positions the acceptor ubiquitin to promote formation of K63-linked polyubiquitin chains (71). The structures of the TRAF6-Ubc13 complex and the Ubc13-Uev1A-ubiquitin complex enable the generation of the model of the quaternary complex of TRAF6, Ubc13, Uev1A, and ubiquitin (Fig. 2C).

Given the aggregation properties of TRAF6 in vitro and in cells, a two-dimensional (2D) lattice model for the infinite oligomerization of TRAF6 was proposed, based on the symmetry mismatch between the dimeric N-terminal region and the trimeric C-terminal region (Fig. 2D) (67). Indeed, endogenous TRAF6 undergoes patch formation at the cell surface in response to TLR stimulation, as shown by immunofluorescence, and disruption of TRAF6 dimerization compromises the ability of TRAF6 to aggregate, as shown by fluorescence resonance energy transfer experiments (67). This proposed 2D lattice of TRAF6 facilitates polyubiquitin synthesis on proteins in the complex, such as IRAK1 and TRAF6 itself, which in turn promotes recruitment of downstream signaling proteins such as the TAK1 and the IKK complexes through interactions with polyubiquitin chains (73).

Interestingly, bacterial chemosensing receptors cluster at distinct regions of the cell and form stable ternary complexes with the histidine autokinase CheA and the adaptor protein CheW as trimer-of-dimer arrays (74) in a manner that is reminiscent of the proposed 2D lattice of TRAF6. This structural parallel may translate into a mechanistic parallel in which both processes are highly cooperative as a result of the high-order oligomerization of the participating proteins (75).

**Recruitment and Activation of the TAK1 and IKK Complexes**

TAK1 (also known as MAP3K7 and MEKK7) plays critical roles in signaling pathways stimulated by TGF-β, IL-1, TLR, TNF-α, LPS, and IL-8 (76, 77). The TAK1 complex contains TAK1 and three kinase-binding proteins (TAB1 to TAB3) (78). The activation of TAK1 requires TAB1, because TAK1 has no observable kinase activity when expressed alone but is active when coexpressed with TAB1 (79, 80). The TAK1-TAB1 complex is crucial for normal embryonic development and morphogenesis of the heart and lung (81). The structure of the complex of TAK1 and TAB1 reveals an extensive interface between a binding pocket on the C-terminal lobe of the kinase domain of TAK1 and an α helix of TAB1 (82). This interaction promotes autophosphorylation of the kinase activation loop of TAK1 (83, 84), likely through an allosteric mechanism (82).

The TAK1-binding proteins TAB2 and TAB3 facilitate TAK1 activation in the TLR–IL-1R signaling pathway by binding to K63-linked polyubiquitin chains generated by TRAF6. TAB2 and TAB3 have similar domain structures, containing an N-terminal ubiquitin-binding domain (CUE), a coiled-coil (CC) region, a TAK1-binding domain, and a C-terminal Npl4 zinc finger (NZF) domain (85). The NZF domain binds to K63-linked polyubiquitin chains much more strongly than it does to K48-linked chains or to linear polyubiquitin (86). The crystal structures of the NZF domains of TAB2 and TAB3 bound to K63-linked diubiquitin have been reported (87, 88). The NZF domains of TAB2 and TAB3 share 79.3% sequence identity and
are functionally redundant (89). Predictably, the structures of the NZF domains of TAB2 and TAB3 bound to diubiquitin are nearly identical. TAB2-NZF binds to K63-linked diubiquitin through two distinct binding sites on adjacent ubiquitin moieties, and multiple NZF domains can recognize longer polyubiquitin chains. The structures reveal that TAB2 preferentially binds to K63-linked chains because it imposes a conformational constraint to the bound ubiquitin chain that cannot be adopted by the linear linkage (88). Presumably, recruitment of the TAK1 complex to the TRAF6-polyubiquitin scaffold brings the kinases into proximity to promote autophosphorylation and activation. Active TAK1 can in turn phosphorylate and activate IKK.

The IKK complex was first identified in HeLa cells and is composed of the catalytic subunits IKKα or IKKβ (or both) and the regulatory subunit NEMO (IKKγ) (90, 91). IKK phosphorylates IkB, tagging it for K48-linked ubiquitination and proteasomal degradation, which enables the nuclear translocation of NF-κB and the expression of target genes (18). IKKα and IKKβ share 52% identity. The IKK complex can consist of an IKKα-IKKβ heterodimer or of homodimers of IKKα and IKKβ (92) associated with NEMO. The C-terminal ends of IKKα and IKKβ contain the NEMO-binding domain (93, 94), which interacts with the N-terminal kinase-binding domain of NEMO (18, 95). The crystal structure of this interacting complex showed that IKKβ and NEMO form a heterotetramer with two molecules of each protein (96). The four molecules pack into a parallel four-helix bundle (96).

The crystal structure of the almost full-length form of IKKβ showed that it contains three recognizable domains: the KD, the ubiquitin-like domain (ULD), and an elongated α-helical scaffold/dimerization domain (SDD) (97) (Fig. 3, A and B). The three domains interact to form a trimodular architecture. Whereas the ULD domain appears to be important for IKKβ activity, the SDD domain mediates IKKβ dimerization and participates in substrate recognition (97). Full-length IKKβ is dimeric in solution, and mutations of the SDD domain disrupt this dimerization (97). Although SDD-mediated dimerization is critical for IKKβ activation but not for its kinase activity, the observed IKKβ dimer does not assume an arrangement that likely facilitates intradimer trans-autophosphorylation. This suggests a role for higher-order oligomerization in the activation of IKKβ. Indeed, IKKβ exists as dimers of dimers in the observed crystal forms, in which the activation loop is potentially within reach of the active site of a neighboring kinase (97). However, this tetramer of IKKβ alone, which is possibly transient, is not observed in solution.

While NEMO mediates the interaction between the IKK complex and a viral IKK activating protein (vFLIP) through the HLX2 domain, it mediates the interaction between the IKK complex and polyubiquitin through the UBAN (ubiquitin binding in ABIN and NEMO) and its C-terminal ZF domain (73, 98–101). The UBAN domain of NEMO binds to linear polyubiquitin chains with higher affinity than it does to K63-linked chains (102, 103). The crystal structures of the UBAN domain alone and in complex with linear diubiquitin and K63-linked diubiquitin have been determined (102–104) (Fig. 3C). In both cases, the UBAN domain forms a parallel, dimeric coiled coil that extends about 130 Å in length. UBAN binds to linear diubiquitin through a conserved hydrophobic patch and the C-terminal tail of the distal ubiquitin and an adjacent surface on the proximal ubiquitin. However, the UBAN domain does not make simultaneous contacts with both moieties of K63-linked diubiquitin. This explains the much lower affinity that NEMO has for K63-linked diubiquitin (104) and provides evidence for the activation of IKK by polyubiquitin. In contrast, the affinity of K63-linked polyubiquitin for UBAN and the C-terminal ZF domain is higher than that for UBAN alone by a factor of ~115 (105). Addition of the ZF domain

---

**Fig. 4.** Overview of TLR and IL-1R signaling. Ligand binding induces both dimerization and higher-order oligomerization of TLRs (blue, top). MyD88 (red) is recruited to the receptors through homotypic TIR-TIR interactions and facilitates Mydosome assembly with IRAK4 (green) and IRAK1 or IRAK2 (orange) through homotypic DD-DD interactions, which leads to phosphorylation and activation of IRAK. Activated IRAK1 or IRAK2 interacts with TRAF6 and promotes the formation of an extended 2D lattice, stimulating the K63-linked ubiquitin ligase activity of TRAF6. Polyubiquitin (gray) is recognized by TAB2 or TAB3 (purple) and NEMO (pink) to enable the recruitment and activation of the kinases TAK1 (purple) and IKK (teal). Activated IKK phosphorylates IkB, promoting its degradation and enabling the nuclear translocation of NF-κB.
has no effect on the affinity of the UBAN domain for linear polyubiquitin. It is likely that both K63-linked and linear polyubiquitin play important roles in different IKK pathways. A model for the full-length IKK complex was proposed as an elongated, flexible dimeric coiled coil structure of NEMO bound to IKKβ (73) (Fig. 3C).

Given that higher-order oligomerization, such as tetramerization, of NEMO has been observed and is important for IKKβ activation (96, 106), it is reasonable to speculate that the IKK complex may be a tetramer or even a higher-order oligomer. A logical scenario might be that in the unstimulated state, the IKK complex is a transient high-order oligomer, which leads to a basal amount of IKK activation and associated NF-kB activity. During TLR or IL-1R signaling, the IKK oligomers may be stabilized through their interaction with K63-linked polyubiquitin chains in the proposed 2D lattice of TRAF6, which leads to robust IKK activation and enhanced NF-kB transcriptional activity.

**Conclusion**

Signal transduction has traditionally been perceived as a linear string of recruitment and allosteric events that transfer and amplify the receptor activation signal to generate the appropriate cellular responses. Surprisingly, for TLR and IL-1R signaling, structural studies are challenging this conventional view. First, it is now known that large oligomers are involved in signaling by these receptors. Some of these oligomers are higher-order but defined, whereas others are open-ended. Oligomerization appears to occur at all different levels of the signaling cascade, from receptors to adaptors, from ubiquitin ligases to kinases, and eventually to the activation of the transcriptional responses of the pathways. Second, instead of being composed of successive signaling complexes, multiple signaling oligomers combine to form gigantic signalosomes (Fig. 4) in which multiple reactions, such as ubiquitination and phosphorylation, can occur simultaneously.

At least three types of intracellular oligomeric scaffold may cooperate in the formation of the TLR or IL-1R signalosomes: (i) the helical DD assembly scaffold, (ii) the infinite TRAF6 aggregation scaffold, and (iii) the polyubiquitin chain scaffold (Fig. 4). Coalescence of these intertwined interactions into gigantic signalosomes may provide at least two implications for signal transduction. First, the intrinsic coopera-

**References and Notes**


Acknowledgments: We apologize for incomplete citations of original work because of space limitations.

Funding: Supported by NIH grants R01AI045937, and R01AI079260.

Citation: R. Ferrao, J. Li, E. Bergamin, H. Wu, Structural insights into the assembly of large oligomeric signalosomes in the Toll-like receptor–interleukin-1 receptor superfamily. Sci. Signal. 5, re3 (2012).