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Phosphorylation modulates direct interactions between the Toll receptor, Pelle kinase and Tube

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SUMMARY

Determination of dorsal/ventral polarity in *Drosophila* requires 12 genetically defined, maternally encoded proteins. These include Toll, a transmembrane receptor, Pelle, a ser/thr protein kinase and Tube, all of which function intracytoplasmically to initiate the cascade that ultimately activates Dorsal, an NF-κB family transcription factor. Here we describe biochemical interactions between recombinant Toll, Pelle and Tube that provide insights into early events in activation of the signaling cascade. We first show that Pelle binds directly to a region within the Toll intracytoplasmic domain, providing the first evidence that these two evolutionarily conserved molecules physically interact. We then demonstrate that Pelle can be autophosphorylated, and that this prevents binding to Toll as well as Tube. Autophosphorylation occurs in the N-terminal, death-domain-containing region of Pelle, which is dispensable for binding to Toll but required for enzymatic activity. We also show that Pelle phosphorylates Toll, within the region required for Pelle interaction, but this phosphorylation can be blocked by a previously characterized inhibitory domain at the Toll C terminus. These and other results allow us to propose a model by which multiple phosphorylation-regulated interactions between these three proteins lead to activation of the Dorsal signaling pathway.

Key words: *Drosophila melanogaster*, Pelle, Tube, Toll, Dorsoventral polarity, autophosphorylation

INTRODUCTION

Activation of the cascade that determines dorsal-ventral polarity in the *Drosophila* embryo requires the activities of 12 maternally expressed genes (reviewed by Belvin and Anderson, 1996; Morisato and Anderson, 1995; Steward and Govind, 1993). The products of the gene *ndl, pip, wind, gd, snk* and *ea* are involved in localized proteolytic processing of the product of *spätzle* in the perivitelline space on the ventral side of the embryo (Stein et al., 1991; Stein and Nüsselein-Volhard, 1992; Morisato and Anderson, 1994; Schneider et al., 1994). Through the binding of the ligand Spätzle to the receptor Toll, an intracytoplasmic pathway requiring the products of the *tube* and *pelle* genes is turned on in the ventral-most and ventral-lateral regions of the embryo. This leads to formation of a nuclear gradient of the transcription factor Dorsal, with the highest levels in the ventral-most region and the lowest in the dorsal-most region (Roth et al., 1989; Steward, 1989; Rushlow et al., 1989). In addition to their roles in early development, Dorsal, Toll, Pelle and Tube are also involved in the *Drosophila* host defense against microorganisms (Lemaitre et al., 1997; reviewed by Meister et al., 1997).

Toll is a large transmembrane receptor protein with several functional domains (Hashimoto et al., 1988). Its 803 residue N-terminal extracellular domain contains two blocks of leucine-rich repeats (LRR) and adjacent cysteine-containing motifs (Hashimoto et al., 1988; Schneider et al., 1991). Deletion of the LRRs leads to ventralization of the embryo (Winans and Hashimoto, 1995). The C-terminal intracytoplasmic region of Toll contains a 200 residue IL-1R (type I interleukin-1 receptor) homologous domain that is essential for Toll activity (Schneider et al., 1991; Norris and Manley, 1992), plus an extra 68 residues of unique sequence at the very C terminus. Deletion of this unique region increases transcriptional activity of Dorsal in cotransfected Schneider cells, suggesting it plays an inhibitory function (Norris and Manley, 1995, 1996). Toll is evenly distributed throughout the membrane at the syncytial blastoderm stage when its activity is needed for dorsal-ventral polarity, suggesting that the activating signal is spatially regulated (Anderson and Nüsslein-Volhard, 1986; Hashimoto et al., 1991).

Genetic studies indicate that the *pelle* and *tube* products are required downstream of Toll. *tube* encodes a protein with an N-terminal death domain (see Feinstein et al., 1995) and a C-terminal domain containing five copies of an 8-residue motif (Letsou et al., 1991). Tube is initially localized to the plasma membrane of the embryo, but it can also be detected in nuclei (Galindo et al., 1995; Towb et al., 1998). Tube can also co-localize with Dorsal in nuclei of cotransfected cells as well as function as a transcriptional activator, perhaps facilitating activation by Dorsal (Norris and Manley, 1995, 1996). Domain mapping has shown that the N-terminal region of Tube is necessary and sufficient to rescue a *tube* null allele (Letsou et

al., 1991, 1993). Pelle is a serine/threonine protein kinase consisting of a C-terminal catalytic domain and an N-terminal putative regulatory domain containing, like Tube, a region with homology to the consensus death domain (Shelton and Wasserman, 1993; Feinstein et al., 1995). Several lines of evidence suggest that Tube and Pelle interact directly. The existence of cold sensitive mutant *pelle* and *tube* alleles suggested that they probably function in a protein complex, and enhanced lethality of double mutants indicates that they function in a common pathway (Hecht and Anderson, 1993). In addition, the N-terminal region of Tube interacts with the putative Pelle regulatory domain in yeast two-hybrid assays, an interaction presumably mediated by the death domains (Edwards et al., 1997). Membrane localization of Pelle or Tube can be sufficient to induce the ventral fate (Großhans et al., 1994; Galindo et al., 1995; Towb et al., 1998). Pelle can phosphorylate Tube in vitro (Großhans et al., 1994), but whether this is a physiological substrate, and the identity of other potential Pelle substrates, is not known.

As suggested initially by the similarity between the Toll and IL-1R intracytoplasmic domains, the Dorsal signaling pathway is highly related to activation of mammalian NF-κB by IL-1. Dorsal is an NF-κB like transcription factor whose activity is regulated by sub-cellular localization (reviewed by Govind and Steward, 1991; Verma et al., 1995; Morisato and Anderson, 1995). Cactus, an I-κB-like protein, inhibits Dorsal activation by retaining Dorsal in the cytoplasm of the early embryo (Roth et al., 1991; Gillespie and Wasserman, 1994; Belvin et al., 1995). I-κB is degraded by a ubiquitin-related proteosome pathway upon phosphorylation by an I-κB kinase (Verma et al., 1995; Chen et al., 1996). Cactus seems to be regulated by a similar mechanism and it forms a cytoplasmic concentration gradient inversely correlated to the nuclear concentration gradient of Dorsal (Reach et al., 1996; Bergmann et al., 1996). Phosphorylation of Dorsal by protein kinase A (PKA) is important for Dorsal transcriptional activity and nuclear localization in transfected cells (Norris and Manley, 1992), while NF-κB is phosphorylated by associated PKA during activation (Zhong et al., 1997). Mouse pelle-like kinase (mPLK), IRAK (human interleukin-1 receptor-associated kinase) and IRAK2 have been cloned and all three share significant homology with Pelle (Trofimova et al., 1996; Cao et al., 1996; Muzio et al., 1997).

Studies employing cotransfected Schneider cells have shown that a Toll derivative lacking its inhibitory domain and Pelle can synergistically activate Dorsal (Norris and Manley, 1996). However, a direct interaction between Toll and Pelle has not yet been detected, perhaps because it is transient and/or indirect, mediated by other factors. In humans, IRAK associates with an IL-1R complex and becomes phosphorylated upon ligand binding (Cao et al., 1996). IL-1R Acp (IL-1R accessory protein) co-immunoprecipitates with IL-1R upon IL-1 stimulation, and is necessary for recruiting IRAK to the IL-1R complex (Huang et al., 1997; Wesche et al., 1997a). MyD88, a macrophage differentiation marker with an N-terminal death domain and a C-terminal IL-1R homologous domain (Hultmark, 1994; Hardiman et al., 1996), is likely an adapter between IL-1R and IRAK (Muzio et al., 1997; Burns et al., 1998). MyD88 differentially interacts with IL-1R Acp and IRAK2, by which IL-1R, IRAK2 and IRAK may be recruited into the same IL-1R complex (Muzio et al., 1997). Thus the mammalian system is complex, and raises the possibility that the Toll-Pelle, and IL-1R-IRAK, interactions may be indirect.

The biochemical interactions between Toll, Tube and Pelle during activation of the signaling pathway are poorly understood. For example, do the proteins all interact directly, or are additional factors required? Are these interactions static, or do they change during activation of the signaling pathway? And what is the initial activating event? Here we have investigated the nature of the interactions between the three proteins in vitro. The data we present show that Toll, through its IL-1R homologous domain, interacts directly with Pelle. Strikingly, this interaction can be detected only when Pelle is unphosphorylated. We show that Pelle can autophosphorylate itself in its regulatory domain, and that this prevents interaction with both Toll and Tube. We show that Pelle can also phosphorylate Toll, and that this phosphorylation can be modulated by the Toll inhibitory domain. We discuss a model whereby these phosphorylation-dependent interactions constitute an early step in activation of the Dorsal signaling cascade.

MATERIALS AND METHODS

DNA constructs

All expression plasmids were constructed by standard subcloning procedures. pGEX-2TK was used to make GST-fusion constructs. pET11aHisAT (provided by Chi Li) was used to construct His-tag expression plasmids. pET3a was used to construct plasmids for in vitro transcription and translation. Gene fragments for subcloning (shown in Fig. 1) were obtained either by PCR amplification or by restriction enzyme digestion from constructs described previously (Norris and Manley, 1992, 1995, 1996).

Protein purification

GST fusion proteins were expressed in *E. coli* JM101. 250 ml of bacterial culture was centrifuged at 6000 rpm for 5 minutes. Cells were resuspended in NETN buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA and 0.5% NP40) supplemented with 1 mM PMSF and sonicated. Suspensions were centrifuged at 14,000 rpm for 10 minutes and the supernatant was incubated with 500 μl of 50% pre-equilibrated glutathione-agarose slurry by rotation at 4°C for at least 1 hour. Beads were washed with NETN or NETN plus 1 M NaCl and then elution buffer (100 mM Tris-HCl pH 8.0, 120 mM NaCl) for three times each. The GST-fusion proteins were eluted twice with 500 μl of elution buffer containing 20 mM reduced glutathione by rotation in a cold room for 30 minutes. Protein concentrations were quantitated by Bradford assays and BSA was used as standard. Intactness, purity and concentration were also monitored by SDS-PAGE. The eluent was then dialyzed against dialysis buffer (20 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.2 mM EDTA, 1 mM DTT and 20% glycerol). Purified proteins were aliquoted and flash frozen at −70°C.

His-tagged proteins were expressed in 250 ml *E. coli* BL21 (DE3). When the OD_{600} was between 0.6-0.7, 0.4 mM IPTG was added and cells were induced for 3 hours at 37°C. Cells were centrifuged and resuspended in 10 ml buffer B (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 0.1% Tween-20) containing 1 mM PMSF. After sonication, extracts were centrifuged at 14,000 rpm for 10 minutes. Supernatants were incubated with 1 ml of pre-equilibrated Ni-agarose at 4°C with rotation for at least 1 hour. Beads were washed with buffer B containing 20 mM imidazole. His-tagged proteins were eluted with 2 ml of buffer E (buffer B and 250 mM imidazole). Protein purity and concentration were verified as above.

Protein binding assays

2-4 μg purified GST-fusion protein was incubated with 20 μl glutathione-agarose (G-agarose) and 100 μl NETN in the cold room with rotation for 1 hour. Beads were then washed twice with NETN. Meanwhile, 0.5-1 μl of in vitro translated [^{35}S]methionine-labeled protein (Promega TNT system) was incubated with 20 μl of G-agarose and 40 μl of NETN for 1 hour. The supernatant was mixed with GST-protein-bound beads for 1-1.5 hours with rotation. Complexes were then washed twice with 100 μl of NETN and eluted with 25 μl of elution buffer containing 20 mM reduced glutathione twice. The eluent was mixed with 50 μl of 2× SDS-sample buffer and subjected to 8-10% SDS-PAGE. Gels were fixed and exposed to X-ray film. For binding assays using His-tagged proteins, 200 ng or 1 μg purified proteins were incubated with GST-proteins bound to beads as above. Bound proteins were detected by anti-pelle immunoblotting (see below).

Antibodies and western blotting

Rabbit anti-Pelle polyclonal antiserum was raised against SDS-PAGE purified GST-PelleK240R by Cocalico Biologicals Inc. Anti-Pelle antiserum was absorbed against a soluble bacterial extract before western blotting. For this, 100 ml bacterial culture expressing GST was centrifuged at 6000 rpm for 5 minutes. The pellet was resuspended in 5 ml of NETN buffer and sonicated. The supernatant was separated by centrifugation at 14000 rpm for 10 minutes. Antiserum and bacterial extract (1:5 V/V) were incubated with rotation at 4°C for 1 hour, centrifuged at 12000 rpm for 5 minutes and the supernatant was used for immunoblotting. Proteins on SDS-PAGE were transferred from SDS-PAGE onto Protran Nitrocellulose paper (0.45 μm). After washing the paper with PBS-T (PBS and 0.1% Tween-20), proteins were detected by chemiluminescence using the ECL detection kit (Amersham Inc). The anti-Pelle antiserum was used at a dilution of 1:1000.

Protein phosphorylation/dephosphorylation

Purified His-Pelle was dephosphorylated by incubation with CIP-agarose (Sigma) in phosphatase buffer (20 mM Tris-HCl pH8.8, 1 mM DTT, 1 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, 5% glycerol and 0.5 mM PMSF) for 30 minutes at 37°C. The dephosphorylated protein was then separated from beads by centrifugation and used for binding assays or SDS-PAGE. For in vitro kinase assays, 20-40 ng of GST-Pelle or His-Pelle and 100-200 ng of the indicated protein substrate were incubated in 30 μl of kinase buffer (25 mM HEPES pH 7.5, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 50 mM β-glycerol phosphate, 10 μCi [γ-^{32}P]ATP) at 25°C for 30-60 minutes. Reactions were terminated by addition of 30 μl 20% TCA with 0.1 mg/ml BSA as carrier. Proteins were precipitated on ice for 30 minutes, collected by centrifugation at 13,000 rpm and washed twice with 10% TCA and once with 95% ethanol. Dried samples were dissolved in SDS-sample buffer and subjected to SDS-PAGE. Phosphorylated proteins were detected by autoradiography.

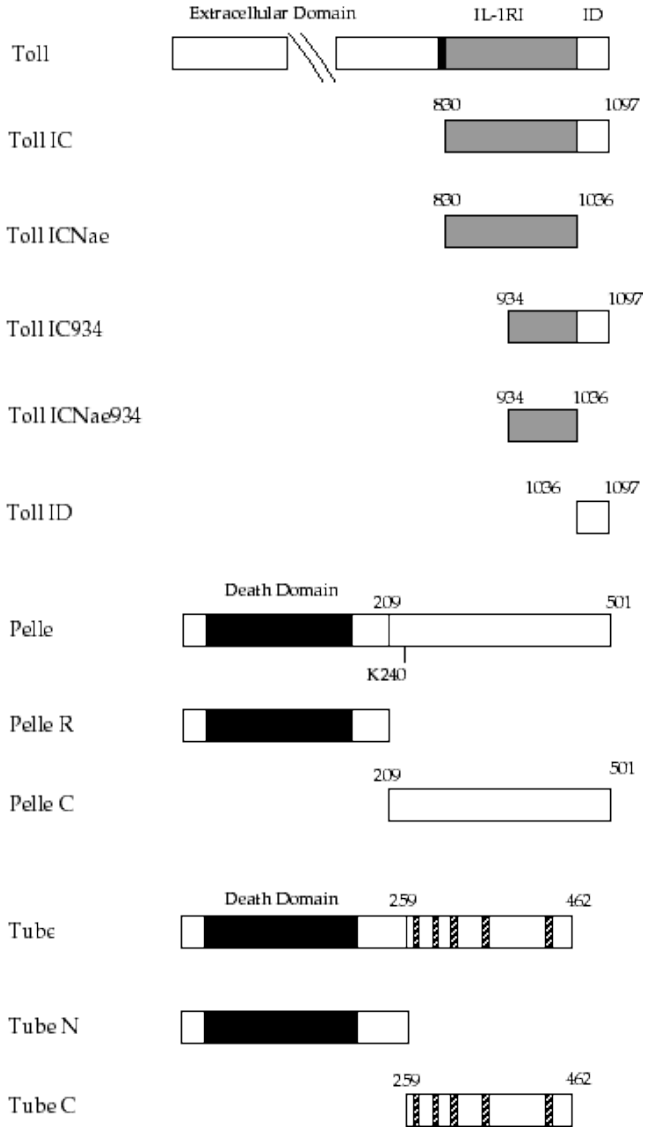

Fig. 1. Schematic diagram of Toll, Pelle, Tube and mutant derivatives. Numbers refer to amino acid residues. The Toll extracellular domain, IL-1R homologous domain (gray region) and inhibitory domain (ID) are indicated. The Pelle catalytic domain includes residue 209-501. Lys 240 was mutated to Arg, inactivating its kinase activity, in PelleK240R. The death domains of Tube and Pelle are indicated. The Tube C-terminal region contains five copies of an 8-residue motif (striped bars).

RESULTS

As described in the Introduction, Pelle and a Toll derivative lacking its C-terminal inhibitory domain (ID) can synergistically activate Dorsal in cotransfected Schneider cells (Norris and Manley, 1996). Additionally, transmembrane localization of Pelle or Tube can be sufficient to induce ventral fate in vivo (Großhans et al., 1994; Galindo et al., 1995). Together these findings suggest that Toll could physically contact Tube and/or Pelle, perhaps facilitating in some manner an early step in the signal transduction cascade resulting in Dorsal activation. In order to investigate the biochemical mechanisms underlying this pathway, we utilized in vitro binding and kinase assays to study interactions between these three proteins. Fig. 1 diagrams different Toll, Pelle and Tube derivatives used in the experiments. Proteins were produced by in vitro translation and/or purified from *E. coli* as GST- or His-tagged fusions.

The Toll cytoplasmic domain interacts with Pelle

We first wished to determine whether Pelle is capable of interacting directly with the Toll intracytoplasmic domain (Toll IC). As an initial test of this, we purified from *E. coli* two GST-Toll IC derivatives and tested their ability to interact with Pelle

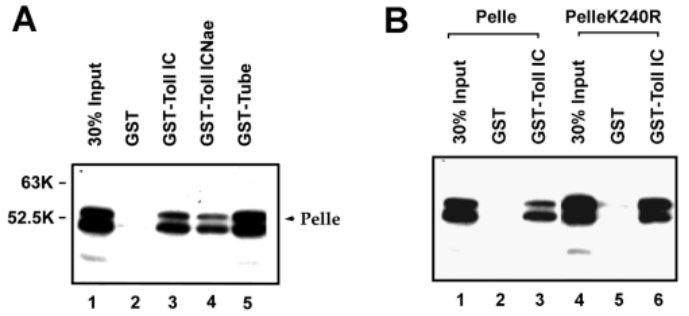

Fig. 2. The Toll cytoplasmic domain interacts with Pelle. 2 μg of GST-Tube or the indicated Toll derivative was immobilized on glutathione-agarose beads, then incubated with in vitro transcribed and translated Pelle or PelleK240R. Protein complexes were eluted with 20 mM glutathione, and binding was detected by autoradiography following SDS-PAGE. (A) Pelle binds the Toll intracytoplasmic domain and Tube with comparable efficiency. Lane 1, 30% of Pelle analyzed in binding reactions (input); lanes 2, 3, 4 and 5; Pelle was incubated with GST, GST-Toll IC, GST-Toll ICNae and GST-Tube, respectively. (B) Both in vitro translated Pelle and PelleK240R interact with the Toll intracytoplasmic domain. Lane 1, 30% of Pelle used in binding; lanes 2 and 3, Pelle was incubated with GST and GST-Toll IC; lane 4, 30% of PelleK240R used in binding reactions; lanes 5 and 6, PelleK240R was incubated with GST and GST-Toll IC.

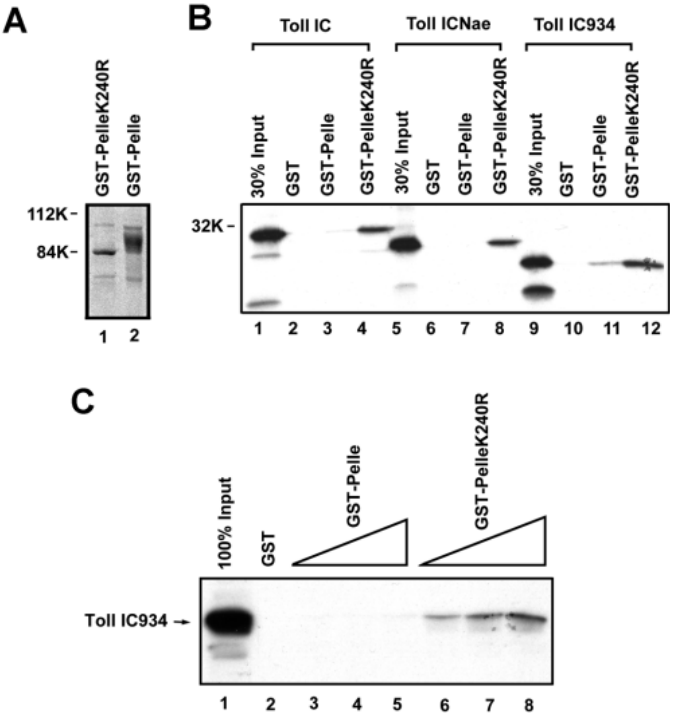

Fig. 3. The Toll cytoplasmic domain interacts with unphosphorylated but not phosphorylated Pelle. (A) GST-Pelle is autophosphorylated in *E. coli*. 2 μg of GST- PelleK240R (lane 1) or GST-Pelle (lane 2) was purified from *E. coli* analyzed by SDS-PAGE and stained with Coomassie blue. (B) 2 μg immobilized GST-fused protein was incubated with in vitro translated protein: lane 1, 30% Toll IC input; lanes 2, 3 and 4. Toll IC was incubated with GST, GST-Pelle and GST-PelleK240R; lane 5, 30% Toll ICNae input; lanes 6, 7 and 8, Toll ICNae was incubated with GST, GST-Pelle and GST-PelleK240R; lane 9, 30% Toll IC934 input; lanes 10, 11 and 12, Toll IC934 incubated with GST, GST-Pelle and GST-PelleK240R. (C) Increasing amounts (1, 2 and 4 μg) of GST-Pelle or GST-PelleK240R were incubated with the same amount of in vitro translated Toll IC934. Lane 1. 100% Toll IC934 input; lane 2, 1 μg GST incubated with Toll IC934; lanes 3, 4, and 5, Toll IC934 incubated with 1, 2 and 4 μg of GST-Pelle; lanes 6, 7 and 8, Toll IC934 incubated with 1, 2 and 4 μg of GST-PelleK240R.

produced by in vitro translation. The GST proteins were bound to glutathione agarose beads, and mixed with the in vitro translated proteins in binding buffer. After binding and washing, the bound proteins were eluted with 20 mM glutathione and analyzed by SDS-PAGE (see Materials and Methods). The results (Fig. 2A) show that both GST-Toll IC and GST-Toll ICNae (a derivative lacking the C-terminal ID but containing the entire IL-1R homology region; Norris and Manley, 1995, 1996; see Fig. 1) bound Pelle with good efficiency (lanes 3 and 4), only slightly less strongly than did a GST-Tube fusion (lane 5). Binding to GST alone was essentially undetectable (lane 2).

In vitro translated Pelle resolved into two species during SDS-PAGE (see Fig. 2A), and the pattern is suggestive of protein modification, such as phosphorylation. We will show below that Pelle is extensively autophosphorylated when produced in *E. coli*, and that this phosphorylation affects its ability to interact with Toll. Fig. 2B presents evidence that the in vitro translated protein is not autophosphorylated. Specifically, wild-type Pelle and Pelle K240R (a catalytically-inactive mutant; see Fig. 1 and below) displayed identical SDS PAGE mobilities (lanes 1 and 4) and also bound indistinguishably to GST-Toll IC. We do not know with certainty the relationship between the two Pelle species. However their mobilities were not affected by phosphatase treatment, and the corresponding unphosphorylated form of Pelle produced in *E. coli* comigrated with the upper form (results not shown), leading us to suspect that the lower band reflects a truncated form of the protein.

The Toll cytoplasmic domain binds directly to unphosphorylated but not phosphorylated Pelle

To extend our studies on Pelle, we wished to purify large amounts of recombinant enzyme for additional interaction experiments and for functional assays. We first expressed GST-Pelle and GST-Pelle K240R fusion proteins in *E. coli* and purified the proteins using glutathione agarose chromatography (see Materials and Methods). Fig. 3A displays a Coomassie-stained SDS PAGE of the two proteins. While the catalytically inactive mutant produced a band of the expected size (approx. 85 kDa; lane 1), GST-Pelle migrated as a diffuse, higher molecular weight species (lane 2). This lower mobility reflects phosphorylation, as the size difference between wild-type and mutant proteins was eliminated by phosphatase treatment (see below), and must have arisen from autophosphorylation as it was detected in *E. coli* and not observed with Pelle K240R.

We first wished to use the GST-Pelle proteins to confirm the results presented in Fig. 2, except using in vitro translated Toll derivatives with the purified GST-Pelle derivatives. Fig. 3B shows that Toll IC, Toll ICNae and Toll IC 934 (a derivative lacking the approx. 100 N-terminal residues of the IL-1R homology region, see Fig. 1) all bound the unphosphorylated

GST-Pelle K240R protein with efficiencies very similar to those seen in Fig. 2 (compare lanes 1 and 4, 5 and 8, and 9 and 12). Most strikingly however, binding of the three Toll derivatives to GST-Pelle was undetectable, or in the case of Toll IC 934 greatly reduced (lanes 3, 7 and 11). These findings raise the possibility that Pelle autophosphorylation regulates the protein's interaction with the Toll IL-1R homology region. Fig. 3C provides evidence that binding of Toll to GST-Pelle was not underestimated because the concentration of GST-Pelle was too low, as higher concentrations also failed to bind significant amounts of Toll IC934.

We next wished to determine whether the Toll-Pelle interaction we detected was direct or, alternatively, mediated by (a) protein(s) present in the reticulocyte lysate. For example, Edwards et al. (1997) showed, using a yeast two hybrid assay, that Toll IC could interact with the structural protein filamin, and it was conceivable that this could provide a bridge between Toll and Pelle. To this end, we purified His-tagged versions of Pelle and Pelle K240R from *E. coli* and used them in binding reactions with purified GST-Toll IC proteins. Fig. 4A shows an SDS-PAGE gel of the purified His-Pelle proteins. Wild-type (lane 1) displayed two bands, while the K240R mutant was detected as essentially only a single species (lane 3), which comigrated with the lower form of the two wild-type species. Importantly, treatment of wild-type His-Pelle with calf intestinal phosphatase (CIP) collapsed the upper form into the lower species (lane 2), confirming that His-Pelle, like GST-Pelle, was autophosphorylated.

Fig. 4B presents the results of in vitro binding assays using His-Pelle and His-Pelle K240R together with GST, GST-Toll IC934 and GST-Toll IC Nae 934 (this later derivative contains only approx. 100 residues of the Toll IC; see Fig. 1). Note that the His-Pelle preparation used in this experiment was completely autophosphorylated (lane 1). Binding assays were done as above, except that glutathione-eluted proteins were detected by western blotting using polyclonal anti-Pelle antibodies (see Materials and Methods). Strikingly, efficient binding of His-Pelle K240R to both GST-Toll derivatives was observed (lanes 5-8), but essentially no binding was detected to His-Pelle (lanes 1-4). These results not only establish that the Toll-Pelle interaction is direct, but also provide additional evidence the Pelle autophosphorylation inhibits binding.

We have interpreted the above results to indicate that His-Pelle K240R bound Toll IC because, unlike wild-type Pelle, it was unphosphorylated. However, an alternative explanation is that the mutant formed a stable interaction with Toll IC only because it was catalytically inactive. Although the ability of wild-type unphosphorylated Pelle produced by in vitro translation to bind Toll IC as efficiently as Pelle K240R (see Fig. 2B) argues against this, we wished to test this possibility with purified proteins. To this end, purified His-Pelle was treated with CIP-agarose beads, the CIP was removed, and phosphorylated and unphosphorylated proteins were tested for their ability to bind GST-Toll IC934, using the same assay as in Fig. 4B. The results, shown in Fig. 4C, confirm that phosphorylated His-Pelle was unable to bind GST-Toll IC934 (lanes 2-4), but most importantly, show that dephosphorylated His-Pelle bound strongly to GST-Toll IC934 (lanes 5-7), comparable to the efficiency observed with His-Pelle K240R (Fig. 4B). These results indicate that Pelle autophosphorylation can regulate Pelle binding to Toll.

The Toll-Pelle interaction requires the Pelle catalytic domain

As mentioned above, Pelle contains an N-terminal putative regulatory domain, consisting largely of a region with significant similarity to the consensus death domain, and a C-terminal catalytic domain. To determine whether either (or both) of these regions was required for the interaction with Toll IC, we produced the Pelle N terminus (Pelle-R) and C terminus (Pelle-C) separately by in vitro translation, and tested whether these molecules could bind any of several GST-Toll IC derivatives. The results (Fig. 5A) indicate that the Pelle N terminus was incapable of binding any of the GST-Toll derivatives (lanes 1-5), whereas the catalytic region bound efficiently to both GST-Toll IC (lane 8) and GST-Toll ICNae (lane 9). The Toll C-terminal inhibitory domain (ID) was neither necessary (lane 9) nor sufficient for (lane 10) this interaction.

To extend these results, we performed similar binding

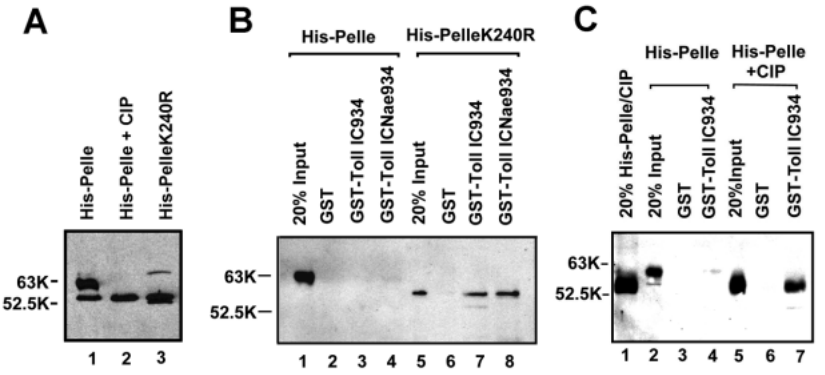

Fig. 4. Pelle autophosphorylation prevents the direct interaction between Pelle and the Toll intracytoplasmic domain. (A) His-Pelle is autophosphorylated in *E. coli*. Purified His-Pelle was dephosphorylated with calf intestinal phosphatase (CIP) conjugated agarose beads. 1 μg of His-Pelle (lane 1), dephosphorylated His-Pelle (lane 2) and His-PelleK240R (lane 3) were loaded on SDS-PAGE and detected by immunoblotting with anti-Pelle antibodies. (B) PelleK240R but not autophosphorylated Pelle directly interacts with Toll IC. 2 μg immobilized GST-fusion protein was incubated with 200 ng of purified His-tagged protein. Bound protein was detected by western blotting with anti-Pelle antibodies; lane 1, 20% His-Pelle input; lanes 2, 3 and 4, His-Pelle incubated with GST, GST-Toll IC934 and GST-Toll ICNae934; lane 5, 20% His-PelleK240R input; lanes 6, 7 and 8, His-PelleK240R incubated with GST, GST-Toll IC934 and GST-Toll ICNae934. (C) CIP- dephosphorylated His-Pelle binds to Toll IC934. 1 μg purified His-Pelle or CIP-dephosphorylated His-Pelle was incubated with 2 μg of immobilized GST-fusion protein. Protein complexes were eluted with 20 mM glutathione and bound His-Pelle was detected by western blotting with anti-Pelle antibodies. Lanes 1 and 5, 200 ng CIP-dephosphorylated His-Pelle; lane 2, 200 ng His-Pelle; lanes 3 and 4, His-Pelle incubated with GST and GST-Toll IC934; lanes 6, and 7, His-Pelle/CIP incubated with GST and GST-Toll IC934.

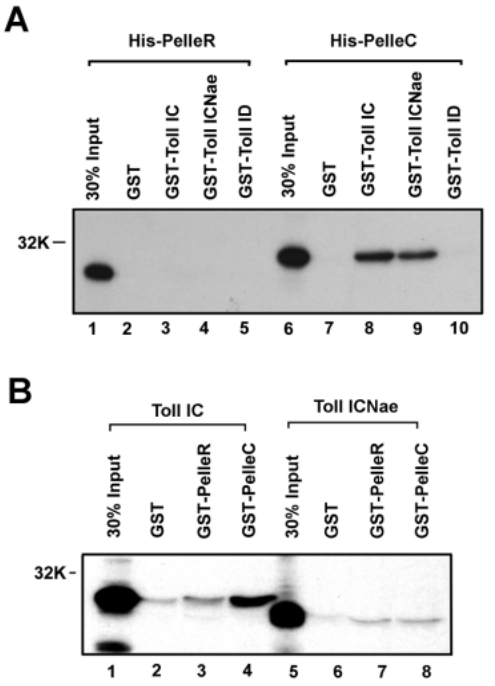

Fig. 5. The Pelle catalytic domain is necessary and sufficient to interact with the Toll IL-1R homologous domain. (A) 2 μg immobilized GST-fusion proteins were incubated with in vitro translated His-tag protein. Lane 1, 30% His-PelleR input; lanes 2, 3, 4 and 5, His-PelleR incubated with GST, GST-Toll IC, GST-Toll ICNae and GST-Toll ID; lane 6, 30% His-PelleC input; lanes 7, 8, 9 and 10, His-PelleC incubated with GST, GST-Toll IC, GST-Toll ICNae and GST-Toll ID. (B) 2 μg immobilized GST-fusion protein was incubated with in vitro translated Toll derivatives. Lane 1, 30% Toll IC input; lanes 2, 3 and 4, Toll IC was incubated with GST, GST-PelleR and GST-PelleC; lane 5, 30% of Toll ICNae input; lanes 6, 7 and 8, Toll ICNae incubated with GST, GST-PelleR and GST-PelleC.

reactions using purified GST-Pelle derivatives and in vitro translated Toll IC and Toll ICNae (Fig. 5B). Although the results obtained were largely consistent with those described above, two differences were observed. First, although Toll IC interacted most strongly with GST-Pelle C (lane 4), a weak interaction was also observed with GST-Pelle R (lane 3). Second, GST-Pelle C bound Toll ICNae only very weakly, barely above the background observed with GST alone (compare lanes 5-8). This contrasts with the rest of our data, which suggest that the Toll ID (which is missing in Toll ICNae) does not contribute to the Pelle-Toll interaction. Taken together, our results indicate that Pelle and Toll interact directly via residues within the Toll IL-1R homology region and the Pelle catalytic domain, although sequences outside these regions, e.g., in the Toll ID, can play a stimulatory or accessory role, at least in the context of GST-PelleC fusion protein.

Pelle phosphorylates itself and Toll in vitro

Previous work has shown that Pelle can phosphorylate Tube in vitro (Großhans et al., 1994). The data presented above raise the possibility that the Toll IC as well as Pelle itself may be Pelle substrates. To investigate this, we utilized purified GST-Pelle and [γ-^{32}P]ATP in in vitro kinase assays with various Toll and Pelle derivatives as potential substrates. Following

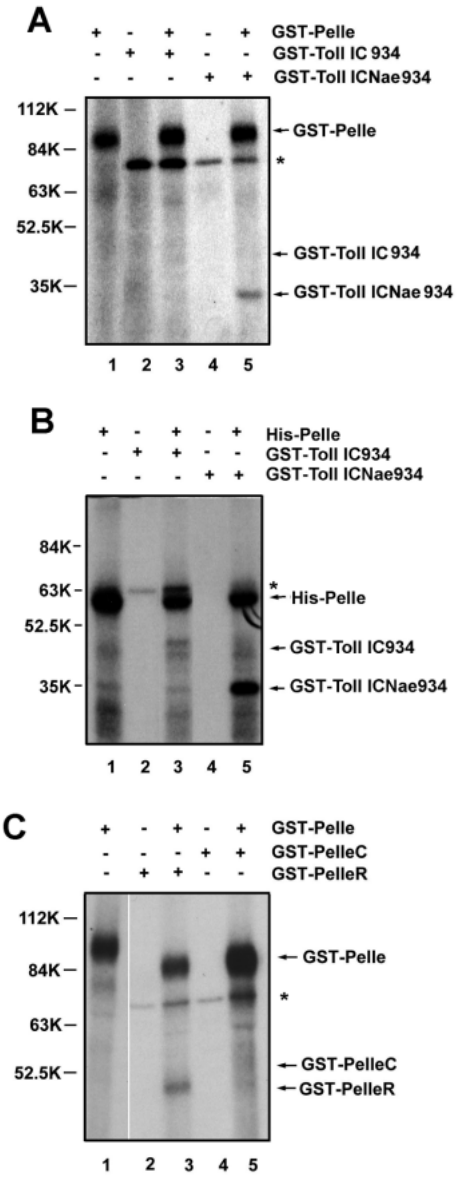

Fig. 6. Pelle phosphorylates itself and Toll in vitro. (A) The Toll inhibitory domain prevents Toll phosphorylation by GST-Pelle. Lane 1, 40 ng GST-Pelle; lane 2, 200 ng GST-Toll IC934; lane 3, 40 ng GST-Pelle and 200 ng GST-Toll IC934; lane 4, 200 ng GST-Toll ICNae934; lane 5, 40 ng GST-Pelle and 200 ng GST-Toll ICNae934. Phosphorylated proteins were analyzed by SDS-PAGE as described in Materials and Methods. In all panels, positions of molecular weight standards are indicated on the left and the proteins on the right. The asterisk indicates a trace contaminating protein that was labeled by ATP in the absence of kinase. (B) Toll inhibitory domain prevents Toll phosphorylation by His-Pelle. Lane 1, 40 ng His-Pelle; lane 2, 200 ng GST-Toll IC934; lane 3, 40 ng His-Pelle and 200 ng GST-Toll IC934; lane 4, 200 ng GST-Toll ICNae934; lane 5, 40 ng His-Pelle and 200 ng GST-Toll ICNae934. (C) The Pelle regulatory domain but not the catalytic domain is phosphorylated by GST-Pelle. Lane 1, 40 ng GST-Pelle; lane 2, 200 ng GST-PelleR; lane 3, 40 ng GST-Pelle and 200 ng GST-PelleR; lane 4, 200 ng GST-PelleC; lane 5, 40 ng GST-Pelle and 200 ng GST-PelleC.

phosphorylation reactions, proteins were TCA-precipitated and resolved by SDS PAGE. Fig. 6A shows the results obtained

with two Toll derivatives. In all reactions containing GST-Pelle, a strong band corresponding to the size expected for GST-Pelle was observed (lanes 1, 3 and 5), confirming that the protein is indeed capable of autophosphorylation. When GST-Pelle was incubated with GST-Toll IC934 (lane 3) or GST-Toll IC (not shown), no evidence that either Toll derivative could be phosphorylated was observed. However, when GST-Toll ICNae 934 was used as a substrate, significant labeling of a band the expected size of the Toll derivative was observed (lane 5). This species was not labeled in reaction mixtures lacking GST-Pelle (lane 4) or containing instead GST-Pelle K240R (results not shown), confirming that labeling was indeed due to the kinase activity of GST-Pelle. Phosphorylation was not due to nonphysiological interactions mediated by the GST moieties, as essentially identical results were obtained when His-Pelle was used instead of GST-Pelle (Fig. 6B). These results indicate that the Toll ID domain, while dispensable for binding of Pelle to Toll, is capable of preventing Toll phosphorylation by Pelle. As discussed below, this suggests an explanation for our previous finding that deletion of the Toll ID allowed synergistic activation of Dorsal by Pelle and Toll in cotransfection assays (Norris and Manley, 1996).

Our results indicate that Pelle efficiently autophosphorylates itself, both in *E. coli* and in vitro. To determine whether this phosphorylation occurred in the regulatory and/or catalytic domains, GST-Pelle was incubated with GST-Pelle R (Fig. 6C, lane 3) and GST-Pelle C (lane 5). Although in neither case was phosphorylation as efficient as the autophosphorylation of the full-length protein, significant phosphorylation of Pelle R, but not Pelle C, was detected. As mentioned above, this region of Pelle consists largely of a death domain, and has been shown to interact with Tube in yeast two hybrid assays (Edwards et al., 1997).

Tube interacts with Toll and unphosphorylated Pelle

As mentioned above, a good deal of previous data supports the existence of an interaction between Pelle and Tube (Großhans et al., 1994; Galindo et al., 1995). In addition, recent two hybrid assays have provided evidence that the Tube-Pelle interaction occurs only with catalytically inactive Pelle (Edwards et al., 1997), similar to the Toll-Pelle in vitro interaction we have described here. We therefore wished to examine possible in vitro interactions between Tube and Pelle or Toll IC. To this end, we again used the GST protein interaction assays together with two Tube derivatives produced by in vitro translation, Tube C, which consists of the C terminus of the protein, which is dispensable for Tube function in Dorsal signaling, and Tube N, which contains the death domain and is sufficient for Tube function in signaling (Letsou et al., 1991, 1993; Norris and Manley, 1995, 1996; Edwards et al., 1997).

The results of protein interaction assays with GST-Toll and GST-Pelle derivatives are shown in Fig. 7. Tube C was unable to interact with any of the fusion proteins (lanes 1-6), consistent with the dispensability of this region for Tube function. In contrast, Tube N interacted strongly with both proteins. Notably, the interaction with Pelle was detected with Pelle K240R and not Pelle wild-type, identical to the requirements for the Pelle-Toll interaction described above and consistent with the yeast two hybrid assays of Edwards et al. (1997). The GST-Toll/Tube N interaction (lanes 11-12) was striking because it was both as strong as the well-established Pelle-Tube interaction and involved the same approx. 100 residue region of the Toll IL-1R homology region shown above to be necessary for the Toll-Pelle interaction. We discuss below how these phosphorylation-regulatable interactions may function during the earliest stages of activation of the Dorsal signaling cascade.

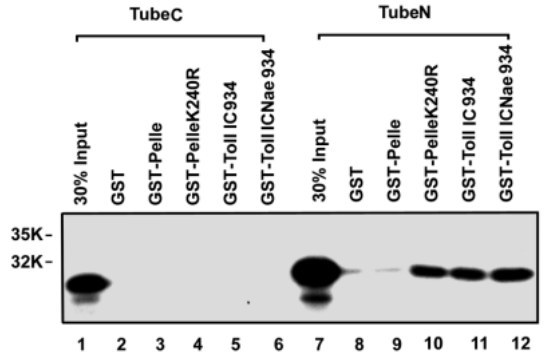

Fig. 7. Tube interacts with the Toll cytoplasmic domain and with unphosphorylated Pelle. Interactions between in vitro translated Tube derivatives and the indicated GST-fusion proteins were examined as above. Lane 1, 30% TubeC input; lanes 2, 3, 4, 5 and 6, TubeC incubated with GST, GST-Pelle, GST-PelleK240R, GST-Toll IC934 and GST-Toll ICNae934; lane 7, 30% TubeN input; lanes 8, 9, 10, 11 and 12, TubeN incubated with GST, GST-Pelle, GST-PelleK240R, GST-Toll IC934 and GST-Toll ICNae934.

DISCUSSION

In early *Drosophila* embryogenesis, localized activation of Toll triggers a series of signaling events through Tube and Pelle, which eventually lead to nuclear translocation and activation of Dorsal. Here we have shown that Pelle directly interacts with the intracytoplasmic domain of Toll, and that Pelle autophosphorylation, which likely occurs within its regulatory death domain, prevents Pelle binding to both Toll and Tube. Given genetic data showing that membrane localization of Pelle and Tube can be sufficient to induce ventral fate (Großhans et al., 1994; Galindo et al., 1995), as well as other findings (discussed below), it is likely that the phosphorylation-regulatable Pelle-Toll interaction is important for activation of the signaling pathway. Unexpectedly, Toll is phosphorylated by Pelle within its Pelle-binding, IL-1R homology region, and the Toll inhibitory domain blocks this phosphorylation. Based on these findings, we propose a model (Fig. 8) for early signaling events in Dorsal activation, and discuss how this may be related to the IL-1 pathway in mammals.

Regulation of Pelle autophosphorylation

An important aspect of our model is that Pelle is unphosphorylated prior to activation of signaling, and this allows association with Toll and Tube in a membrane-bound ternary complex. Given that Pelle is capable of autophosphorylation, this requires a mechanism for repression of premature autophosphorylation. Our results suggest two possibilities. Specifically, the data we presented indicated that Pelle is autophosphorylated when produced in *E. coli*, but

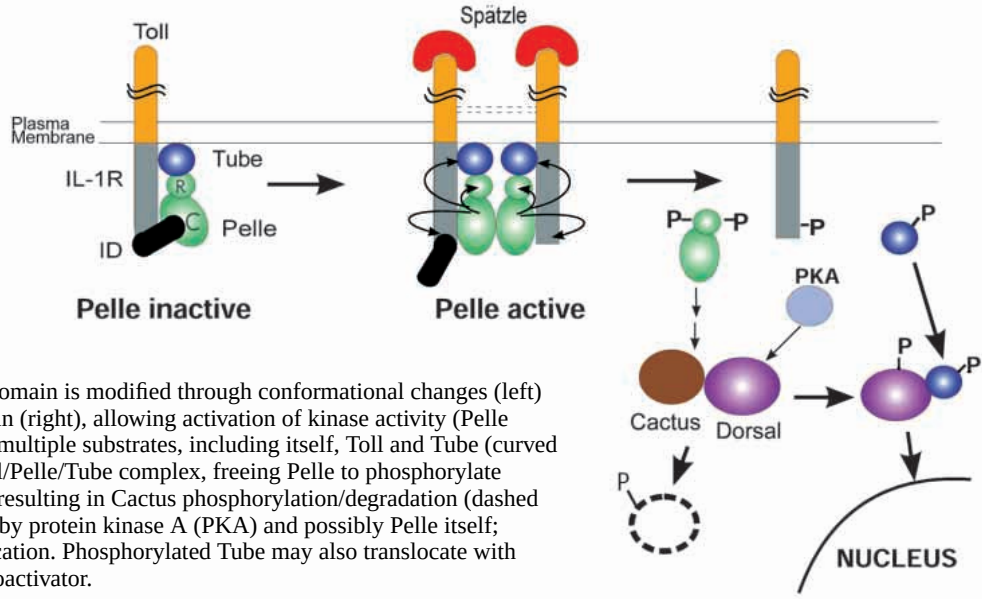

Fig. 8. Model for activation of the Dorsal signaling pathway. The intracytoplasmic IL-1R homology domain of the Toll receptor initially interacts directly with unphosphorylated Pelle, and the Toll ID helps down regulate kinase activity. Tube is also recruited to the complex through its interaction with Pelle and/or Toll, forming the Toll/Pelle/Tube ternary complex (Pelle inactive). Binding of Spätzle to Toll induces Toll dimerization (dashed lines), and the Toll cytoplasmic domain is modified through conformational changes (left) or proteolysis of the Toll inhibitory domain (right), allowing activation of kinase activity (Pelle active). Active Pelle then phosphorylates multiple substrates, including itself, Toll and Tube (curved arrows). This causes disruption of the Toll/Pelle/Tube complex, freeing Pelle to phosphorylate unknown downstream targets, eventually resulting in Cactus phosphorylation/degradation (dashed circle) and Dorsal phosphorylation (e. g., by protein kinase A (PKA) and possibly Pelle itself; Edwards et al., 1997) and nuclear translocation. Phosphorylated Tube may also translocate with Dorsal and function as a transcriptional coactivator.

unphosphorylated when synthesized in vitro in reticulocyte lysate. Additionally, Pelle is unphosphorylated in transfected Schneider cells when expressed at low levels, but phosphorylated at higher levels (unpublished data). One explanation consistent with this data is that Pelle must dimerize to become active. The kinase would dimerize, and autophosphorylate when expressed at high levels in bacteria or Schneider cells, but would be an inactive monomer when produced at low levels in vitro or in vivo. During signaling, we suggest that Pelle dimerization is induced in the Toll/Tube/Pelle ternary complex upon ligand binding, and we discuss below possible mechanisms by which this could occur. Another possibility is that a specific evolutionarily conserved inhibitor of Pelle autophosphorylation is present in reticulocyte lysate and Schneider cells, but not in *E. coli*. (When Pelle is expressed at high levels in Schneider cells, the putative inhibitor would become limiting.) This model envisions that the inhibitor would be displaced or inactivated upon activation of the signaling pathway, perhaps by a conformational change in the Toll intracytoplasmic domain. As there is no genetic (or biochemical) evidence to support the existence of a specific Pelle inhibitor, we favor the simpler dimerization model.

The Toll-Pelle interaction

Our data has established both that Pelle and the Toll intracytoplasmic domain interact directly and also that this region of Toll can be a substrate for Pelle kinase activity. These findings are likely relevant to IL-1 signaling in mammals, as they both involve the IL-1R homology region of Toll. Although a number of previous studies have suggested the existence of a Toll/Pelle/Tube complex, there has been no indication of a direct interaction between Toll and Pelle (or Tube). Indeed, a yeast two hybrid assay specifically failed to obtain evidence for such an interaction (Galindo et al., 1995). We suggest this is because Pelle was phosphorylated in yeast and this prevented the interaction. Supporting this, subsequent work by the same authors showed that the Pelle-Tube interaction was very weak unless catalytically-inactive Pelle was used (Edwards et al., 1997), which is also consistent with our in vitro data. In humans, IL-1R is known to associate with IRAK2, and IL-1RAcp with IRAK, but it is unclear whether these interactions are direct (Cao et al., 1996; Muzio et al., 1997). Interestingly, however, IRAK becomes phosphorylated in this complex upon stimulation with IL-1 (Cao et al., 1996). Although it has not been proved that this is autophosphorylation, this is remarkably similar to the model suggested by our data for activation of Pelle. It will be of interest to determine if IL-1R also becomes phosphorylated following activation of signaling. Our data also showed that Tube and Toll can interact in vitro. However, this may be indirect, as we have to date been unable to confirm this with purified proteins (unpublished data). It may be that the two proteins are bridged by a factor in the reticulocyte lysate, with possibilities including the structural protein filamin (Edwards et al., 1997) or a Pelle-like kinase.

What is the function of Toll phosphorylation? One possibility is that it contributes to the dissociation of the Toll-Pelle complex, which our data suggests occurs upon Pelle autophosphorylation. Another is that it helps to desensitize the receptor, by preventing formation of additional Pelle/Tube/Toll complexes. It is also conceivable that phosphorylated Toll has a function subsequent to Pelle activation. Although genetic epistasis data showed that *pelle* and *tube* function is required downstream of *Toll* (Hecht and Anderson, 1993), *pelle* and *tube* gain-of-function alleles (Großhans et al., 1994; Galindo et al., 1995) have not been tested in the context of a *Toll* null allele, and it is thus possible that Toll plays an additional role in the Dorsal activation pathway.

Function of the Toll inhibitory domain

We proposed previously that the 60 C-terminal residues of Toll constitute an inhibitory domain (ID) because deletion of the ID resulted in enhanced activity in transient transfection assays, especially when Toll and Pelle were coexpressed (Norris and Manley, 1995, 1996). Deletion of this region also allowed the Toll intracytoplasmic domain to select an interacting protein, filamin, in a yeast two hybrid screen (Edwards et al., 1997). The functional significance of this interaction, however, remains to be determined. Our data provide insights into how the ID might

participate in activation of Dorsal signaling. Our experiments showed that the presence or absence of the ID had no effect on the Toll-Pelle interaction, except in the context of a mutant Pelle lacking its N-terminal regulatory domain, in which case the presence of the ID enhanced binding, suggesting that the ID can influence the interaction between Toll and the Pelle catalytic domain. More strikingly, deletion of the ID was required to detect phosphorylation of Toll by Pelle. These results suggest a model whereby the ID blocks Pelle catalytic activity, and activation of signaling causes a change in Toll conformation or structure (see below) such that the ID no longer interferes with kinase activity. Whether this interference affects only the ability of Pelle to phosphorylate Toll, or more generally negatively regulates the overall activity of Pelle in the Toll/Pelle/Tube complex, remains to be determined.

A family of Toll-like receptors has recently been discovered in human (Medzhitov et al., 1997; Rock et al., 1998) and together with the IL-1 receptors, there are now a dozen or so receptors containing intracytoplasmic IL-1R, or Toll, homology domains. However, only two, Toll and a second, *Drosophila* receptor, 18 Wheeler (18W; Eldon et al., 1994) contain significant extensions C-terminal to the IL-1R homology domain, and these two regions are divergent. Thus the mechanism we have suggested for ID function appears not to be conserved throughout evolution (although the Toll ID is highly conserved in different *Drosophila* species; Yamagata et al., 1994). However, it may be that the same type of control over signaling is achieved in different ways. For example, residues within the IL-1R homology region of the mammalian receptors may modulate the conformation of the domain, analogous to the way we envision the ID functioning in Toll. Alternatively, it may be that in mammals a *trans*-acting factor fulfills the function of the Toll ID. In this regard, it may be significant that mammals also appear to lack a homologue of Tube. Perhaps a single factor has in some fashion combined the function of Tube and the Toll ID. A candidate is MyD88 (which does not have a known Drosophila counterpart), which contains an N-terminal death domain (like Tube) and a C-terminal IL-1R homology domain (like Toll), and upon IL-1 stimulation is recruited into a stable, active complex with IL-1R, IRAK and other factors (Wesche et al., 1997a,b; Burns et al., 1998). Conceivably, heterotypic interactions between IL-1R homology domains in IL-1R, MyD88 and IL-1RAcp (Muzio et al., 1997) leads to a conformation analogous to that in the Toll complex following Spätzle binding, allowing activation of IRAK, with MyD88-IRAK death domain interactions stabilizing the complex. Based on the data presented here, we suggest activation of the pathway, in both flies and mammals, involves phosphorylation of multiple components, disruption of the complex, and initiation of downstream signaling.

Initiation of Dorsal signaling through a Toll/Pelle/Tube ternary complex

The direct interactions between unphosphorylated Pelle, Toll and Tube we described are consistent with the existence of a ternary complex at the plasma membrane. Pelle interacts with Toll via residues in its catalytic domain and with Tube via its N-terminal death domain, so both interactions can occur simultaneously. An important question is whether the ternary complex forms independently of signaling. Previous studies have shown that the artificial recruitment of Pelle or Tube to the plasma membrane can initiate the signaling pathway independent of ligand binding (Großhans et al., 1994; Galindo et al., 1995). But it is not clear in these studies whether it was recruitment to the membrane *per se* that resulted in activation, or the dimerization of the Torso fusion proteins employed in both studies. As discussed above, a possible mechanism for Pelle activation is simply dimerization, induced naturally, we suggest, by conformational changes in the ternary complex that occur following ligand binding.

How might such changes be induced? There is considerable indirect evidence suggesting that Toll molecules interact (e.g., Anderson et al., 1985, Norris and Manley, 1992), and an attractive model is that ligand binding induces dimerization or even aggregation. We suggest that this leads to activation of signaling, i.e., of Pelle activity, by either (or both) of two related mechanisms (Fig. 8). First, oligomerization of Toll receptors increases the local ternary complex concentration and hence Pelle concentration, thereby favoring Pelle dimerization and activation by simple mass action. Second, ligand-induced Toll self-association causes a conformational change in the intracytoplasmic domain such that the ID is displaced, thereby facilitating Pelle activation, again perhaps by dimerization. A speculative possibility is that the ID is actually cleaved upon activation. The product of the strong dominant gain-of-function allele *Toll*^{10b}, which contains a single C→Y change in its extracellular domain (Schneider et al., 1991), has been found in a partially proteolyzed form, such that full-length Toll^{10b} is associated with a truncated form lacking most or all of its extracellular domain as well as likely sequences from the very C terminus, i.e., the ID (Winans and Hashimoto, 1995). Perhaps relevant to this, a putative PEST degradation sequence is situated between the IL-1R homology region and the ID (Yamagata et al., 1994). It is intriguing that the structure of this truncated product is similar to mammalian IL-1RAcp, which functions in IRAK activation during IL-1 signaling (Weschel et al., 1997a; Muzio et al., 1997). In any event, we propose that IL-1R homology domain interactions activate Pelle via the direct, phosphorylation-sensitive protein-protein interactions we have described here.

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