Small GTPases 2:2, 95-98; March/April 2011; © 2011 Landes Bioscience

Regulation of Toll and Toll-like receptor signaling by the endocytic pathway

Viktor K. Lund[1] and Robert DeLotto[1,2,*]

[1]Department of Biology; University of Copenhagen; Denmark; [2]Department of Biological Sciences; Rutgers University-Newark; Life Sciences Center; Newark, NJ USA

Key words: Rab5, ESCRT0, mop, NFκB, TLR

Abbreviations: IRF, interferon regulatory factor; JNK, jun N-terminal kinase; PI3K, phosphatidyl inositol-3-kinase; IRAK, interleukin-1 receptor-associated kinase; TRAF, TNF receptor associated factor; MyD88, myeloid differentiation primary response gene 88; TRIF, TIR domain-containing adaptor-inducing interferon-beta; TIR, toll/interleukin 1 receptor; TIRAP, TIR domain containing adaptor protein; TRAM, TRIF-related adaptor molecule; EGFR, epidermal growth factor receptor; ESCRT, endosomal sorting complex required for transport; NFkappaB, nuclear factor kappaB; Rab, Ras related in brain; Rho, Ras homolog gene family; MVB, multivesicular body; Mop, myopic; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; PLC-d1, phospholipase C-d1; MAPK, mitogen activated protein kinase; MAPKK, mitogen activated protein kinase kinase; TLR, toll-like receptor; Cbl, casitas B-lineage lymphoma; ER, endoplasmic reticulum

Submitted: 01/12/11

Revised: 03/02/11

Accepted: 03/07/11

DOI: 10.4161/sgtp.2.2.15378

*Correspondence to: Robert DeLotto;
Email: rdelotto@bio.ku.dk

Extra View to: Lund VK, DeLotto Y, DeLotto R. Endocytosis is required for Toll signaling and shaping of the Dorsal/NF-kappaB morphogen gradient during Drosophila embryogenesis. Proc Natl Acad Sci USA 2010; 107:18028–33; PMID: 20921412; DOI: 10.1073/pnas.1009157107.

The Toll/TLR receptor family plays a central role in both vertebrate and insect immunity, driving the activation of humoral immunity in response to pathogens. In Drosophila, Toll is also responsible for directing the formation of the Dorsal/NFkappaB gradient specifying dorsoventral patterning of the embryo. Two recent studies have revealed that endocytosis and elements of the molecular machinery governing endosomal progression are required for Drosophila Toll signaling in development and immunity. We demonstrated that Toll is not only present at the plasma membrane but also in a Rab5^{+} early endosomal compartment in the embryo and that the distribution of constitutively active Toll^{10B} is shifted towards endosomes. Localized inhibition of Rab5 function on the ventral side leads to a reduction of nuclear Dorsal levels, while locally increasing Rab5 function leads to potentiation of signaling. Independently, another laboratory identified the endosomal protein Mop as a potentiator of Toll signaling in Drosophila cell culture and fat-body tissue. Mop functions together with the ESCRT 0 component, Hrs, previously reported to stimulate endosomal progression and the signaling ability of internalized EGFR. We discuss these studies and briefly summarize the most significant findings concerning the role of intracellular localization and trafficking in mammalian TLR function.

Toll and Toll Like Receptors

The Toll/Toll Like Receptor (TLR) protein family is a large metazoan transmembrane receptor family that plays prominent roles in development and defense against pathogens. Its discovery about a decade ago led to a renaissance in the field of non-antibody mediated humoral immunity. Drosophila Toll, the first identified member of this large receptor family, was originally identified as a plasma membrane receptor required for dorsoventral patterning of the early embryo, reviewed by Moussian and Roth.[1] In contrast, mammalian Toll Like Receptors serve primarily as pattern recognition receptors in B cells and in a large variety of innate immune cell types.[2] Toll is also responsible for the activation of the anti-fungal/anti-Gram positive branch of the innate immune response in Drosophila fat-body tissue, however in this case it functions as a cytokine receptor rather than a pattern recognition receptor, through recognition of the Spätzle ligand generated in response to microbial insult.[3] Eight other Toll paralogs are encoded in the fly genome.[4] However, with the exception of 18-wheeler which plays an important role in salivary gland morphogenesis by controlling Rho signaling as well as follicle cell migration and fat body development, very little is known about their function.[5,6]

Drosophila Toll signals through adaptor proteins DMyD88 and Tube and the IRAK-related kinase, Pelle, to effect the degradation of the IkappaB factor Cactus and nuclear accumulation and activation of the NFkappaB transcription factors, Dorsal and Dif. This pathway appears to be related to the mammalian MyD88 dependent NFkappaB activating pathway employed to some extent by most

mammalian TLRs[2,7] and may represent the ancestral signaling mechanism of the TLR family. In addition, mammalian TLR3 and TLR4 signal through a pathway that depends upon the adaptor protein TRIF, activating Interferon Regulatory Factor 3 (IRF3) as well as NFkappaB. Both the MyD88 and TRIF-dependent pathways can also direct activation of Map Kinases ERK1/2, p38 and JNK through the TAK1 MAPKK.[2] Activation of NFkappaB generally leads to the production of inflammatory cytokines, whereas activation of IRF3 and 7 induces type I interferon.

A perennial theme in transmembrane receptor biology is that subcellular localization and endocytic trafficking has a critical impact upon receptor function and signaling activity. Intracellular trafficking and endocytosis were initially shown to exert control over receptor function through receptor silencing and degradation. Subsequently they were also shown to regulate accessibility to ligand and control the ability of receptors to signal through different pathways by determining the availability of effectors necessary for signal transduction. For instance, many receptors have now been reported to require endocytosis and trafficking to particular endosomal compartments to initiate normal signal transduction after ligand binding.[8-11] Though some aspects of mammalian TLR signal transduction appear to be controlled by the endocytic pathway,[11,12] until now nothing has been known about the role of endocytosis and intracellular trafficking in Toll function.

Drosophila Toll: A Requirement for Endosomal Localization

Recent work from two different laboratories provides strong evidence that signal transduction in the Drosophila Toll pathway requires Toll to be present in an endosomal compartment. In a report from Huang and coworkers[13] the authors employ both genetic and biochemical methods to identify the early endosomal sorting complex ESCRT 0 components Myopic (Mop) and Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) as indispensible for Toll signaling in Schneider S2 cell culture and in fat-body tissue during the anti-gram positive response. By screening cells with a dsRNA library coding for all known and predicted Drosophila kinases and phosphatases using Cactus-Luciferase degradation in response to recombinant activated Spätzle as a readout, the authors identify the previously known Toll signal transducer Pelle and the novel putative protein tyrosine phosphatase, Myopic, as potentiators of Toll signaling. Assaying the expression of anti-microbial peptide genes either in response to Spz or LPS, the authors establish that the effect of Mop knockdown is specific to the Toll (anti-fungal/anti-gram positive) pathway and does not perturb the anti-gram negative IMD pathway. Epistasis experiments using RNAi and overexpression of Toll pathway components in cell culture place Mop upstream of MyD88 and Pelle and at the level of Toll in the hierarchy of the signaling pathway. As *mop* excision alleles are homozygously lethal, the authors test the role of Mop in the adult fly immune response by fat-body specific knock-down of Mop during Gram positive bacterial challenge and in doing so demonstrate that a reduction in Mop levels leads to a defect in the induction of anti-bacterial peptides controlled by the Toll pathway. Mop is also shown to localize to an intracellular vesicular compartment overlapping with the early endosomal marker Rab5, Toll and the ESCRT 0 component, Hrs. The authors demonstrate that Toll, Mop and Hrs exist in a biochemical complex and that Hrs is also required for Toll signaling. These data indicate that endocytosis and subsequent trafficking through the endosomal system is indispensable for Toll signaling in vitro in S2 cells as well as in fat-body tissue in vivo.

An independent study addressing the role of endocytosis in classical Toll signaling during embryonic development was published by us in reference 14. During early embryogenesis, activation of Toll signaling by processed Spatzle establishes a ventral-to-dorsal gradient of Dorsal across nuclei in the blastoderm.[1] Using fluorescent chimeras in conjunction with live imaging we demonstrate that Toll not only localizes to the plasma membrane as has been previously known from antibody staining but also to cytoplasmic vesicles overlapping with Rab5 positive early endosomes in syncitial and cellular blastoderm stage embryos. The constitutively active Toll variant Toll[10b] is shifted towards internal endosomal compartments compared to wild type Toll, suggesting that the subcellular localization of the Toll receptor is a function of its state of activation. We also demonstrate that a local disruption of entry to the early endosomal compartment on the ventral side of the embryo by injection of dominant negative Rab5 synthetic mRNA leads to a strong attenuation of Dorsal nuclear accumulation proximally and expansion of the Dorsal nuclear gradient distally. In extreme cases this leads to the complete inversion of the Dorsal gradient. Similar localized effects were achieved by injection of Dynasore, a small molecule Dynamin inhibitor.[15] These results suggest that the endocytic pathway and specifically the Rab5 compartment are necessary for embryonic Toll signaling and that endocytosis might act to restrict the propagation of a ventralizing factor. This factor is likely to be activated Spätzle ligand or perhaps an active form of one of the upstream proteases, as we show that the mobility of Toll receptor within the plasma membrane is much too low to explain the distal effects upon signaling.[14] This interpretation is consistent with the previously published observations that the ligand part of processed Spatzle is quickly removed from the extracellular space during embryonic patterning.[16] Conversely, increasing endosomal trafficking by microinjection of mRNA encoding wild type Rab5 or dominant constitutively active Rab5 mRNA leads to a local stimulation of Toll signaling and Dorsal nuclear accumulation, in agreement with what has previously been observed in Wingless and Notch signaling, which also require endocytosis.[8,10]

Although each study employs different approaches both studies converge upon the conclusion that the endocytic machinery is essential for Toll signaling in both its embryonic and adult functions. Our results suggest that the Rab5 positive compartment is necessary for normal Toll signaling. However, what is the mechanism to explain this requirement? As the early endosome functions as a sorting compartment,[17] communicating both with late endosomal, recycling and trans-Golgi

compartments, the effects observed upon Rab5 inhibition may reflect a failure to recycle the receptor or to reach another compartment that is required for the signaling process. However, failure to recycle back to the plasma membrane is not a likely explanation, as Toll is not depleted from the plasma membrane on the ventral side upon injection of dominant negative Rab5 and injection of a dominant negative form of Rab11 does not perturb the Dorsal gradient. Failure to reach another endocytic compartment remains a possibility.

The work of Huang et al. offers an interesting insight into the mechanistic basis for the endocytic requirement by identifying Mop and Hrs as potentiators of Toll signaling in humoral immunity. Hrs is a subunit of the ESCRT 0 complex, regulating the initial stages of the sorting process targeting endocytosed membrane proteins to intralumenal vesicles of late endosomes/multivesicular bodies (MVB).[18-20] Interestingly, both Hrs and Mop, as well as components of the downstream ESCRT I complex are also necessary for proper Drosophila EGFR signaling in imaginal disc patterning and cell culture.[21,22] Miura et al. further demonstrate that receptor progression from early to late endosomes has a positive effect on EGFR signaling and that the absence of Hrs or Mop disrupts this process. However, the positive role of Hrs and the ESCRT machinery in Drosophila EGFR signaling is by no means universal, as Hrs appears to promote EGFR downregulation in early embryonic development.[20,22] In addition, Hrs is required for efficient FGFR and JAK/STAT signaling in flies,[9,22] but is a negative regulator of Wingless signaling, although the latter also requires endocytosis.[10]

Hrs is thought to recognize mono-ubiquitinated membrane proteins, recruiting them to Clathrin coated microdomains of early endosomes and ultimately committing them to the MVB pathway.[18-20] In the case of EGFR, ubiquitination is catalyzed by the Cbl E3 Ubiquitin ligase that also promotes endocytosis of activated receptors.[23] Although Toll is not a known Cbl substrate, K-63 linked polyubiquitination does play a central role in the signal transduction process of the MyD88/IRAK/TRAF6 pathway employed by mammalian TLRs[7] and the putative K-63 E3 ubiquitin ligase DTRAF2, the Drosophila ortholog of TRAF6, is indispensable in Toll signaling in the fat-body.[24] K-63 polyubiquitination has also been shown to target internalized membrane proteins to the MVB pathway[25] and Hrs exhibits a strong binding preference for K-63 polyubiquitin chains over monoubiquitin in vitro.[26] It is possible that Toll binds to the Mop/Hrs complex indirectly, through polyubiquitinated signaling components. However, Hrs has also been reported to interact with sorting substrates in a ubiquitin independent manner.[27]

Surprisingly, Hrs appears to be dispensable for Toll signaling during embryonic patterning, as embryos maternally lacking Hrs only exhibit a weakly ventralized phenotype, apparently due to a defect in EGFR attenuation.[20,22] While it is currently unknown whether Mop is necessary for embryonic Toll signaling, it is possible that the mechanistic basis for the endocytic requirement in adult and embryonic Toll function is different. This may in part reflect more profound differences in the signaling mechanism of the Toll pathway between embryos and adults, as the two versions of the pathway only have some, but not all, components in common. For instance, DTRAF2 is not needed in embryonic signaling,[24] while the novel signal transducer, Weckle, that does not appear to play any role in Toll signaling in fat-body and S2 cells, is indispensable for dorsoventral patterning.[28]

Notwithstanding the specific mechanisms governing internalization and trafficking of Toll in the immune response, the question remains as to why the targeting of Toll to an endocytic compartment is a requirement for embryonic signaling. It is unlikely that ligand recognition takes place in the endosomal system, as work in early embryos has shown that functional Toll is necessary to remove activated Spz form the perivitelline space.[16] However, it is possible that the ligand induced conformational change that allows Toll to dimerize[29] is stimulated by cofactors only present in an endosomal compartment. Alternatively, vital signal transducers might preferentially or exclusively be recruited to a subset of endosomal surfaces. The involvement of Hrs in Toll-dependent NFkappaB activation suggests that, at least in fat body and S2 cells, the signaling permissive compartment is the MVB or an Hrs positive subdomain of the early endosome. Here, Toll is presumably allowed to signal until it is sequestered into intralumenal vesicles through the action of the ESCRT complexes I, II and III.[30]

The Role of Intracellular Trafficking in Mammalian TLR Function

Although there would appear to be no mechanistic requirement limiting MAP Kinase and NFkappaB activation by TLR signaling to endosomal compartments, recent work by Kagan et al. has suggested that IRF activation through the TRIF dependent pathway is obligately endocytic.[11] The mechanistic basis for this appears to be the restriction of the signal transducer TRAF3 to the surfaces of endosomes. Consequently, targeting TRAF3 to the plasma membrane through a PLC-d1 pleckstrin homology domain can support activation of the IRF pathway by plasma membrane localized TLR2 which does not normally do so.[11] Kagan et al. demonstrate that TLR4 activated by LPS signals through the MyD88 dependent pathway from the plasma membrane to activate NFkappaB, but later switches to the TRIF dependent pathway to activate IRF3 upon endocytosis to an early endosomal compartment. The mechanism behind this switch, apart from the endocytic localization of TRAF3, appears to be the differential affinity of TIRAP/MyD88 and TRAM/TRIF adaptor pairs for the lipid composition of the plasma and endosomal membranes.[11]

In accordance with the obligate endocytic nature of TRAF3-dependent IRF3 and IRF7 activation, the entire class of nucleic acid sensing TLRs, comprising TLR3, TLR7, TLR8 and TLR9, all localize to intracellular compartments.[2,12] TLR3 signals through the TRIF dependent pathway to activate IRF3 and NFkappaB in response to dsRNA, while TLR7 and TLR9 activate IRF7 and NFkappaB in a MyD88 dependent manner in response to ssRNA and non-methylated CpG DNA respectively. Recent reports also indicate that TLR9 and TLR7 must be processed by endolysosomal proteases to become

fully functional.[31,32] Curiously, TLR9 and TLR7 exclusively localize to the ER in unstimulated cells, but quickly shuttle to endolysosomes upon ligand stimulation from where they signal.[33,34] Consequently, defects in the molecular machinery controlling this translocation can lead to susceptibility to viral and bacterial infections and have been shown to be involved in human disease.[35-37] Altered TLR trafficking has also been implicated in maintenance of anergy in B cells. Anergic B cells fail to translocate the B cell receptor (BCR) and TLR9 from early to late endosomes following BCR stimulation leading to a block in TLR signaling.[38]

Though it is presently unknown to what extent the role of endosomal trafficking in mammalian TLR function parallels the newly discovered endocytic requirement in Drosophila Toll signaling, it is becoming increasingly clear that the regulation of the Toll/TLR receptor family signaling is intimately tied to the endocytic pathway. Elucidating the basis for this requirement in the future will provide valuable insights into general properties of this widespread and important family of transmembrane receptors.

Acknowledgements

V.K.L. would like to thank the Carlsberg Fund and the Novo Nordisk Fund for support and R.D. would like to thank the Lundbeck Foundation and Novo Nordisk for research support.

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