Ligand-Receptor and Receptor-Receptor Interactions Act in Concert to Activate Signaling in the Drosophila Toll Pathway* ♦

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In Drosophila, the signaling pathway mediated by the Toll receptor is critical for the establishment of embryonic dorso-ventral pattern and for innate immune responses to bacterial and fungal pathogens. Toll is activated by high affinity binding of the cytokine Spätzle, a dimeric ligand of the cysteine knot family. In vertebrates, a related family of Toll-like receptors play a critical role in innate immune responses. Despite the importance of this family of receptors, little is known about the biochemical events that lead to receptor activation and signaling. Here, we show that Spätzle binds to the N-terminal region of Toll and, using biophysical methods, that the binding is complex. The two binding events that cause formation of the cross-linked complex are non-equivalent: the first Toll ectodomain binds Spätzle with an affinity 3-fold higher than the second molecule suggesting that pathway activation involves negative cooperativity. We further show that the Toll ectodomains are able to form low affinity dimers in solution and that juxtacrine sequences of Toll are critical for the activation or derepression of the pathway. These results, taken together, suggest a mechanism of signal transduction that requires both ligand-receptor and receptor-receptor interactions.

The Toll family of type I transmembrane receptors mediate cellular signal transduction pathways that are central to innate immune responses in both vertebrates and invertebrates (1). These receptors are responsible for detecting microbial molecular patterns, for example bacterial lipoproteins and lipopolysaccharides, and activating a cellular defense response. The overall structure of Toll receptors is evolutionarily conserved, with characteristic ectodomains consisting mainly of sequences of leucine rich repeats (LRRs)1 flanked by cysteine-rich capping structures and an intracellular TIR (Toll/IL-1R) domain (2) (see Fig. 4B). Despite this structural conservation, different mechanisms have arisen for the detection of pathogen patterns in invertebrates and vertebrates (3). In insects, for example, peptidoglycan is bound by a specific recognition protein and this complex then stimulates a cascade of serine proteases the terminal member of which processes an inactive proprotein form of the cytokine Spätzle to form an active protein ligand (4–6). By contrast, in mammals Toll-like receptors are thought to directly interact with microbial compounds (7, 8). Despite these differences, Toll receptors probably require ligand-induced dimerization or cross-linking to establish intracellular signal transduction, a feature shared with other type I transmembrane receptors (9). Such a mode of action was recently illustrated for the founding member of the family, Drosophila Toll (5).

In Drosophila, the Toll receptor is involved in dorso-ventral patterning of the embryo as well as innate immune responses (10, 11). For example, female flies carrying loss-of-function mutations in Toll lay eggs that develop into embryos in which all cells adopt dorsal fates. In addition, these flies fail to express antimicrobial peptides in response to infection by Gram-positive bacteria and fungi and poorly resist these infections. Both processes require the same post-receptor components and the activating Spätzle ligand. However, differently regulated protease cascades are responsible for ligand generation in embryonic development and innate immune responses in adult flies (12). In the 1990s, genetic studies identified two classes of Toll gain-of-function alleles that cause a dominant ventralization of the embryo (13). The first class (type I mutants) are point mutations in the extracellular domain of Toll involving specific cysteine residues in the juxtacrine, cysteine-rich flank of the second block of LRRs. These cysteine residues are thought to be involved in disulfide bonds and disruption of these is believed to cause constitutive activation of the receptor. The second class of mutations (type II mutants) produces truncated ectodomains that are secreted from the embryo. The available evidence suggests that these mutations act by binding and then redistributing active Spätzle ligand in the perivitelline space of the embryo, causing inappropriate activation of receptors located at lateral and dorsal positions.

The basic principle that signal transduction by Type I transmembrane receptors requires dimerization is long established (9). This has led to an expectation that active signaling complexes should be structurally symmetrical and that the sites of ligand interaction would be essentially equivalent. However, more recent structural and biophysical studies suggest that the function as a function of sedimentation coefficient; c(M), concentration as a function of molecular mass.

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events leading to ligand binding are complex and that some signaling assemblies are in fact asymmetrical. For example, cross-linking of the epidermal growth factor (EGF) receptor requires binding of two monomeric EGF molecules. After formation of ligand-receptor complexes, subsequent receptor dimerization may involve either second separate sites on ligand and receptor or a conformational rearrangement in the receptor allowing self-association (14). The latter view has gained considerable support from structural analysis of EGF-receptor complexes (15–17). In another example, structures of the fibroblast growth factor (FGF) receptor in complex with FGF show that a molecule of heparin links together two heterodimers of FGF and FGF-R into a highly asymmetrical pentameric complex (18).

Unlike the EGF and FGF pathways Drosophila Toll is activated by a dimeric ligand. The structure adopted by the active C-terminal fragment of Spätzle (C106) is a cystine knot, a fold held in common with neurotrophins such as NGF, and two monomers are held together as a covalent dimer by a single intermolecular disulfide bond (4, 19). Both in vivo and in vitro, C106 can form a heterotrimer with two molecules of the Toll receptor ectodomains (5). Thus in contrast to EGF, Spätzle binding alone should be sufficient to cause receptor cross-linking. In this paper we report that Toll pathway activation probably requires receptor-receptor interactions as well as ligand induced cross-linking.

MATERIALS AND METHODS

Protein Production and Purification—Recombinant His-tagged Toll, Toll5B, and C106 proteins were produced in the baculovirus system and purified using Ni-NTA affinity chromatography as described in detail in (5). In the reverse setup, C106 at a concentration of 45.5 μM was injected into Toll or Toll5B at 5.16 or 4.19 nM, respectively, into the measuring cell (15.7-Å spacer arm) were added from 10 mM stocks (in dimethyl sulfoxide) to 148 or 105 μM, respectively, into the measuring cell containing C106 fragment at concentration of 4.98 μM. Data were analyzed using SEDPHAT software (21).

Sedimentation equilibrium experiments were performed with an Optima XL-A/1 (Beckman Coulter) using double-sector centripetals and the interference optical system for data acquisition. For the analysis of the Toll-C106 1:1 complex and the Toll monomer-dimer equilibrium sedimentation velocity runs were performed at 55,000 rpm (3 or 2 min between scans, respectively) at 20°C. The sample volume was 400 μl. Data were analyzed using SEDINTERP software (20).

Luciferase Assay—HEK (human embryonic kidney) 293 cells were grown in DMEM medium (Sigma) supplemented with 10% fetal calf serum and 1-glutamine (2 mM) and transfected in 60-mm dishes at a density of 4 × 10⁵ cells. Cells were transfected using Lipofectamine 2000 (Invitrogen) and either 2 μg of empty pcDNA3.1 (Invitrogen) or pcDNA3.1 plasmids expressing Toll, the Toll-hTLR4 chimera 1 (Toll residues 1–845 fused to hTLR4 residues 622–799), or 2 (Toll residues 1–802 fused to hTLR5 590–799), respectively, as well as 0.2 μg of a luciferase reporter gene under the control of the NFκB (nuclear factor κB) promoter (Promega). Cells were seeded into white 96-well plates and the medium was replaced with media, Spätzle, or human IL-1 at 10 ng/ml for 6 (data not shown) and 24 h. Luciferase production was measured after washing with phosphate-buffered saline and phosphate-buffered saline containing 1 mM Ca²⁺ and Mg²⁺ ions and subsequent addition of LucLite luciferase lysis and reporter buffer (PerkinElmer Life Sciences). Sample analysis was carried out on a Microbeta counter (PerkinElmer Life Sciences) in triplicate.

Sequence Alignments—Sequences for residues 754–883 of Toll and 542–660 of human TLR4 were aligned using ClustalW, formatted using JOY software (22).

RESULTS

Isothermal Titration Calorimetry Studies Indicate a Complex Binding Reaction between Toll and Spätzle—We used ITC to study the thermodynamics and the stoichiometry of binding between full-length and a truncated Toll ectodomain and active, dimeric Spätzle ligand (Spz C106). Purified Toll ectodomain was titrated into the ITC measuring cell containing Spätzle C106 protein (Fig. 1A). This results in the formation of a complex of two ectodomains binding 1 Spätzle dimer with a measured dissociation constant of 82 nM. By contrast, in the reciprocal titration in which C106 was injected into Toll ectodomain, a complex with a 1:1 stoichiometry and a significantly higher dissociation constant of ~30 μM was observed (Fig. 1B). This difference in affinity shows that the binding of a first Toll molecule reduces the affinity for the second binding event and therefore that the two binding events are non-equivalent. Other thermodynamic parameters were also recorded and are summarized in Table I. The changes in Gibbs free energy are the same in both experiments, implying that both titrations describe the same binding process. This is also supported by the measured changes of enthalpy for which a factor of 2, due to the divalence of the C106 ligand, has to be taken into account.

ITC experiments were also performed using Toll5B, a truncated ectodomain lacking 133 amino acids of sequence including a substantial portion of the second LRR block and the Cys-rich C-terminal flank (see Fig. 4), a motif known to be important for signaling. Titrations with Toll5B gave the same values as the full-length ectodomains, which indicates that LRR block II and the C-terminal Cys-rich flank do not make contributions to the binding of the ligand.

Cross-linking Studies and Analytical Ultracentrifugation

Confirm the Existence of Both 2:1 and 1:1 Complexes of Toll and Spätzle—To validate the existence of the 2 different stoichiometries found for complexes between Toll and Spätzle, we first sought to confirm the existence of a 2:1 complex using chemical cross-linking. Bifunctional reagents were added to a Toll → C106 mixture at increasing concentrations and incu-
bated for 45 min at ambient temperature, and the mixtures were then analyzed by SDS-PAGE (Fig. 2A). The addition of Sulfo-GMBS cross-linker to the Toll-Spa¨tzle sample results in the appearance of a 240-kDa band. A similar result is obtained when another cross-linking reagent, Sulfo-KMUS, is used (Fig. 2A). The 240-kDa band is likely to correspond to a protein complex consisting of two Toll monomers of $\frac{110}{110}$ kDa and one C106 dimer with 24 kDa.

To confirm the existence of a 1:1 complex we had to use an experimental technique in which the excess of C106 necessary to maintain a 1:1 stoichiometry was not removed, as occurs in gel filtration. We therefore performed sedimentation velocity analytical ultracentrifugation on a mixture of Toll with a molar excess of C106. Analysis of the data for the Toll-Spa¨tzle complex showed a predominant species with a sedimentation behavior equivalent to a molecular mass of 134 kDa (Fig. 2B), correlating well with the estimated mass of a 1:1 complex.

**Full-length Toll Ectodomain Forms a Weakly Associated Dimer in Solution**—It has been shown that some receptor ectodomains exist as preformed dimers (Ref. 23 and references therein). To test whether the complex binding of Toll to C106 could potentially be influenced by such a phenomenon, we again used analytical ultracentrifugation (sedimentation velocity) to analyze the oligomerization state of Toll ectodomain in solution. As shown from a $\text{c(S)}$ analysis (Fig. 2A), there are two species present in solution, having sedimentation coefficients of 5 and 6 S, respectively. The corresponding $\text{c(M)}$ analysis revealed that the molecular masses of these species are $\frac{90}{90}$ and $\frac{170}{170}$ kDa, respectively. The first is consistent with a Toll monomer and the second with a Toll dimer. The slightly lower mass for the dimers probably reflects a difference in the diffusion coefficient for the monomer and dimer, which is not taken into account in the analysis.

We then characterized the association of Toll using sedimentation equilibrium experiments. These were conducted at three different concentrations of Toll ectodomain, 2, 16, and 42 $\mu$M. The results, as shown in Fig. 3B, were fit to a monomer-dimer model. The quality of the data is excellent and the local root mean square deviation for each fit was 0.01. The recovered monomer molecular mass was $\frac{87,472}{87,472}$ Da and the dissociation constant, $K_d$, is $\frac{2}{2}$ M. This result suggests that, in contrast to C106 binding, dimerization of Toll is of low affinity but comparable with p75, a receptor for the neurotrophin NGF (24), a structural homologue of Spa¨tzle C106. Thus, it is possible that binding of the ligand displaces binding interfaces and that receptor dimerization modulates C106 binding.
FIG. 2. Cross-linking and analytical ultracentrifugation studies of Toll-Spätzle complexes. A, a Toll-Spätzle mixture with an excess of Toll shows a 2:1 complex that can be cross-linked. Toll alone or a Toll → C106 mixture were incubated with different concentrations of Sulfo-GMBS (10.2-Å spacer arm) and Sulfo-KMUS (15.7-Å spacer arm) cross-linking reagent for 45 min at room temperature. Samples were then analyzed by SDS-PAGE on 7% Tris-Acetate gels and the gels silver stained. The protein concentration for both protein solutions was 1 mg/ml. B, ultracentrifugation verifies the presence of a 1:1 complex in a mixture containing excess Spätzle. Left panel, representation of consecutive scans that were fitted using the Sedfit program. Center panel, residuals representation. Right-hand panel, distribution of molecular mass showing the c(M) fit.

Critical Role of the Transmembrane and Juxtamembrane Sequences in the Regulation of Toll Signaling—Given that the analysis of the TollΔN fragment suggested that the C-terminal residues of Toll are important in Toll-Toll interactions, we next sought to determine whether other regions of the receptor, such as the juxtamembrane or transmembrane regions also have an influence on signaling. As these sequences are not involved in ligand binding any effect must be secondary in nature, for example by allowing receptor-receptor interactions to occur. To determine whether the transmembrane region of Drosophila Toll and that of the human Toll-like receptor 4 fulfil a comparable functional role, receptor chimeras were prepared as illustrated in Fig. 5A. Chimera 1 consists of the Drosophila Toll ectodomain and transmembrane segment and the hTLR4 TIR domain, and chimera 2 consists of the Drosophila Toll ectodo-
main but the hTLR4 transmembrane and TIR domains (Fig. 5C). As shown in Fig. 5B in both cases Spz C106 is able to stimulate activation of an NFκB reporter gene in transfected HEK293 cells and the dose-response curve is comparable with that seen in Drosophila cells (5). However, in the case of chimera 2 there is a substantial level of constitutive activation. This suggests that there is an important role for the transmembrane α-helix in suppressing signaling activity and that there are significant mechanistic differences in the activation of the Drosophila Toll and hTLR 4 pathways. This may be indicated by the sequence divergence evident in this region when the two sequences are aligned (Fig. 5C).

**DISCUSSION**

In this paper we present biophysical analyses of the interaction between the ectodomain of Toll and the dimeric ligand Spätzle. Our ITC data show that two thermodynamically sim-
FIG. 6. Model of signal transduction through the Toll receptor. A, on the cell surface, unbound Toll preformed dimers are in equilibrium with monomeric Toll receptor. Contacts within the dimer are mediated by interactions involving C-terminal structures in the Toll ectodomains. B, binding of a Spa¨tzle C106 dimer to the N-terminal half of one Toll molecule results in a conformational change that is transmitted to C-terminal receptor regions and causes the receptor to switch to an active conformation. C, Spa¨tzle binding the receptor results in a reduction in the affinity of the second binding site of C106. D, another Toll molecule binds to the C106 dimer, and this complex is further stabilized by interactions between the C-terminal ectodomain regions of the receptors. The intracellular domains in the complex are brought into proximity and are allowed to transmit a signal.

ilar modes of binding exist in solution: a 1:1 complex existing when Spa¨tzle C106 is in excess and a 2:1 complex predominating when Toll is in excess. Interpretation of the dissociation constants ($K_d$) measured in these titrations indicates that the first and the second binding event are non-equivalent and that the first event has a significantly higher affinity of binding than the second. This suggests negative cooperativity, the first binding event could for example cause structural changes in the symmetrical binding sites in the C106 dimer. Interestingly, a structural study has recently been published of NGF, a dimeric cystine knot ligand structurally related to Spa¨tzle (19), bound to a single molecule of the receptor p75 (24). In this report, the authors show that p75 interacts with two discontinuous sites at the interface of the NGF homodimer and that this first binding event induces a certain amount of structural distortion in the ligand, which disables the second putative p75-binding site in the NGF homodimer. The authors also present biophysical data supporting the 1:1 stoichiometry for NGF:p75, notably an ITC titration of NGF into a concentrated solution of p75, an experiment comparable with that presented in Fig. 1, A and C, which also gives a 1:1 stoichiometry (the reciprocal titration was not presented). Our results suggest that allosteric changes occur in the C106:Toll complex and point to similarities between C106 and NGF in receptor interaction. However, in contrast to NGF:p75, the allosteric changes in the C106:Toll complex do not preclude the recruitment of a second receptor molecule. Negative cooperation may help explain the phenotype of the class II of dominant alleles mentioned above. In wild-type embryos, C106 generated in close proximity of the membrane is immediately trapped by Toll (C106 can only be isolated from the perivitelline fluid of Toll−/−embryos, not wild-type embryos (25)). By contrast, in type II mutants, C106 encountering a soluble Toll receptor is less likely to interact at the cell surface with full-length receptor, as this second interaction occurs with a lower affinity, thus increasing the ability of C106 to diffuse before eliciting a signal.

The ITC experiments show that the binding of C106 to the truncated Type II ectodomain Toll$^{34C}$ is indistinguishable from that of the full-length protein. Although the exact residues involved in ligand binding remain to be defined more precisely, this implies that the second block of LRRs and the membrane proximal cysteine-rich flank are not involved in ligand binding. This is consistent with the earlier notion that Toll type II dominant alleles bind to Spa¨tzle C106 despite the fact that they are truncated ectodomains (13) (5). In fact, the shortest of these, Toll$^{46C}$ (truncation at residue 464; see schematic in Fig. 4B), comprises only Toll N-terminal features and the first 13 LRRs, and this suggests that the binding site for Spa¨tzle is contained within this region. The extracellular LRRs of Toll are likely to adopt a concave structure similar to that seen in glycoprotein Ib (26), and it is interesting to note that the NGF binding sites of p75 are found in a similar extended concave structure formed, in that case, by cysteine rich regions (27). It is plausible that, like NGF, Spa¨tzle has two discontinuous binding sites arranged tangentially to the LRR superhelix in the Toll ectodomain.

Localization of the Spz binding site to the first block of LRRs suggests that the second block, the cysteine-rich flank, and transmembrane α-helix play an indirect role in receptor activation or regulation. Our experiments shed some light on their respective functions. We present evidence suggesting that LRR block 2 and other C-terminal features are involved in mediating receptor-receptor contacts and that removal of the Toll N terminus results in a strong association between these regions. This suggests that in the absence of C106, the N-terminal ligand binding domain is required to suppress strong Toll-Toll interaction and thus prevents receptor signaling in the absence of ligand. We suggest that ligand binding relieves this constraint by introducing conformational changes that subsequently bring Toll receptors into closer proximity and thereby allow signaling through their intracytoplasmic domains. This is similar to a proposal based on time resolved fluorescence imaging microscopy studies for the EGF receptor (28). These experiments indicate that the EGF receptor undergoes a substantial structural rearrangement upon ligand binding, involving the rotation of the ectodomains.

Previous findings that Toll transmembrane and intracellular domains on their own are constitutively active (29–31) and that there is an unstable association of Toll ectodomains as shown in this study highlight the importance of the relative spatial arrangement of extra- and intracellular domains determined by juxta- and transmembrane regions. This is demonstrated by the properties of the constitutively active Type 1 dominant Toll receptors and second by the experiments described above, which show that swapping of the transmembrane domain derepresses the receptor. This indicates that in the inactive state, these motifs are arranged in a way that prevents receptor self-association together with N-terminal
features (see above). The disulfide bonds of the C-terminal flank may confer a conformational rigidity on the juxtamembrane region that is disrupted in the Type 1 mutants or re- 

related in the Type 1 dominant mutants is activated by forma-

tion of a covalent cross-link with another receptor. However, we were unable to show that one of these dominant alleles (Toll10B) is cross-linked in this way.2

It is likely that repression of signaling function associated with the C-terminal flank is strongly influenced by the transmembrane domain of the receptor. Thus, the replacement of sequences including just 4 amino acids predicted to lie outside the membrane causes a substantial level of constitutive acti-

vation in Toll-TLR4 chimeric receptors (Fig. 4B). This points to differences in the role of juxta- and transmembrane regions in the regulation and activation of Drosophila and human Toll receptors signal transduction. Taken together our results suggest a model of activation for Drosophila Toll which is illustrated in Fig. 6. This mechanism has features in common with that elucidated for the EGF receptor (see Introduction). However, in that case the binding of monovalent EGF to two receptor molecules facilitates receptor self-association, whereas we propose that ligand induced dimerization causes secondary receptor-receptor interactions, which stabilize the signaling complex. Further structural studies of Toll ectodomains or Toll-Spatzle complexes, as well as experiments addressing Toll-Spatzle interactions on the cell surface, will be required to validate this model. It will also be interesting to investigate the relevance of this model for the activation of mammalian TLRs. The best characterized TLR at this stage is TLR4, which is activated by lipopolysaccharides from Gram-nega-

tive bacteria. Recent results indicate that activation of TLR4 requires the secreted accessory molecule MD-2 and that binding of lipopolysaccharides induces a conformational change in the TLR4-MD-2 complex (33–35).

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