Review Article

Toll-Dorsal Pathway Regulates Immunity as well as Dorso-Ventral Patterning in Drosophila

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The identification of the Toll receptor in Drosophila was a major discovery which restructured the paradigm in immunity as well as developmental biology. Toll homologues are present in mammals where they regulate only immunity. However, in flies, same Toll pathway controls two disparate biological processes like embryonic dorso-ventral patterning and humoral immune response. Toll activation by Spätzle is preceded by a proteolytic cascade which is differently regulated in immunity and embryonic development. Signalling downstream to Toll is similar in both processes and eventually leads to the activation of Drosophila homologues of NF-κB proteins; Dorsal and DIF. Toll pathway, in the early embryo results in graded expression of Dorsal protein leading to Dorso-Ventral axis patterning. On the other hand, activation of Toll-Dorsal pathway in the fat-body leads to induction of antibacterial proteins in larval and adult stages. Here, we review the current status of our knowledge of Dorsal pathway in embryonic development and immunity with special emphasis to regulation of target gene/s of the two pathways by Dorsal.

Key Words: Dorsal; Toll; DNA Geometry; Dorso-Ventral Patterning; Insect Immunity

Introduction

Drosophila has been used to dissect genetic basis of different phenotypes and pathways which has helped us in understanding many biological paradigms (Ashburner and Carson, 1986; Fortini et al., 2000). One such paradigm was the discovery of primary role of Toll receptors in embryonic development (Nusslein-Volhard and Wieschaus, 1980; Stein et al., 1991). Subsequent study led to the finding of rest of the downstream genes of dorsal-ventral (D-V) patterning of the embryo. These genes are collectively called as ‘the dorsal group of genes’ which includes Toll, tube, pelle, cactus, the NFκB homolog dorsal. In the year 1996, it was conclusively shown that ‘dorsal group of genes’ from the Toll ligand spätzle (Spz) to cactus, is involved in the anti-fungal response in Drosophila (Lemaitre et al., 1996). This set a platform for the discovery and characterization of Toll-like receptors in humans (Medzhitov et al., 1997) establishing the role of the Drosophila Toll-pathway as an evolutionarily conserved mechanism. However, mammalian TLRs are believed to have no role in development (Kimbell and Beutler, 1998), whereas the Drosophila Toll-pathway is involved both in immunity and developmental processes (Halfon et al., 1995; Lemaitre et al., 1996). Here, we try to understand, how Drosophila Toll-pathway controls both embryonic patterning and immune response.

Toll receptor of Drosophila

The Toll gene was discovered as a component of D-V patterning pathway in Drosophila early embryos (Anderson and Nüsslein-Volhard, 1984; Anderson et al., 1985). Drosophila Toll belongs to a family of...
nine paralogues. These are Toll, 18 wheeler (18w), Toll-3 (also called MstPrx), Toll-4, Toll-5 (also called Tehao), Toll-6, Toll-7, Toll-8 (also called Tollo), and Toll-9. Nearly all of the Drosophila paralogues share the same protein architecture. The main difference between the Toll paralogs is in the number of LRRs. The exception is Toll-9, which has a single block of LRRs in the ectodomain, compared to the two blocks seen in the other Tolls (Imler and Hoffmann, 2001). Toll-9 is more closely related to the vertebrate Toll-like receptors (TLRs) than other Drosophila Tolls. Of all Toll proteins of Drosophila, Toll-1 is well studied and is the regulator of D-V patterning and immune response.

Toll expresses maternally in early embryos, and zygotically during late embryonic development. Zygotic Toll is expressed in the gut, salivary glands and epidermis (Gerttula et al., 1988). Toll is expressed in migrating cells, and also localises to the sites of contact between the fused epidermal layers at the dorsal midline (Hashimoto et al., 1991). Drosophila Toll receptor is a type I transmembrane protein. It comprises of an N-terminal ecto-domain, middle transmembrane domain, and the C-terminal intracellular domain. The ecto-domain is involved in interaction with other proteins through leucine-rich repeats (LRRs). The LRR domain in Toll is interrupted by two Cysteine rich domains (CRD), thus dividing the LRRs into two blocks. Second CRD is sandwiched between the C-terminal LRR and the transmembrane domain. The intracellular region of Toll contains a Toll/Interleukin-1 Receptor (TIR) domain, which initiates intracellular signalling upon recruitment of adapter proteins.

**Toll in Dorso-Ventral Patterning**

Bilateral symmetry in animals results from the presence of the Anterior-Posterior (A/P), and, Dorso-Ventral (D/V) axis. In Drosophila, D-V patterning pathway gets activated in the maternal egg chamber itself. This pathway is triggered upon somatic expression of Nudel, Pipe and Windbeutel proteins which results in the establishment of ventral cell fates (Stein et al., 1991; Dissing et al., 2001). Pipe is expressed on the ventral side of the follicular epithelium, while Nudel, a protease, is uniformly expressed, (LeMosy et al., 1998; Zhu et al., 2005). Next step is activation of proteolytic cascade involving three proteases, Gastrulation defective (Gd), Snake and Easter, in the perivitelline space (Han et al., 2000; Dissing et al., 2001; Cho et al., 2010). The final proteolytic step releases mature Spz from its inactive pro-peptide form by Easter, after which it binds Toll (Stein et al., 1991; Schneider et al., 1994; DeLotto and DeLotto, 1998; Weber et al., 2003). Cleaved Spz is a dimer, and binding of one Spz dimer to Toll induces receptor homo-dimerization (Gangloff et al., 2008). Recently it has been shown that Toll dimerization is accompanied by endocytosis of this complex (Huang et al., 2010).

On the inner side of the cell membrane, the intracellular TIR domain of Toll interacts with proteins containing Death-domain, which are collectively called as adapter proteins. The three adaptor proteins of Toll pathway are MyD88, Tube and Pelle (Hecht and Anderson 1993; Shen et al., 2001; Kambris et al., 2003). Upon binding of Spz, the TIR-domain of Toll protein forms a homotypic interaction with the TIR domain of MyD88, resulting in the recruitment and activation of the other two adapter proteins (Kambris et al., 2003). Subsequent step in the Toll signalling is the activation of transcription factor, Dorsal. In the cytoplasm Dorsal remains bound to its inhibitor protein called Cactus (Whalen and Steward, 1993). Upon Toll activation, Cactus gets phosphorylated ultimately leading to its degradation by the ubiquitin-proteasome pathway (Bergmann et al., 1996, Reach et al., 1996). In the process, Dorsal gets released and this free Dorsal enters the nucleus to induce or repress target genes.

Regulation of target gene expression by Dorsal is concentration dependent. On the ventral side, concentration of Dorsal is maximum which leads to activation of genes like twist and snail, which specify the mesoderm. On the lateral surface, Dorsal expression tapers down and in this region, it activates sim which specifies meso-ectoderm and rho and sog which specify neuro-ectoderm (Stathopoulos et al., 2002; Stathopoulos et al., 2004; Biemar et al., 2006). On the dorsal surface, there is no expression of Dorsal
at all, and here *zen* is transcribed. Transcription of *zen* on the ventral and lateral surface is inhibited by Dorsal (Huang et al., 1997; Stathopoulos and Levine, 2002). The induction and repression of target genes by Dorsal in a concentration dependent manner is regulated by affinity of Dorsal to its binding sequence (κB motif) (Biemar et al., 2006). It has been proposed that genes like *twist* and *snail* which are expressed on the ventral surface (where Dorsal expression is highest), possess low affinity κB motifs, while genes like *dpp* and *zen* which are expressed on the dorso-lateral surface (where Dorsal expression is low) have high affinity κB motifs (Ip et al., 1991; Jiang et al., 1992; Kirov et al., 1994; Dubnicoff et al., 1997; Huang et al., 1997; Stathopoulos et al., 2004; Hong et al., 2008). This is how the gradient of nuclear Dorsal, triggered by ventrally restricted Toll signalling, establishes the dorso-ventral axis in *Drosophila* embryo.

**Toll in Innate Immunity**

*Drosophila* was shown to have an inducible antibacterial system way back in 1970s (Bakula, 1970; Boman et al., 1972; Boman et al., 1974). Since *Drosophila* does not have an adaptive immune arm hence this offers the cleanest system to explore humoral response (Tzou et al., 2002). Humoral immune response in *Drosophila* is mediated by Anti-Microbial Proteins (AMPs) which are produced in the fat body (analogous to mammalian liver) and secreted into the hemocoel (Boman et al., 1996; Carton et al., 1997; Lemaître et al., 1997; Basset et al., 2000; Imler and Bullet, 2005). Post bacterial challenge AMP response persists for several days and gives protection against microbes. *Drosophila* secretes a total of 55 AMPs upon infection by diverse pathogens, which are grouped into seven major classes based on amino acid compositions (Basset et al., 2000; Hultmark, 2003). Prophenoloxidase activation precedes AMP activation and is very critical for insect immunity (Soderhall and Cerenius, 1998). These AMPs are not microbe specific e.g. drosomycin and metchnikownik are active against fungi; attacin, cecropin, diptericin and drosocin are active against Gram-negative bacteria while defensins are active against Gram-positive bacteria (Boman et al., 1974; Lemaître et al., 1996; Lemaître et al., 1997; Levashina et al., 1999; Hoffmann, 2003; Brennan and Anderson, 2004; Vierstraete et al., 2004). Activation of these genes requires triggering of the Toll or IMD pathway. Flies deficient for both the Imd and Toll pathways fail to induce any of the known antimicrobial peptides and succumb readily to infection (Lemaître et al., 1996; Tzou et al., 2002; Hoffmann, 2003; Hultmark, 2003; Brennan and Anderson, 2004). Moreover, activation of either pathway, for example; by over-expressing a pathway component, is sufficient to trigger AMP expression in the absence of infection (Levashina et al., 1999; Georgel et al., 2001; Imler et al., 2004). In this review we will restrict to Toll pathway only.

**Extracellular Toll Signalling**

Pathogens on their surfaces express molecules called as Pathogen-Associated-Molecular-Patterns (PAMPs), which are recognized by the host for mounting immune response (Irving et al., 2001). The molecular system in the host that recognizes these PAMPs are called as Pathogen Recognition Receptors or PRRs. *Drosophila* has two families of PRRs: Peptidoglycan (PGN) recognition proteins (PGRP) and Gram-negative binding proteins (GNBP), which identify distinct PAMPs associated with Gram-positive (LYS-type PGN); Gram-negative bacteria (DAP-type PGN); or fungi (Beta-1,3-glucan) (Gottar et al., 2006; Ferrandon et al., 2007). Circulating GNBP3 in hemolymph directly recognizes Beta-1,3-glucan of fungi and triggers downstream signalling, that finally activates the Toll pathway (Buchon et al., 2009; Levashina et al., 1999). In contrast, the PGN of Gram positive bacteria are recognised by PGRPSA/PGRP-SD and GNBP1. Formation of PAMP and PRR complex activates ModSP (modular of serine proteases) which, in turn, triggers a proteolytic cascade that results in the activation of Spz, the ligand of Toll (Kambris et al., 2006). However, the proteolytic pathway that gets activated in immunity is different from the one that is used in dorso-ventral patterning and this is how the two processes distinctly regulate Toll activation (Fig. 1). The first serine protease in the immune cascade is ModSP, and recognizes both bacterial and fungal PAMPs (Buchon
et al., 2009). Parallel to ModSP is the protease Persephone (Levashina et al., 1999; Ligoxygakis et al., 2002). Interestingly, Persephone can activate Toll independently of PRRs and is said to respond to ‘danger signals’ associated with infection (El Chamy, 2006; Kambris et al., 2006). Next protein in the pathway is Sphieroide, a serine protease, which further regulates Spatzle Processing Enzyme (SPE). SPE directly cleaves Spz, releasing the Cys-knot in the Spz, which is critical for binding Toll (Jang et al., 2006).

**Intracellular Toll Signalling**

Unlike the extracellular proteolytic cascade, the intracellular signalling cascade activated by Toll during immune response is similar to that in the D-V patterning (Gay and Gangloff, 2007). In spite of the similarity in the intracellular signaling, the effector molecule induced in immunity is not Dorsal but its homologue named DIF (Dorsal related Immunity Factor) (Manfruelli et al., 1999; Meng et al., 1999). In the larva, DIF and Dorsal act redundantly, but in the adult, DIF is alone required for mounting immune response and not Dorsal (Rutschmann et al., 2000). The target genes of the Toll-Dorsal signalling pathway during immunity include anti-microbial peptides like drosomycin (Lemaitre et al., 1996).

Toll has an intracellular TIR domain, which recruits adapter proteins, MyD88, Tube and Pelle, to the Spz bound activated Toll receptor (Fig. 1). All three adaptor proteins contain a Death-domain, which is involved in protein-protein interactions leading to formation of a trimer complex (Hecht and Anderson, 1993; Kambris et al., 2003). MyD88 and Tube dimer is localised to the plasma membrane. The Death-domain of Pelle then binds that of Tube (Sun et al., 2002). Pelle also carries a kinase domain and triggers the activation of Dorsal, possibly through interaction with dTRAF2 (Shen et al., 2001). Interestingly, dTRAF2 is required for Toll signalling in immunity, but is not essential for embryogenesis: as dTRAF2 null mutant flies survive to adulthood (Cha et al., 2003).

In the absence of signalling, Dorsal is found in the cytoplasm in complex with IκB homologue Cactus (Whalen and Steward, 1993). Activation of Toll signalling leads to the degradation of Cactus and the nuclear localisation of Dorsal to direct gene expression (Bergmann et al., 1996). The phosphorylation of both Dorsal and Cactus is required...
for the nuclear localisation of Dorsal (Gillespie and Wasserman, 1994; Drier et al., 1999). Phosphorylated Dorsal interacts with dNTF2 (Drosophila Nuclear Translocation Factor 2) and Nup88 to localise to the nucleus.

Once inside the nucleus, Dorsal/DIF rapidly activates AMPs, which are later released in the hemolymph (Hoffmann, 2003). AMPs are small, secreted, cationic peptides that can kill microbes or block their growth by disrupting membrane integrity (Hoffmann, 2003; Tanzi et al., 2010). AMP gene loci regulated by the Toll pathway have a single κB site characterized by a GGGAA consensus half site. While the regulation of target AMP genes by Dorsal/DIF upon microbial infection is well established, transcriptional regulation of Dorsal or DIF is not known. We found that in dl1 mutant, expression of Dorsal protein was not detected (Mrinal and Nagaraju, 2010). This indicated towards a possible auto-regulation of dl gene by Dorsal itself. Recently, we have explained the mechanism of auto-regulation for Dorsal (Mrinal and Nagaraju, 2010). We found that early in immune response, Dorsal induces transcription of its own gene by binding to a κB motif present in the first intron of dl gene. However, in the later stages of immune response, Dorsal repositions itself to another binding site in the promoter of dl gene (Mrinal and Nagaraju, 2010). Interestingly, Dorsal binding to the promoter κB motif results in transcriptional repression of dl. We have shown that the repressor function of Dorsal is due to co-operative interactions with a co-regulator AP-1 (Drosophila homologue) protein. This established the temporally regulated feedback loop in Dorsal auto-regulation (Mrinal and Nagaraju, 2010). Auto-activation and auto-repression of genes has been known for a long time. However, Dorsal auto-regulation is the first example of its kind, where both auto-activation and auto-repression of a gene is affected by the same transcription factor. We were puzzled to notice that the two κB motifs in the dl gene bind the same transcription factor and still produce opposite outcomes. This prompted us to further explore the mechanism of dl auto-regulation by Dorsal protein.

**Mechanism of Gene Activation and Repression by Dorsal**

Dorsal is a transcription factor belonging to NFκB family of proteins. TFs of this family can act both as activator and/or repressor of transcription. In Drosophila, Torso receptor tyrosine kinase signalling pathway, which is activated at the poles, selectively masks the ability of Dorsal to function as a transcriptional repressor at these positions (Rusch and Levine, 1994; Rusch and Levine 1996). Torso signalling modulates the ability of Capicua (Cic), Cut, and Dri, proteins that consequently influence the ability of Dorsal to function as a transcriptional repressor (Valentine et al., 1998; Jimenez et al., 2000). This suggests that activation or repression by Dorsal is dependent on a potential crosstalk with other pathways.

First, classical study based on the analysis of cis-regulatory sequences showed that Dorsal can function either as an activator or a repressor to control gene expression along the D-V axis (Jiang et al., 1993, Kirov et al., 1994). Dorsal, by default, is an activator of transcription in Drosophila and its repressor function is context-dependent, e.g., repression of zen by Dorsal was ascribed to cooperative interactions with DNA-binding proteins occupying associated AT-rich sequences upstream of Dorsal binding site in the zen promoter (Kirov et al., 1993). It was also shown that repressor activity of Dorsal requires Groucho, a global co-repressor (Dubnicoff et al., 1997). We have already shown that, repressor activity of Dorsal was co-repressor dependent (Mrinal and Nagaraju, 2010). Both these studies have suggested that repressor function of Dorsal is co-regulator dependent. However, it has raised another query as to why Dorsal interaction with co-regulator is possible at few κB motifs but not on others?

**Role of Combinatorial Code in Target Gene Regulation by Dorsal**

Combinatorial interactions between Dorsal and other transcription factors were found to be important in spatio-temporal regulation of target gene expression. Synergetic DNA binding between Dorsal and Twist permits gene expression in more lateral regions of
the embryo where, neither Dorsal nor Twist is capable of inducing gene expression alone (Gonzalez-Crespo and Levine, 1993 and 1994; Jiang et al., 1993; Stathopoulos and Levine, 2002). Furthermore, transcriptional repressors function to refine the expression domains produced by activators. For instance, to restrict rho expression to ventro-lateral stripes in the embryo, Dorsal and Twist activation is antagonized in ventral regions by the Snail repressor, resulting in the lateral stripe pattern of gene expression exhibited by these genes (Kosman et al., 1991; Ip et al., 1992). Advent of genomics has allowed scientists to dig further this concept of combinatorial regulation of Dorsal. Such studies have identified a set of 25 cis-regulatory sequences, which act in concert with Dorsal along the D-V axis (Stathopoulos and Levine, 2004; Markstein et al., 2004). Genome-wide studies employing ChIP-chip identified hundreds of binding sequences, including hitherto unknown binding motifs, for Dorsal and associated regulatory proteins in the D-V patterning genes (Sandmann et al., 2007; Zeitlinger et al., 2007). For instance, apart from Dorsal-binding sequence in the target genes, the motif GCTGGY A was identified in enhancers that control expression in a broad lateral stripe. On the contrary, genes that expressed in the ventro-lateral stripes had CACA TGT and stripe. On the contrary, genes that expressed in the ventro-lateral stripes had CACA TGT motif sequences

is not without a reason and perhaps the sequence of the Dorsal-binding motif may have ‘a’ role in gene regulation and Dorsal function.

Role of κB Motif Sequence and Geometry in Gene Regulation by Dorsal

In recent times, we have started to develop understanding towards the factors that discriminate activator function of Rel proteins from its repressor function. While studying the auto-regulation of Dorsal, we performed motif swap experiment and found that Dorsal interaction with AP-1 was specific for AGAAAAACA motif present in the promoter (Mrinal and Nagaraju, 2010). When this motif was replaced with canonical GGAAATTC activator motif, co-repressor function of AP-1 was lost. The Dorsal-API complex was not recruited, either in vitro or in vivo, with the canonical activator motif GGAAATTC. This indicated that sequence of κB motif perhaps had some role to play in Dorsal interaction with its co-regulator (Mrinal and Nagaraju, 2010).

We explored this question further and showed that composition of the κB motif sequence has a key role to play in the interaction of co-regulator with Dorsal. We found that all known Dorsal target genes which are repressed by Dorsal, have an A-tract in the binding motif while none of the activator motifs had A-tract. Through molecular simulation studies, we have shown that κB core with A-tract (which is present in repressor motifs) is more deformable than activator κB core, which always lacks A-tract. We further showed that presence of ‘A’ or ‘T’ at the 6th position in the nonameric κB motif is critical for imparting activator or repressor function (Mrinal and Nagaraju, 2010; Mrinal et al., 2011). While different activator motifs had comparable major groove geometry, the repressor motifs had distinct major groove geometry. Thus, binding of Dorsal to different repressor motifs is decided by the sequence of κB motif and the co-repressor (Mrinal et al., 2011). This suggests that DNA sequence can induce allosteric changes in the TF to enable its binding to the DNA motif. This led us to conclude that κB motifs in Drosophila exist in activator and repressor conformations, implying that there is a sequence
encoded geometry in DNA binding motifs (Fig. 2). Later on, similar findings were made by Wang et al. as they found that sequence at the central position of the κB motif affects not only transcriptional activity of Rel proteins but also its ability to heterodimerize with other Rel proteins (Wang et al., 2012).

**Conclusion**

Study of Toll pathway in last three decades has greatly enhanced our knowledge of molecular mechanisms in D-V signalling and its regulation. In last few years, research in this field has focussed on understanding the Dorsal gradient formation and its gene regulatory network using high throughput technologies. This has expanded the number of target genes downstream to Toll and brought Dorsal into focus. The precise question to be answered for now is: How does Dorsal decide the qualitative and quantitative nature of target gene expression?

It is evident that ability of Dorsal to induce or repress gene expression is context-based. Understanding this ‘context’ is very crucial, since this will help in decoding the molecular basis of target gene recognition by transcription factors in general. Differential ability of Dorsal to bind many variants of κB motifs leading to different degrees of transcriptional expression of target genes indicates that, there cannot be a simple model of recognition codes of DNA-binding by Dorsal. Flexibility in protein-DNA interactions, selective role of co-regulators and other effects of local and non-local parameters confound straightforward descriptions of DNA base preferences in Dorsal binding (Mrinal et al., 2011). Dorsal acts as a global regulator and the co-regulators act in gene specific manner. Since, Dorsal can interact with many co-regulators, this adds tremendously to its DNA-binding diversity (Stathopoulos and Levine, 2004; Markstein et al., 2004). Hence, to completely decipher the DNA-recognition codes of Dorsal, high throughput technologies would be required which will help to gain insights into gene targeting in vivo. This will also help in understanding the role of DNA induced allostery, co-operative recruitment or combinatorial codes in Dorsal binding which will in turn help us to understand how specificity is imparted in gene regulation.

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