INTRODUCTION

Dorsal/ventral polarity of the Drosophila embryo is determined by the asymmetric nuclear translocation of the Dorsal protein, an NF-κB like transcription factor (reviewed by Drier and Steward, 1997). The intracellular signaling cascade responsible for activation of Dorsal is highly conserved evolutionarily, and is similar to the mammalian IL-1 receptor/Toll pathway that leads to NF-κB activation in innate immunity (reviewed by Kopp and Medzhitov, 1999; Anderson, 2000). Consistent with this function in mammals, the Drosophila Toll pathway also plays important roles in anti-fungal defense (reviewed by Imler and Hoffmann, 2000).

Dorsal activation in embryos requires the function of 11 maternally expressed genes, among which Toll, tube and pelle encode proteins involved in signaling across the membrane to the cytoplasm. Although these genes have been well studied genetically (reviewed by Belvin and Anderson, 1996), the biochemical mechanism by which signaling is activated is poorly understood. Toll is a large transmembrane receptor, with an N-terminal extracellular leucine-rich repeat (LRR) region, a C-terminal intracellular domain that contains significant similarity with the corresponding region of the IL-1 receptor (also called a Tir domain), and a unique short inhibitory region (Hashimoto et al., 1988; Norris and Manley, 1996). Tir domains have now been found in many species, including flies, humans and plants, and define the Toll-like receptor (TLR) family (reviewed by O’Neill and Greene, 1998; Kopp and Medzhitov, 1999). Tube contains an essential N-terminal death domain (Feinstein et al., 1995) and interacts with Pelle (Galindo et al., 1995), although its exact role in signaling is not known. Pelle is a serine/threonine kinase that also contains an N-terminal death domain, as well as a C-terminal catalytic domain (Shelton and Wasserman, 1993). Several studies support the existence of a Toll/Tube/Pelle complex. Pelle has been shown to interact directly with Toll through its Tir domain in vitro (Shen and Manley, 1998). As Pelle and Tube interact via their death domains (Galindo et al., 1995), and Pelle binds Toll via its catalytic domain (Shen and Manley, 1998), a heterotrimeric protein complex can be formed. Supporting the significance of the Toll-Pelle interaction, Toll derivatives and Pelle can synergistically activate Dorsal in co-transfected Schneider cells (Norris and Manley, 1996). Pelle can co-localize to the membrane with the dominant gain-of-function mutant Toll10b protein in transgenic flies in which Toll10b is artificially overexpressed at the anterior tip of the embryo (Towb et al., 1998). Indeed, artificial membrane localization of Pelle can induce ventral fate, even in the absence of Tube (Galindo et al., 1995; Großhans et al., 1994; Großhans et al., 1999; Towb et al., 1998). These data suggest that Pelle may be recruited to the membrane and form a protein complex with Toll and Tube. Similarly, the IL-1R-
associated kinase (IRAK), a mammalian Pelle homolog, has been shown to associate with the IL-1R complex during IL-1 signaling (Cao et al., 1996a).

How is Pelle (or IRAK) regulated, and hence signaling activated, within the Toll complex? Is it simply by recruitment to the membrane, or might it require activation, or modification, of kinase activity? Oligomerization contributes to activation of an artificially membrane-localized Pelle (Großhans et al., 1999), which suggests that Pelle may naturally be activated in association with the Toll receptor complex after Toll oligomerization induced by ligand binding. Although nothing is known regarding the phosphorylation status of Pelle in vivo, IRAK is known to be phosphorylated upon IL-1 induction (Cao et al., 1996a; Cao et al., 1996b), consistent with the possibility that IRAK might be activated by phosphorylation. Both Pelle and IRAK can be autophosphorylated in vitro (Shen and Manley, 1998; Maschera et al., 1999). Pelle autophosphorylation abrogates its ability to bind both Tube and Toll in vitro (Shen and Manley, 1998), suggesting that Pelle might be released from the complex upon autophosphorylation. However, the relevance of these interactions to Pelle function is unknown. Likewise, whether IRAK phosphorylation following IL-1 stimulation is due to autophosphorylation, or what effect this modification has on activity, if any, is unclear. It has in fact been controversial whether the kinase activity of IRAK is actually required for IL-1R signaling (Knop and Martin, 1999; Li et al., 1999; Maschera et al., 1999; Vig et al., 1999). Taken together, more studies are necessary to elucidate the activation mechanism and function of Pelle/IRAK kinase in TLR signaling.

We have shown previously that Pelle directly interacts with Toll in vitro, and that this interaction, as well as the Tube-Pelle interaction, is disrupted by Pelle autophosphorylation (Shen and Manley, 1998). We provide evidence that Pelle is transiently phosphorylated in early embryos in a manner strictly dependent upon activation of the Toll signaling pathway. We show that in both transfected cells and in vitro with purified proteins Pelle autophosphorylation is induced at high concentrations. Strikingly, only the autophosphorylated form displays significant kinase activity. Based on these and other results, we present a model for activation of TLR signaling that involves Toll-mediated induction of Pelle autophosphorylation.

MATERIALS AND METHODS

Plasmids, fly stocks and antibodies

The HisPelle-encoding plasmid and the rabbit anti-Pelle polyclonal antibody were described previously (Shen and Manley, 1998). Plasmids for transfections have also been described previously (Norris and Manley, 1992; Norris and Manley, 1995; Norris and Manley, 1996). Wild-type flies were a gift from Dr D. Kalderon (Columbia University). Toll mutant flies, QRXrX^B3 (Toll knockout) (Hashimoto et al., 1988), Toll10b and r444 (Schneider et al., 1991) were kindly provided by Dr K. Anderson (Sloan Kettering Cancer Institute). Gastrulation-defective (ggf) stock was a gift from Dr S. Wasserman (UC San Diego). The rabbit anti-Toll cytoplasmic domain polyclonal antibody was a gift from Dr C. Hashimoto (Yale University). Rat anti-Dorsal polyclonal antibody was a gift from Dr C. Rushlow (New York University).

Whole-embryo extracts

Flies were allowed to deposit eggs on fresh apple-juice-agar plates with yeast paste for 1 hour, and then removed. After 0-5 hours, embryos on the plates were collected, washed and dechorionated. Embryos were homogenized in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS and protease inhibitors) with or without phosphatase inhibitors (50 mM NaF and 50 mM β-glycerol phosphate), and centrifuged at 30,000 g for 15 minutes. The clear protein supernatant was carefully separated from floating lipid and precipitate, and quantitated by Bradford assay. Equivalent amounts of embryo extracts were subjected to SDS-PAGE and western blotting (Olahmeyer and Kalderon, 1998). For dephosphorylation assays, freshly made whole embryo extracts without phosphatase inhibitors were either incubated at 30°C for 1 hour, or incubated with CIP-beads in CIP buffer at 37°C for 1 hour, before analyzed on SDS-PAGE and western blotting.

Transfection of SL2 cells and CAT assays

Transient transfection of Drosophila Schneider line 2(SL2) cells was performed as described previously (Han et al., 1989; Norris and Manley, 1996).

Affinity purification of anti-Pelle antibodies

HisPelleK240R was purified from E. coli as described (Manley, 1998), dia lyzed against Buffer A (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 0.1% Tween 20 and 5% glycerol), and then loaded on a 1 ml MonoQ column (Pharmacia). The flow-through was collected and conjugated to Affi-Prep 10 beads (BioRad). Anti-Pelle antibody was purified by HisPelleK240R affinity chromatography as described (Harlow and Lane, 1988).

Purification of different forms of HisPelle

Autophosphorylated HisPelle was directly purified from E. coli as before (Shen and Manley, 1998). HisPelle was dephosphorylated by calf intestinal phosphatase (CIP) in CIP buffer (Shen and Manley, 1998) at 37°C for 1 hour. Beads were then extensively washed with 20 volumes of 1 M NaCl in buffer B (20 mM Tris-HCl pH 8.0, 0.5% Tween 20 and 5% glycerol), and then loaded on a 1 ml MonoQ column (Pharmacia). The flow-through was collected and conjugated to Affi-Prep 10 beads (BioRad). Anti-Pelle antibody was purified by HisPelleK240R affinity chromatography as described (Harlow and Lane, 1988).

In vitro kinase assays

The indicated amounts of purified proteins were incubated in 30 µl of kinase buffer (Shen and Manley, 1998) containing 10-20 µM ATP and 5-10 µCi of [γ-32P]ATP (NEN or Amersham) at 30°C for 30-60 minutes. Reactions were stopped by TCA precipitation in the presence of 0.3 µg/µl bovine serum albumin (BSA) on ice for at least 30 minutes. Washed precipitates were analyzed by SDS-PAGE, and phosphorylated proteins were detected by autoradiography and/or PhosphorImager analysis (Shen and Manley, 1998). In Pelle activation assays, increasing concentrations of CIP-Pelle (25-100 µg/ml) were preincubated in kinase buffer containing 30 µM ATP and 0.3 µCi/µl [γ-32P]ATP at 30°C for 1 hour. Identical amounts of such Pelle proteins were then removed and incubated at the same concentration (5 µg/ml) with 5 µg/ml HisTubeN for 30 minutes. Reactions were normalized to contain identical amounts of ATP. Reactions were stopped by adding SDS sample buffer and directly analyzed by SDS-PAGE.

GST pull-down assays

GST (2 µg) or GST fusion proteins purified from E. coli were immobilized on glutathione agarose, and then incubated with 1 µg of CIP-Pelle in 40 µl of NETN buffer at room temperature for 1.5 hours.
RESULTS

Pelle is phosphorylated in early embryos concomitant with signaling

Our previous studies indicated that recombinant Pelle is capable of efficient autophosphorylation (Shen and Manley, 1998). But nothing is known about the phosphorylation status of Pelle in vivo. For example, is Pelle phosphorylated in early embryos, and if so does phosphorylation change during signaling? To investigate this, we prepared whole-embryo extracts from staged embryos and subjected them to immunoblotting using affinity-purified anti-Pelle polyclonal antibodies (see Materials and Methods). The results with extracts from wild-type embryos are shown in Fig. 1A. The majority of Pelle was detected as a single species, which based on its mobility compared with known standards (e.g. recombinant kinase-inactive Pelle K240R; data not shown) appears to be unmodified. But a small amount of protein could be detected migrating slightly slower than the major species, consistent with possible phosphorylation. Significantly, appearance of this minor species was transient, peaking between 2-4 hours after egg laying, which corresponds to the time when Toll signaling is activated.

To extend these results, we examined Pelle in mutant backgrounds that result in either enhanced (Toll10b; Fig. 1B) or reduced (Tollr444, Gd– and 9QRX; Fig. 1C) signaling. Strikingly, the strong Toll10b gain-of-function mutation (Schneider et al., 1991) resulted in a dramatic increase in the amount of modified Pelle. Appearance of the modified form was again transient, peaking between 2 and 4 hours post-fertilization and fading to background levels by 4-5 hours. By sharp contrast, the loss-of-function mutants all showed significantly reduced or undetectable levels of the modified form. [Note the absence of Toll in the 9QRX embryos, which contain a Toll null allele (Hashimoto et al., 1988).]

To determine whether the low-mobility species indeed represented a phosphorylated form of Pelle, whole-embryo extracts were prepared in the absence of phosphatase inhibitors from Toll10b embryos. Such embryo extracts were previously shown to be able to dephosphorylate Dorsal by the activity of endogenous phosphatases (Gillespie and Wasserman, 1994). We thus incubated Toll10b extracts at 30°C for 1 hour, before electrophoresis and western blotting. Fig. 1D shows that the low mobility species was eliminated in the absence (compare lane 1 with lane 2) but not the presence (lane 3) of phosphatase inhibitors. Similar results were observed in embryo extracts incubated with calf intestinal phosphatase (CIP) (Fig. 1D, lanes 4 and 5). Together, these results indicate that a fraction of Pelle present in early embryos is transiently phosphorylated in response to Toll signaling.

Pelle can be autophosphorylated in transfected Schneider L2 cells

We next wished to learn more about the nature and function of Pelle phosphorylation. For example, does Pelle undergo autophosphorylation? What is responsible for inducing phosphorylation? To address these questions, we first employed transient transfection assays in Drosophila Schneider L2 (SL2) cells. This assay allows manipulation of analyzed by SDS-PAGE and western blot as in A. (D) Pelle is phosphorylated in Toll10b embryos. For dephosphorylation by endogenous phosphatases, Toll10b whole embryo extract from the second to fourth hours after egg laying (lane 1) was incubated at 30°C with or without NaF (lane 2, 3) for 1 hour. The same extract was also incubated with immobilized calf intestine phosphatase (CIP-beads), with or without NaF (lane 4, 5), at 37°C for 1 hour. Pelle protein was analyzed by SDS-PAGE followed by anti-Pelle immunoblotting.

Fig. 1. Signal-dependent phosphorylation of Pelle during early embryogenesis. (A) Pelle is transiently modified in early embryos. Whole embryo extract was prepared from wild-type embryos of different stages (see Materials and Methods), and equivalent amounts of extract were subjected to SDS-PAGE. Pelle was detected by anti-Pelle immunoblotting (top). Anti-Toll immunoblotting was used as control (bottom). Lanes 1-6, embryo extracts were from 1- to 6-hour-old embryos as indicated. (B) Pelle modification is enhanced in gain-of-function Toll mutant embryos. Wild-type or mutant embryos with an activated Toll allele (Toll10b) were collected and analyzed as in A. The top panel is an anti-Pelle blot, and the bottom panel is a blot with anti-Toll antiserum. Lanes 1-5, wild-type embryos between 0 and 5 hours after egg laying; lanes 6-10, Toll10b embryos between 0 and 5 hours after egg laying. (C) Pelle modification is decreased in loss-of-function Toll mutant embryos. Wild-type, Toll10b, gastrulation defective null mutant (Gd+), weak loss-of-function Toll mutant (Tollr444) and Toll knockout (9QRX) embryos were collected 2-4 hours after egg laying and
the amounts and combinations of specific proteins, and we have used it previously to gain insights into the molecular interactions involved in Toll signaling (Norris and Manley, 1992; Norris and Manley, 1995; Norris and Manley, 1996). In the experiments described here, we have used expression vectors encoding wild-type or mutant derivatives of Pelle and/or Toll, as well as Dorsal plus a Dorsal-responsive CAT reporter plasmid (see Materials and Methods). This allowed us to monitor both Pelle phosphorylation status (by western blotting) and function (by Dorsal phosphorylation or activation of CAT activity) under a variety of conditions.

We showed previously that expression of Pelle in the absence of Toll was sufficient to bring about moderate activation of Dorsal in SL2 cells (Norris and Manley, 1996). To examine the phosphorylation status of Pelle in SL2 cells, cells were transfected with increasing concentrations of expression vector encoding either wild-type Pelle or the catalytically inactive mutant, PelleK240R (Shelton and Wasserman, 1993; Norris and Manley, 1996), plus the vector expressing Dorsal and the reporter plasmid. Fig. 2A (top) displays a western blot of whole-cell lysates prepared from the transfected cells probed with the anti-Pelle antibodies. Not unexpectedly, the amount of both wild-type and mutant Pelle increased with the amount of expression vector (PelleK240R, lanes 2-5; Pelle, lanes 6-9). Note that cells lacking a Pelle expression vector contained detectable levels of Pelle (lane 1). This endogenous expression varies and is not always detected (Norris and Manley, 1996). At higher concentrations, several low mobility, phosphorylated forms were detected with wild-type but not mutant kinase. Strikingly, the ratio of phosphorylated to unphosphorylated Pelle isoforms increased significantly as the concentration of Pelle increased. At the lowest concentrations (lanes 6 and 7), low mobility forms were essentially undetectable. At an intermediate concentration (lane 8), phosphorylated forms were readily apparent, but represented less than half of the total Pelle detected. At the highest concentration (lane 9), significantly more than half of the total Pelle was phosphorylated. These data indicate that Pelle becomes phosphorylated in a concentration-dependent manner when expressed in SL2 cells. Given that this phosphorylation requires Pelle kinase activity, and the ability of the protein to autophosphorylate itself efficiently when produced in Escherichia coli (Shen and Manley, 1998), we conclude that the observed modification reflects concentration-dependent autophosphorylation. Consistent with the view that this
phosphorylation is functionally significant, Dorsal is activated in a similar concentration-dependent manner by wild-type but not mutant Pelle, as measured either by Dorsal phosphorylation (Fig. 2A, bottom) or CAT activity (Fig. 2B). Note that although Dorsal phosphorylation in embryos is in part signal dependent (Gillespie and Wasserman, 1994), it is not known whether this is directly or indirectly mediated by Pelle.

The concentration of Pelle does not change significantly during embryogenesis (Fig. 1A), so signal-dependent phosphorylation of Pelle in embryos probably does not result simply from increasing concentration. But a related idea is that a signal-activated Toll/Tube/Pelle complex might induce Pelle autophosphorylation by transiently creating a high local concentration of Pelle. To provide support for this, we asked whether Toll expression could enhance Pelle autophosphorylation in SL2 cells (note that Tube but not Toll is expressed endogenously in these cells (Norris and Manley, 1995)). Cells were transfected with expression vectors encoding Toll10b, Pelle and Dorsal, using an amount of the Pelle plasmid that would not produce sufficient Pelle by itself to allow detectable phosphorylation. Fig. 3A displays anti-Pelle western blots of cell lysates from the transfected cells. Pelle phosphorylation was detected in the presence but not the absence of Toll10b, and the pattern closely resembled that observed in early embryos during signaling (Fig. 1). This combination also produced high CAT activity, indicative of activated Dorsal (Fig. 3B). No phosphorylation, or increased CAT activity, was observed when Pelle K240R was expressed instead of Pelle.

**Pelle autophosphorylation is necessary for kinase activity in vitro**

The above experiments provide evidence that Pelle becomes autophosphorylated during Toll-dependent signaling, and that this modification can be induced by increased Pelle concentration. But what if anything might be the effect of this on Pelle activity? And what is the mechanism responsible for the concentration-dependent phosphorylation observed in Schneider cells? To address these questions, we purified recombinant his-tagged Pelle (HisPelle) from E. coli, which, as shown before, is autophosphorylated and displays considerable activity in in vitro kinase assays (Shen and Manley, 1998). To compare its properties with those of unphosphorylated Pelle, we needed to obtain purified unphosphorylated Pelle. The most effective means to accomplish this was to treat HisPelle with CIP and then repurify the dephosphorylated enzyme (CIP-Pelle; see Fig. 4A, lane 1 and Materials and Methods). The two kinase preparations were then used in in vitro kinase assays, analyzing both autophosphorylation and phosphorylation of a Tube derivative, HisTubeN (Shen and Manley, 1998). Strikingly, while HisPelle displayed the high activity anticipated from our previous study, an equivalent amount of CIP-Pelle (6.7 g/ml) was in comparison nearly inactive (Fig. 4B; the bottom panel verifies that reactions contained the same amounts of kinase and substrate). This inactivity was not due to the presence of contaminating CIP in the repurified CIP-Pelle, because addition of the phosphatase inhibitor NaF did not affect activity, and a mixture of CIP-Pelle and HisPelle displayed high activity (results not shown). Furthermore, the CIP treatment did not irreversibly damage the kinase because, as shown in the next section, the dephosphorylated enzyme could be reactivated.

**Concentration-dependent autophosphorylation and activation of dephosphorylated Pelle in vitro**

The above results allowed us to test the idea that the concentration-dependent autophosphorylation we observed in SL2 cells might reflect activation of the kinase induced by multimerization and subsequent transphosphorylation. Specifically, we next tested whether incubation of increasing concentrations of CIP-Pelle under phosphorylation conditions could induce autophosphorylation and kinase activity. In the experiment shown in Fig. 5A, increasing amounts of CIP-Pelle (25-100 µg/ml, lanes 1-4) were first preincubated in the presence of ATP. After this initial incubation, 50 ng was removed from each sample and incubated at a concentration of 5 µg/ml with HisTubeN as a substrate. Strikingly, over this relatively small concentration range during preincubation, a significant increase in kinase specific activity (more than fourfold), in both autophosphorylation and Tube phosphorylation, was observed. The western blots in Fig. 5B show two things: first, equivalent amounts of Pelle and Tube were in fact present in each of the phosphorylation assays shown in Fig. 5A; second, low but increasing amounts of slow-mobility forms of Pelle (upper arrows) were detected with increasing Pelle concentration during preincubation. One or both of these probably represent the kinase-active form of the...
enzyme. These results provide strong biochemical evidence that Pelle kinase activity is activated by concentration-dependent autophosphorylation.

We next wished to provide further evidence that activation of Pelle reflects self-association and transphosphorylation. We first examined whether unphosphorylated Pelle is in fact capable of transphosphorylation (Fig. 6A). To this end, a low but active concentration of CIP-Pelle was incubated in phosphorylation conditions either alone (lane 1) or together with catalytically inactive GST-PelleK240R (lane 2). The results indicate that unphosphorylated Pelle was capable not only of increased autophosphorylation (compare lanes 1 and 2), but also of phosphorylating GST-PelleK240R (which as expected was by itself inactive; lane 3). To determine whether unphosphorylated Pelle can stably self-associate, we performed GST ‘pull-down’ assays using GST-PelleK240R and CIP-Pelle (Fig. 6B). CIP-Pelle was incubated either with GST or GST-PelleK240R, and after washing, proteins were eluted with glutathione and detected by western blotting. The results indicate that CIP-Pelle bound to GST-PelleK240R but not to GST, indicating that Pelle is in fact capable of self-association. Together, these findings indicate that Pelle is autophosphorylated and activated by concentration-dependent self-association and resultant transphosphorylation.

**DISCUSSION**

We have shown here that Pelle is transiently phosphorylated in early *Drosophila* embryos, concomitant with signaling and dependent on upstream components. Co-transfection assays provided the first evidence that Pelle becomes autophosphorylated at high concentrations, and also showed that at a low concentration Pelle phosphorylation is induced by an activating signal from Toll. Significantly, in vitro kinase assays showed directly that Pelle can be autophosphorylated, and activated, by incubation at high concentrations. We present a model in which Pelle is activated by a transient high local Pelle concentration that is created by interactions within a signal-dependent Toll receptor complex (Fig. 7). Below we discuss this model and its implications for Tir domain signaling more generally.

**Formation and function of Toll/Tube/Pelle complex**

As mentioned in the Introduction, several lines of evidence indicate that Pelle must be recruited to the membrane for signaling, and have even suggested that such localization might be sufficient to activate the pathway. But an important question regards the timing of this recruitment: is Pelle recruited to Toll activation? In normal embryos, a mesh-like distribution of Pelle throughout the embryo reveals no dorsal/ventral asymmetry or enhanced membrane localization at the syncytial blastoderm (i.e. activated) stage (Towb et al., 1998), suggesting that Pelle membrane localization does not depend on Toll activation. This may indicate that the interactions between Toll and Pelle are weak or transient, but sufficient for signaling, and/or that only a small amount of Pelle needs to be membrane localized, and activated. Indeed, the amount of Pelle in wild-type embryos appears to be in excess over the levels required to activate signaling (Muller-Holtkamp et al., 1985). Consistent with this, our data show that only a small fraction of Pelle is phosphorylated in response to Toll signaling in early embryos, implying that a small amount of Pelle is sufficient for signaling. We thus suggest that Pelle interacts with Toll, or is membrane localized, prior to Toll
Activation, but such complexes are essentially monomeric and thus inactive (Fig. 7). In the absence of ligand, steric hinderance of Toll extracellular domains has even been suggested to block Tir-domain association, preventing spontaneous Pelle activation that is due to transient Toll-Pelle interactions (Winans and Hashimoto, 1995). Ligand binding, however, induces Toll multimerization, leading to activation of Tube and Pelle for signaling.

In mammalian systems, it is generally believed that IRAK is recruited to IL-1R upon IL-1 induction. But it is nonetheless possible that IRAK is membrane associated via interaction with a Tir domain protein before signaling. In contrast to the direct interaction of Pelle with Toll (Shen and Manley, 1998), IRAK has been shown to interact with IL-1RacP (IL-1R Accessory Protein), not IL-1R (Muzio et al., 1997). However, IL-1RacP is similar in structure to IL-1R, containing a Tir domain and related extracellular domain, and is an essential component of the IL-1R complex (Greenfeder et al., 1995; Cullinan et al., 1998). Significantly, an apparently direct interaction between IL-1RacP and IRAK was detected in yeast two-hybrid assays (Voippe et al., 1997). These findings suggest that IRAK might also membrane localized, via its interaction with IL-1RacP, before ligand binding. Upon IL-1 induction, IRAK, together with IL-1RacP and another accessory protein, MyD88, is recruited to the IL-1R complex (Huang et al., 1997), which we suggest leads to IRAK autophosphorylation and activation. The affinity of IL-1R for IL-1 is increased by IL-1RacP (Cullinan et al., 1998), supporting the significance of the IL-1RacP association.

**Activation mechanism of Pelle kinase**

How is Pelle regulated within the Toll receptor complex? We have shown here that Pelle is activated by transphosphorylation at high concentrations in vitro. Studies with chimeric Pelle constructs demonstrated that Pelle fused to a Torso receptor mutant capable of ligand-independent oligomerization, but not to wild-type Torso, was capable of inducing ventral fate in *snake* mutant embryos, suggesting that Pelle multimerization, not simply membrane localization, is necessary to activate Pelle (Großhans et al., 1999). Similar to the behavior of this chimeric Pelle, dimerization of Pelle bound to Toll could be facilitated by Toll dimerization. Indeed, we have obtained in vitro evidence that Toll Tir domains are capable of self-association (B. S. and J. L. M., unpublished). Moreover, comparative modeling suggests that the Toll ligand, Spätzle, forms a dimer containing a single, intermolecular disulphide bridge (Mizuguchi et al., 1998), and processed Spätzle has in fact been shown to be a dimer (DeLotto and DeLotto, 1998). Hence, a plausible mechanism is that Spätzle dimersizes and activates Toll by inducing receptor dimerization. This proposed mechanism suggests that Toll-Tir domains and Tube (curved arrows), Pelle (and Tube) are released from Toll, and Pelle phosphorylates downstream targets. See text for additional details.
phosphorylation, and activation, requires Toll signaling. We also observed that the timing of Pelle phosphorylation is indistinguishable in wild-type and gain-of-function Toll (Toll) embryos. This suggests that generation of the same threshold of activating signal upstream of Toll is necessary to potentiate efficient signaling, i.e., activation of Pelle, in both cases. Consistent with this, in mRNA microinjection assays, easter null embryos require fivefold more Toll (Toll) transcript to induce ventral fate than do Toll null embryos (Schneider et al., 1991). Likewise, phosphorylated Pelle disappears in the fifth hour after egg laying in both wild-type and Toll (Toll) embryos, indicating a downstream regulation of signaling. Autophosphorylated Pelle could directly de-sensitize signaling (Towb et al., 2001), perhaps by phosphorylating Toll (Shen and Manley, 1998) and/or signaling Pelle degradation. Indeed, evidence has been presented that IRAK is degraded by the proteasome after phosphorylation (Yamin and Miller, 1997). Other mechanisms may also contribute to Pelle regulation. For example, Raf1, a kinase of the same superfamily as Pelle, can be regulated at multiple levels, including phosphorylation by other kinases and protein-protein interactions (reviewed by Morrison and Cutler, 1997; Sun et al., 2000). Recently, a novel protein, Tollip, was found to interact with IRAK and IL-1RαCp before their recruitment to the IL-1R complex, and to display an inhibitory effect (Burns et al., 2000). Whether related proteins exist in Drosophila is not known.

Function of activated Pelle

Whether the kinase activity of IRAK is necessary for IL-1R signaling or not is controversial. For example, IL-1-induced NF-κB activation is reduced, but not completely abrogated, in IRAK-deficient mice (Thomas et al., 1999). Transient transfection assays with kinase-inactive IRAK mutants show that such mutants still induce NF-κB activation in response to IL-1 (Knop and Martin, 1999; Li et al., 1999; Maschera et al., 1999; Vig et al., 1999). It is possible that different IRAKs (IRAK, IRAK2 and IRAKM) (Cao et al., 1996a; Muzio et al., 1997; Wesche et al., 1999) are functionally redundant, and our results raise the possibility that transfected IRAK mutants could facilitate activation by associating with endogenous IRAKs. In Drosophila, the kinase inactive PelleK240R mutant is not only incapable of activating Dorsal, but also dominantly inhibits Toll-mediated activation in transfected Schneider cells (Norriss and Manley, 1996) (Fig. 3A). Additionally, PelleK240R has been shown to be unable to rescue ventral fate in pelle mutant embryos (Shelton and Wasserman, 1993). Taken together, Pelle kinase activity is required for dorsal/ventral pattern formation in embryos.

What is the function of activated Pelle? We previously showed that autophosphorylation allows Pelle to dissociate from both the Toll receptor and Tube (Shen and Manley, 1998). On the one hand, Pelle function might thus be to phosphorylate Toll and Tube to facilitate Pelle dissociation following activation (Shen and Manley, 1998). Phosphorylated Toll might then recruit other factors, analogous to cytokine receptors, that recruit STAT after being phosphorylated by JAK kinase (reviewed by Schindler and Darnell, 1995), although there is currently no evidence for this. On the other hand, it seems highly likely that autophosphorylated, activated Pelle phosphorylates specific downstream targets. For example, Pellino and Dorsal were both shown to bind phosphorylated wild-type Pelle but not PelleK240R (Großhans et al., 1999; Edwards et al., 1997), suggesting that one or both proteins might be a Pelle target. In addition, Pelle can phosphorylate Cactus in vitro (Großhans et al., 1994) and mouse Pelle (IRAK) is able to phosphorylate IkBα (Trofimova et al., 1996), suggesting the possibility of direct physical and functional interactions between Pelle and the Cactus/Dorsal complex (Edwards et al., 1997). Moreover, the TNF receptor-associated factor TRAF6 functions in NF-κB activation by mammalian TLRs, and interacts with IRAK upon IL-1 induction (Cao et al., 1996b). Drosophila TRAF2, an apparent TRAF6 homolog (Liu et al., 1999), is a target of Pelle phosphorylation in vitro, and contributes strongly to Dorsal activation in transfected Schneider cells (Shen et al., 2001).

In summary, we have shown here that Pelle is transiently phosphorylated upon Toll activation in Drosophila embryos. Pelle autophosphorylation is concentration dependent and can be induced by activated Toll in co-transfected Schneider cells. More importantly, Pelle autophosphorylation is necessary for kinase activity, and dephosphorylated Pelle can be activated by autophosphorylation at high concentrations. Based on these results, we have presented a model in which Toll-bound, unphosphorylated Pelle is activated by transphosphorylation at high local concentrations, which are created by Toll multimerization induced by ligand binding. Available data suggest that this model is probably relevant to the function of TLR proteins generally. A next important question is how activated Pelle transmits its signal downstream, and ultimately to the Dorsal/Cactus complex. Analysis of Pelle-interacting factors and target proteins will help to fill the gap between the Toll receptor complex and the Dorsal/Cactus complex. But our results have provided a molecular mechanism for the first intracellular steps in the Toll pathway.

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