Structural Insight into the Mechanism of Activation of the Toll Receptor by the Dimeric Ligand Spätzle

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The Drosophila Toll receptor, which functions in both embryonic patterning and innate immunity to fungi and Gram-positive bacteria, is activated by a dimeric cytokine ligand, Spätzle (Spz). Previous studies have suggested that one Spz cross-links two Toll receptor molecules to form an activated complex. Here we report electron microscopy structures of the Toll ectodomain in the absence and presence of Spz. Contrary to expectations, Spz does not directly cross-link two Toll ectodomains. Instead, Spz binding at the N-terminal end of Toll predominantly induces the formation of a 2:2 complex, with two sites of interaction between the ectodomain chains, one located near to the N terminus of the solenoid and the other between the C-terminal juxtamembrane sequences. Moreover, Toll undergoes a ligand-induced conformational change, becoming more tightly curved than in the apo form. The unexpected 2:2 complex was confirmed by mass spectrometry under native conditions. These results suggest that activation of Toll is an allosteric mechanism induced by an end-on binding mode of its ligand Spz.

Toll and Toll-like receptors (TLRs)4 are essential for innate immunity in both vertebrates and invertebrates. They are activated by a variety of molecules derived from microbial pathogens ranging from peptides, lipids, and carbohydrates to nucleic acids (1). These pathogen-associated molecules are recognized either directly or indirectly by the extracellular domain, or ectodomain, of these receptors (2). In the case of Drosophila Toll, peptidoglycan from Gram-positive bacteria stimulates the production of an endogenous protein ligand, Spz, which then binds to the Toll ectodomain and establishes signal transduction (3). The Toll and TLR ectodomains consist of leucine-rich repeats (LRRs) that form an extended solenoid flanked by N- and C-terminal capping structures. The ectodomains are linked to a cytoplasmic signaling module (the TIR domain), by a single transmembrane segment (4, 5). The Drosophila Toll protein differs from the vertebrate TLRs, since the ectodomain contains two blocks of terminally capped LRRs that are separated by a linker region. The first block has 19 LRRs, but the second has only three repeats.

The Spz ligand is a homodimer with a cystine knot structure similar to that of vertebrate nerve growth factor (6, 7). Since Spz has two equivalent, symmetry-related binding sites, it was proposed to dimerize two Toll molecules during the signaling process. Previous studies using biophysical methods showed that Spz binds with high affinity to soluble Toll ectodomain in solution (8). These studies also indicated a two-step binding reaction with initial formation of a 1:1 complex consisting of one Spz dimer and one Toll ectodomain. The second binding event was proposed to lead to the formation of a cross-linked complex of Spz with two receptor ectodomains that can activate transmembrane signal transduction (9). This work also showed that the complex is stabilized by both receptor-ligand and receptor-receptor interactions. In particular, the immediate juxtamembrane sequences of the ectodomain mediate interchain contacts in the activated complex (10).

In this paper, we report the electron microscopy structures for isolated Toll ectodomain (at 22 Å resolution) and Toll-Spz complexes of 1:1 and 2:2 stoichiometries (at 30 and 32 Å resolution, respectively). We also carried out mass spectrometry studies that revealed that the 2:2 complex was predominant over the 2:1 described earlier (9). We show that Toll dimerization induced by Spz is indirect. Binding of Spz triggers a conformational change in Toll, which in turn allows two discrete regions of the receptor ectodomains, one near the N termini and the other involving the juxtamembrane sequences, to interact.
Spätzle Binding Mode to Drosophila Toll Receptor

MATERIALS AND METHODS

Toll Ectodomain Preparation—Toll wild-type ectodomain expression and purification has been described elsewhere (8). Briefly, for baculovirus expression, a 3-liter suspension culture of SF9 cells at a density of 1 × 10⁶ cells/ml in serum-free SF900 medium (Invitrogen) was infected at a multiplicity of infection of 10.0 with virus expressing Toll ectodomain (Met¹–Ala⁸⁰¹). The supernatant was harvested 2 days postinfection after removing the cells by centrifugation at 3000 rpm for 10 min and filtered on a Sartobran P sterile capsule of 0.45 µm (Sartorius). The supernatant was concentrated with a Centramate tangential flow filtration system (Pall Filtron) in 500 ml of buffer A (300 mM NaCl, 20 mM Tris-Cl, 20 mM imidazole, pH 7.5), centrifuged at 10,000 rpm for 1 h to remove debris, and loaded on a 5-ml Ni²⁺-nitrilotriacetic acid Superflow column (Qiagen) by absorption at 280 nm. The yield of the reverse phase step was 106 cells/ml in serum-free SF900 medium (Invitrogen) was infected at a multiplicity of infection (1.0) and harvested 2.5 days postinfection. The supernatant was harvested 2 days postinfection after removing the cells by centrifugation at 3000 rpm for 10 min and filtered on a Sartobran P sterile capsule of 0.45 µm (Sartorius). The supernatant was concentrated with a Centramate tangential flow filtration system (Pall Filtron) in 500 ml of buffer A containing 250 mM imidazole. Peak fractions were pooled and purified by size exclusion chromatography on HiLoad 16/60 Superdex 200 column (GE Healthcare) at 1 ml/min in buffer C: 150 mM NaCl, 20 mM Tris-Cl, pH 7.5. Fractions were analyzed by Coomassie-stained SDS-PAGE. Protein concentration was quantified by absorption at 280 nm. A typical yield was 10 mg of purified protein per liter of cell culture.

Spz C106 Preparation—We have used the N-His-TEV-C106 construct (11) that contains a His tag and a TEV protease cleavage site between the N-terminal pro-domain (Met¹–Arg¹⁶⁴) and the C-terminal active fragment C106 (Val¹⁶⁵–Gly²⁷⁰). Briefly, 6-liter suspension cultures of SF9 cells were infected at a multiplicity of infection of 1.0 and harvested 2.5 days postinfection. The supernatant was processed as described for the Toll ectodomain and yielded 3 mg/liter purified Spz full-length protein. Spz was then incubated overnight at 4 °C with recombinant TEV protease (12) at a 1:10 ratio. The pro-domain was separated from C106 by reverse phase chromatography on a C8 column of 25-cm length and 4.6-mm diameter (HiChrom) at 3 ml/min using a Varian HPLC. The sample was adjusted to 10% acetonitrile, 0.1% trifluoroacetic acid prior to injection. Elution was performed in a gradient from 10 to 50% acetonitrile, 0.1% trifluoroacetic acid over 50 min. Fractions corresponding to pure Spz C106 were pooled and lyophilized overnight in an Eppendorf concentrator 5301. Spz C106 protein was then dissolved in 50 mM NaCl, 100 mM Tris-Cl, pH 7.5, and quantified by absorption at 280 nm. The yield of the reverse phase step was ~10%. The 6-liter baculovirus/insect cell Spz preparation provided about 2 mg of purified Spz protein.

Toll-Spz Complex Preparation—Different ratios were tested for Toll-Spz complex preparation. A 3-fold molar excess of Spz compared with Toll was found to be optimal in terms of sample homogeneity. Indeed, Spz was readily separated from the complex by size exclusion chromatography given the difference in size and shape of the proteins. In contrast, it was found that unliganded Toll and Toll-Spz complex co-eluted. Typically, 2 mg of Spz C106 were mixed with 3 mg of Toll ectodomain. The purified complex was concentrated to 200 µl (17.4 mg/ml) and purified by size exclusion chromatography on Superdex 200 10/300 GL column (GE Healthcare) at 0.5 ml/min in buffer C. The complex isolated from the C106 excess was concentrated to 1 mg/ml for structural characterization.

Electron Microscopy—Three-dimensional structures were reconstructed using transmission electron microscopy and single particle analysis as described by Sun et al. (13). Briefly, the proteins were diluted into a solution of 20 mM Tris-Cl, pH 7.5, and 150 mM NaCl and then adsorbed to freshly glow-discharged carbon-coated copper grids. Following staining with 1% uranyl acetate solution, the grids were examined using a JEOL 1200 transmission electron microscopy operated at 100 kV at a calibrated magnification of ×39,000. Electron micrographs were digitized using an Epson Projection 3200 scanner at 1200 dpi, corresponding to 5.5 Å/pixel at the specimen level. Three-dimensional reconstructions were performed using the EMAN software package (14). For each sample, more than 3000 particles were selected using the BOXER routine, filtered, and centered. The class averages, with no assumed symmetry, were calculated by using the “refine2d” routine of the EMAN program. An initial three-dimensional model was reconstructed with no assumed symmetry. The reconstructions were iteratively refined until the structure was stable as judged by Fourier shell correlation. Three-dimensional reconstructions were visualized using the UCSF Chimera software package (15).

Cryo-EM data were collected at the National Center for Macromolecular Imaging at Houston. Briefly, 2.5 µl of purified Toll-Spz complex solution (0.2 mg/ml in 20 mM Tris, pH 7.5, 150 mM NaCl) was applied to a glow-discharged holey carbon grid (Quantifoil R2-1), blotted with filter paper, and flash-frozen with liquid ethane. A JEOL 2010F electron microscopy (JEOL, Tokyo, Japan) was used for data collection with a Gatan model 626 cryostage (Gatan, Pleasanton, CA). Each area was imaged twice on a 4000 × 4000 CCD camera (Gatan US4000) at ×60,000 magnification, corresponding to 1.81 Å/pixel.

The images used to select particles semiautomatically with the “boxer” program of the EMAN software package. The coordinates of the particles were then applied to the closed-to-focus image. The “Alignhuge” program was used to align the two images. More than 400 particles were selected from 10 independently obtained images to generate class averages.

Molecular Modeling—In the absence of high resolution x-ray crystal structures for Spz C-106 and Toll, computational modeling was performed to provide a most reasonable approximation of the respective structures that were also compatible with the electron density maps obtained for the proteins. The structure templates for the target proteins used for homology modeling were searched using a profile hidden Markov model-based algorithm HHpred (16) and a threading algorithm Spark (17). The statistically most significant alignments from both methods were evaluated and used as basis for rigorous pairwise sequence alignment with the aid of a profile-based approach using PRALINE (18) and manual refinement.

The refined sequence alignments of Spz and Toll ECD were used to direct a three-dimensional modeling of the protein structures with a modeling software, Modeler (19), which generated a model including both conserved regions and loops and subsequently refined it with built-in energy minimization fea-
tures. Individual domains of the Toll ectodomain that had a high level of similarity matches with different crystal structures were modeled separately and subsequently combined in silico based on the shape of the Toll ectodomain determined by transmission electron microscopy negative staining. The manipulation of the fragments with reference to the EM structure was performed using the modeling feature of Chimera (15). The gaps between the domains were joined to form continuous loops using the Modloop program (20).

The Spz protein is known to exist as a dimer that interacts with one Toll receptor. The modeling of the Spz dimer was achieved by manually docking two monomers according to the dimeric conformation of its closest structural homolog human neurotrophin 3 (21) and further verified using a fast Fourier transformation docking method (22). The complex model of a Spz dimer with the N-terminal end of Toll was manually docked based on the negatively stained EM structure. As per the EM study, two monomers of Toll could be docked to form a dimer that interacts at the C termini. The known dimeric conformation of CD14 (23) was used to direct the manual docking of Toll ectodomain in the process of fitting the structures to the EM density maps.

**Mass Spectrometry**—Toll ectodomain alone, and the Toll-Spz complex were equilibrated in 200 mM ammonium acetate, pH 7.0, using Bio-Spin columns (Bio-Rad). Where indicated, DMSO was added to 10% (by volume) after buffer exchange. Nanoflow electrospray ionization capillaries were prepared as described previously (24).

Ion mobility mass spectrometry (IM-MS) was performed on a Synapt HDMS Q-IMS-ToF-type instrument (25). Other mass spectra were acquired on a Q-Star XL mass spectrometer (MDS-Analytical Technologies) and on a Q-ToF 2 mass spectrometer (Waters), both of which were adapted for high mass operation by means of a reduced frequency quadrupole (26).

For the Q-Star XL, a pressure-restricting sleeve around the first quadrupole ion guide was used to aid the radial focusing of high mass ions (27). A similar effect was achieved on the Synapt and Q-ToF2 by throttling the pumping efficiency of the first vacuum stages. Typically, nanoflow electrospray ionization was performed using 2–3 μl of aqueous protein solution, using a capillary voltage of 1.5–1.8 kV, and with the aid of a backing pressure of ~0.5 bar. [CsI]n+ clusters were used as an external calibrant. Drift time versus m/z data were analyzed in Driftscope 1.0/Masslynx 4.1 (Waters). Q-Star XL data were acquired using Analyst 1.0 software (MDS-Sciex) and exported to MassLynx 4.0 (Waters) for processing. Q-ToF 2 data were acquired and analyzed using MassLynx.
Toll–Spz complex, resulting in a structure at 30 Å resolution. The observed complexes have masses, at the peak apexes, of 132,802 Da (Toll–Spz), and 265,733 ± 642 Da (Toll2Spz2). Minor overlapping signals for Toll, and Toll–Spz, were also observed, which were more abundant in the absence of DMSO; these ions were resolved and identified by IM-MS (see supplemental materials). MS of Toll1 alone (see supplemental Fig. S5) gave a mass of 108,342 Da, compared with 90.1 kDa predicted from the unglycosylated amino acid sequence.

Due to the broad peaks in the spectra, the observed masses were measured both at the peak apexes (boldface type) and at the half-height peak onsets (italic type); these were as follows: Toll monomers in 200 mM NH₄OAc = 108,342 ± 155 Da (107,052 ± 284 Da); the Toll–Spz 1:1 complex in 200 mM NH₄OAc = 133,030 ± 87 Da (131,519 ± 87 Da), in 10% DMSO = 132,802 ± 131 Da (131,286 ± 166 Da); the Toll–Spz 2:2 complex in 200 mM NH₄OAc = 266,799 ± 99 Da (peak onsets insufficiently resolved), and in 10% DMSO = 265,733 ± 642 Da (262,995 ± 686 Da).

RESULTS

Electron Microscopy of Toll and Toll–Spz Complexes—To understand the mechanism of signal transduction by the Toll receptor in more detail, we visualized the Toll ectodomain under conditions that retain native materials. This analysis has the advantage of not relying on the use of stains and can result in more accurate measurements of the dimensions of molecules (Fig. S1). Of the particles observed, there is good agreement with the structure of the negatively stained 2:2 Toll–Spz complexes (Fig. S4).

To further confirm the stoichiometry seen in the reconstructions, we analyzed Toll in its apo form and the Toll–Spz complex by mass spectrometry under conditions that retain native contacts in noncovalent complexes (31). Experiments were carried out at protein concentrations between 1 and 10 μM. The Toll ectodomain was predominantly found as a monomer (108.2 kDa), although at higher concentrations, dimers were also observed (216.6 kDa) (data not shown). The spectra of the Toll ectodomain but with an additional electron density corresponding to the bound Spz molecule that can be seen at the broader end of the Toll solenoid (Figs. 1F, S1, and S2). Spz has been assigned by genetic and biochemical studies to bind to the N terminus of Toll (29). Interestingly, Spz binding revealed a notch in the solenoid that could correspond to the junction of the two LRR blocks in Toll (Fig. 1F, right-hand panel). To gain further insights into the arrangement of the molecules, we docked models of Toll and Spz into these low resolution structures (Fig. 1, E and F) (see “Materials and Methods”). This shows that the elongated Spz dimer binds to the Toll solenoid in an end-on configuration, although at this resolution, we are not able to determine the orientation of the Spz molecule. The appearance of this feature suggests that the ectodomain undergoes a twist, transmitted from the N-terminal cap, the site of Spz binding, toward the C-terminal juxtamembrane region.

Next we reconstructed images of the Toll–Spz ternary complex. Contrary to expectations, the reconstructed Toll–Spz ternary complex did not contain one Spz dimer and two Toll ectodomains. Instead, the reconstructed Toll–Spz dimer shows a symmetrical, 2:2 complex stabilized by two regions of protein–protein interaction between the receptor chains (Figs. 1G, S1, S2, and S3). The first of these is close to the N terminus, probably in the first LRR block, and involves the lateral rather than the concave or convex surfaces of the solenoids. The second region is at the C terminus, as predicted by published studies (9, 30). This result suggests that the transmembrane segments of the receptor chains will be forced into close proximity during the signaling process.

The Toll–Spz Signaling Complex Has a 2:2 Stoichiometry—Given the unexpected arrangement of the 2:2 Toll–Spz complex, we imaged its structure using cryoelectron microscopy. This analysis has the advantage of not relying on the use of stains and can result in more accurate measurements of the dimensions of molecules (Fig. S1). Of the particles observed, there is good agreement with the structure of the negatively stained 2:2 Toll–Spz complexes (Fig. S4).
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Toll-Spz complex show two series of ions; the first series is predominantly due to the Toll-Spz 1:1 heterodimer, with a mass of 132 kDa, whereas the second is predominantly due to the Toll-Spz 2:2 complex, with a mass of 266 kDa (Figs. 2 and S5). These ion series overlapped with minor signals from Toll monomers lacking Spz and also from the 2:1 complex. These overlapping ion series were revealed by IM-MS, which separates ions first by their cross-sectional shape and charge and then subsequently by mass/charge (32) (Fig. 3). The IM-MS analysis revealed heterogeneity in the Toll1- and Toll2-containing complexes (Fig. 3). Therefore, the ions were collisionally activated in the transfer region (post-IM separation) to maximize desolvation but with minimum activating voltages (prior the IM cell) to minimize gas phase conformational changes. In the case of the Toll1-containing group, the ions were resolved in their drift time versus m/z trends into a major Toll1:Spz2 component (Fig. 3B, iii) and a minor, less well resolved, Toll2:Spz1 component (Fig. 3B, iv).

It is interesting to note that the Toll1 and Toll1:Spz1 ions show bimodal arrival time distributions in the ion mobility cell (indicated by the vertical axis of Fig. 3B), whereas the Toll1:Spz2 and Toll2:Spz1 ions show unimodal distributions. This observation indicates the existence of two conformations of the Toll1-containing ions in the gas phase. Using this two-dimensional approach, the different populations within these ion series could be resolved, confirming the 2:2 complex as the predominant stoichiometry. This result is consistent with the observed conformational change observed in the EM reconstructions.

Attempts to measure stoichiometry from collision-induced dissociation products in tandem mass spectra were unsuccessful. Covalent fragmentation products from oligosaccharides were observed at high collision energies, but dissociation of intact protein subunits was not observed, even at the highest collision energies available. This may reflect a high degree of internal disulfide bonding within both the Toll and C106 subunits, which could stabilize the complexes against an unfolding-driven gas phase dissociation process (33, 34).

Spz Binding Induces a Change in the Curvature of the Toll Solenoid—
Superimposition of the EM envelopes for the ectodomains and the 1:1 complex reveals that the ectodomain solenoid in the latter has a tighter curvature (Fig. 4). Assembly of two 1:1 heterodimers may induce further changes in the curvature and relative orientation of the ectodomain, and these conformational changes may explain the observed cooperativity of the signaling process (10). In this regard, recent experimental data (35) and molecular dynamics simulations of the TLR3 ectodomain crystal structure and TLR2 and -4 ectodomain models suggest that ligand binding may induce a switch from a relaxed, lower energy state to a tighter, higher energy conformation.5

DISCUSSION

The low resolution structural study reported here reveals an indirect allosteric mechanism of signal transduction by the Toll receptor. Previous work using chemical cross-linking and isothermal titration calorimetry suggested that the signaling complex consisted of one Spz dimer and two receptor chains (9). We have reexamined these data and find that they are consistent with both a 2:1 and a 2:2 complex. Furthermore, the

5 A. N. Weber, submitted for publication.
cryo-EM analysis and the nondissociating mass spectrometry reported here confirm that the predominant complex is a heterotetramer of two Toll receptor molecules and two Spz molecules. A recent paper describes the structure of a heterodimer containing human TLR1 and 2 fusion proteins cross-linked by a triacylated lipid (36). In this case, two acyl chains of the lipid bind into a cavity in the LRR solenoid of TLR2 with the third chain associating with a hydrophobic cleft on the surface of TLR1. Binding of the lipid induces the formation of extensive protein–protein interactions between the lateral surfaces of the TLR1 and -2 solenoids (Fig. 5A). In addition, the C termini of the ectodomains are brought into close proximity (≈8 Å), although this arrangement is nonnative, since the structural analysis used truncated ectodomains fused to heterologous VLR sequences. The topology of the TLR1 and -2 ectodomains is similar to that described here for Toll and Spz in that association of the ligand promotes interactions between the lateral surfaces of the receptor ectodomain (Fig. 5B).

Our complex formed from native proteins shows that the C termini of the two Toll ectodomains are closely associated. This observation confirms previous proposals that initial binding of Spz releases a constitutive autoinhibition and allows dimerization of the juxtamembrane sequences of the Toll ectodomain (30). This arrangement is also seen in CD14 dimers that interact through the C termini of two LRR ectodomains (23). It is likely that activation of TLR4/MD-2 by endotoxin also involves induced interactions between lateral surfaces of two ectodomain molecules. In other work, we have shown that specific residues in LRRs 14–17 are required for signaling.6 Furthermore, docking studies predict that two heterodimers of TLR4 and MD-2 (37) will form a heterotetramer stabilized by both receptor-receptor interactions between the lateral surfaces of TLR4 at LRR14–17 and extended TLR4-MD-2 contacts (Fig. 5C). Thus, the association of lateral surfaces of the receptor solenoid induced by binding of ligand appears to be a common feature of Toll and TLR activation.

The indirect activation mechanism we propose here for Toll has features in common with that of the epidermal growth factor (EGF) receptor. The EGF receptor has four subdomains and is activated by a dimeric, cystine knot ligand, EGF. EGF binds to the first and the third of the subdomains causing a conformational change that enables a homotypic protein–protein interaction to occur between subdomain II of two receptor molecules. Mutagenesis studies are under way to characterize further the allosteric mechanism of Toll activation by N-terminal end-on binding of Spz. As with Spz, EGF binds to EGFR in a 2:2 stoichiometry in the active complex (38–40). It is interesting to note that the anti-cancer biologic Herceptin inhibits EGF signaling in breast cancer by binding to the site of receptor-receptor interaction (41).

In conclusion, this work reveals a novel and unexpected mode of dimerization for the Drosophila Toll receptor induced indirectly by an endogenous protein ligand. This basic mechanism may be conserved in human TLRs and could be the basis for therapeutic intervention.

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