The protein kinase Pelle mediates feedback regulation in the *Drosophila* Toll signaling pathway

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**SUMMARY**

Dorsoventral polarity in the *Drosophila* embryo is established through a signal transduction cascade triggered in ventral and ventrolateral regions. Activation of a transmembrane receptor, Toll, leads to localized recruitment of the adaptor protein Tube and protein kinase Pelle. Signaling through these components directs degradation of the IκB-like inhibitor Cactus and nuclear translocation of the Rel protein Dorsal. Here we show through confocal immunofluorescence microscopy that Pelle functions to downregulate the signal-dependent relocation of Tube. Inactivation of the Pelle kinase domain, or elimination of the Tube-Pelle interaction, dramatically increases Tube recruitment to the ventral plasma membrane in regions of active signaling. We also characterize a large collection of *pelle* alleles, identifying the molecular lesions in these alleles and their effects on Pelle autophosphorylation, Tube phosphorylation and Tube relocation. Our results point to a mechanism operating to modulate the domain or duration of signaling downstream from Tube and Pelle.

Key words: Patterning, Dorsal, Pelle, Dorsoventral polarity, *Drosophila melanogaster*

**INTRODUCTION**

In *Drosophila*, regulation of an intracellular signal-transduction system defines the embryonic dorsoventral axis. Activation of a transmembrane receptor, Toll, leads to the nuclear translocation of a Rel-related transcription factor, Dorsal (Belvin and Anderson, 1996). Levels of Toll and Dorsal are uniform throughout the syncytial embryo, but generation of the Toll ligand is asymmetric. Since production of the ligand is highest along the ventral midline and negligible on the dorsal side, Toll signaling generates a ventral-to-dorsal gradient of nuclear Dorsal. Here we show through confocal immunofluorescence microscopy that Pelle functions to downregulate the signal-dependent relocation of Tube. Inactivation of the Pelle kinase domain, or elimination of the Tube-Pelle interaction, dramatically increases Tube recruitment to the ventral plasma membrane in regions of active signaling. We also characterize a large collection of *pelle* alleles, identifying the molecular lesions in these alleles and their effects on Pelle autophosphorylation, Tube phosphorylation and Tube relocation. Our results point to a mechanism operating to modulate the domain or duration of signaling downstream from Tube and Pelle.

Key words: Patterning, Dorsal, Pelle, Dorsoventral polarity, *Drosophila melanogaster*
inactivation of the Pelle kinase domain resulting in a 30-fold increase in the strength of the Tube-Pelle interaction (Edwards et al., 1997). Since Tube and Pelle also bind directly to Dorsal (Edwards et al., 1997; Yang and Steward, 1997), the components of the Toll pathway are likely to function in a multiprotein complex. Consistent with this hypothesis, there is evidence that multimerization activates Tube and Pelle and that Tube aggregates at sites of active signaling (Grosshans et al., 1999; Towb et al., 1998).

One question not well understood in the Drosophila Toll pathway is that of feedback regulation. Once Dorsal enters nuclei a pathway operates to downregulate the production of the Toll ligand (Morisato and Anderson, 1994; Misra et al., 1998). It is not known, however, whether there is an additional mechanism to reset Toll, Tube, and Pelle to an inactive state after signaling. Here we demonstrate that Pelle modifies Tube localization and thereby downregulates Tube clustering. Through molecular characterization of pelle alleles and biochemical analysis of Pelle mutants, we show that a Pelle mutant protein that is unable to phosphorylate Tube, yet retains autophosphorylation activity, is severely compromised in signaling ability. These results point to a novel mechanism for adjusting the activity of the dorsoventral signal transduction pathway.

MATERIALS AND METHODS

Stocks

Drosophila melanogaster stocks were maintained on standard cornmeal-yeast agar medium (Ashburner, 1989) at 18 or 25°C. Oregon R was used as the wild-type stock. Many of the pelle alleles used in the sequencing project were generously provided by Kathryn Anderson and all except pl166, a gift from Dave Stein, have been described previously (Hecht and Anderson, 1993). Characterization of the tube alleles tub2 and tub4 has been reported previously (Letsou et al., 1991; Letsou et al., 1993). In all experiments, tube mutations were assayed in trans to the deficiency Df(3R)XM3 and pelle mutations were assayed in trans to the deficiency Df(3R)IR16 or Df(3R)D605 (Letsou et al., 1991; Shelton and Wasserman, 1993; Thomas et al., 1991). Since the tube, pelle and dorsal genes are all maternal effect loci, we refer to the defective embryos generated by mutant females as tube, pelle or dorsal embryos.

Immunolocalization studies

Embryo collection, fixation and staining were carried out as previously reported (Galindo et al., 1995; Towb et al., 1998). The Tube antisem has been described previously (Letsou et al., 1993). The polyclonal Toll antisem, raised in rabbit against amino acids 830-1097 of Toll, was a gift from C. Lavoie. Images were collected using a Zeiss LSM 410 or a BioRad 1024 confocal microscope; final image manipulation was done using Adobe PhotoShop software.

Sequencing of pelle mutations

Males heterozygous for a pelle mutation and the pelle deficiency Df(3R)IR16 were used as the source of genomic DNA, prepared as described (Gloor and Engels, 1992). We used the Expand High-Fidelity PCR system (Roche) to amplify the pelle-coding region from these genomic DNA preparations. We carried out four separate PCR reactions for each pelle allele and pooled these reactions. We then recovered the amplified DNA on a DNA affinity column (Qiagen), resuspended the sample in water, and carried out sequencing with four primers distributed across the gene in the forward and reverse direction.

RESULTS

Inactivating mutations in Tube or Pelle enhance the signal-dependent Tube localization gradient

The distribution of Tube within the syncytial embryo reflects the localized activity of the Toll signaling pathway. Toll activation on the ventral side of embryos causes a recruitment or clustering of Tube that is proportional to signal strength (Towb et al., 1998). The result is a shallow gradient of Tube protein, with highest levels ventrally (Fig. 1B).

To determine what role the interaction of Tube with Pelle might play in the signal-dependent Tube localization gradient, we examined the distribution of the Tub2 protein in embryos. The tub2 mutation blocks both the Tube/Pelle interaction and signaling from Toll to Dorsal (Letsou et al., 1993; Edwards et al., 1997). Surface views show that the Tub2 protein concentrates strongly in a broad band down the presumptive ventral midline (Fig. 1D). The Tube localization gradient is thus greatly enhanced in tub2 embryos relative to the wild type (compare Fig. 1C and 1D), although protein levels in the two genetic backgrounds are comparable (Letsou et al., 1993). The partial loss-of-function tub4 mutation, which also maps to the Pelle interaction domain of Tube, had similar effects (S. Gillespie and S. A. W., unpublished data).

By counting nuclei, we determined that the intense ventral Tube membrane localization spans approximately one fourth of the circumference of tube mutant embryos. Similarly, Dorsal is exclusively nuclear in the ventral-most quarter of wild-type embryos, where Toll activation is highest (Belvin and Anderson, 1996). Both patterns have graded boundaries. The simplest interpretation of these observations is that the domain

Immunoblotting and kinase assays of pelle mutations

Zero- to four-hour old embryos were collected from mothers that were transheterozygous for the mutant pl1 allele and the deficiency Df(3R)D605. For immunoblotting, frozen embryos were homogenized in Laemmli loading buffer using a Branson sonifier with microtip setting 4. The homogenate from the equivalent of 30 embryos was separated by SDS-PAGE, blotted and analyzed using the affinity-purified anti-Pelle antibody (Grosshans et al., 1994). For in vitro kinase assays, frozen embryos were lysed in IP buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1% glycerol, with protease and phosphatase inhibitors) using a dounce homogenizer. The volume of the IP buffer was determined in such a way that a concentration of 2 embryos/µl resulted. The lysate was spun for 1 minute and the supernatant extracted with 1 volume trifluoro-trichloro-ethane to remove lipids. Following centrifugation at 14,000 rpm for 15 minutes, 2 µg of affinity-purified anti-Pelle antibody pre-bound to Protein A Sepharose beads (Pharmacia) was added to the cleared extract and incubated for 2-3 hours at 4°C with rocking. The beads were gently washed five times in IP buffer, twice in kinase buffer (25 mM Hepes, pH 7.5, 25 mM glycerol phosphate, 50 mM KCl, 1 mM DTT, 10 mM MnCl2, 2 mM MgCl2, 5% glycerol) and suspended in 30 µl kinase buffer. The kinase reaction was started by adding 100 ng of bacterially expressed and renatured Tube-Hiss protein and 10 µCi of [γ-32P]ATP. After incubation for 30 minutes at 25°C the beads were removed from the supernatant, washed four times in IP buffer and analyzed by SDS-PAGE and autoradiography to determine Pelle autophosphorylation. The supernatant was subject to immunoprecipitation using anti-Tube antibody as described above, followed by SDS-PAGE and autoradiography.
Pelle mediates feedback regulation of high level Toll activation defines the extent of the enhanced Tube gradient. Since two tube mutations that prevent interaction with Pelle enhance the Tube localization gradient, we wondered whether inactivating Pelle similarly enhances the Tube gradient. To test this hypothesis, we examined how Tube localization is affected by pll^{25}, one of the strongest pelle alleles, by phenotypic criteria (Hecht and Anderson, 1993). The mutation entirely eliminates the Pelle catalytic domain and truncates 36 of the approx. 100 amino acids constituting the Tube interaction domain (see below). As shown in Fig. 2A, Tube clustering along the ventral midline is greatly enhanced in pll^{25} embryos, similar to the enhancement seen in tub^{2} embryos (compare with Fig. 1C,D). Other strongly inactivating pelle mutations, for example pll^{078} (Fig. 2B) and pll^{074} (Fig. 2D), also enhance the Tube localization gradient. Thus either inactivating Pelle or blocking its interaction with Tube causes an increased accumulation of Tube in regions of active signaling.

**Effects on Tube clustering are proximate consequences of the Tube/Pelle interaction**

In analyzing the effects of tube and pelle mutations on Tube localization, we considered several possible underlying mechanisms. Since pelle and tube mutations block signaling, we postulated that the change in Tube localization could be the consequence of the failure to translocate Dorsal from the cytoplasm to the nucleus. To test this hypothesis, we examined Tube localization in a dorsal null background (dl^{1}). Tube localization in dl^{1} was indistinguishable from that in the wild type (Fig. 3A, compare with Fig. 1C). Signaling from Toll to Dorsal is therefore not necessary for the establishment of the Tube localization gradient, nor does the absence of such signaling cause the enhancement observed in tub^{2} and pll^{25} embryos.

We also considered the possibility that Tube aggregation reflects redistribution of the Toll receptor upon ligand binding. Studies from other systems indicate that multimerization or clustering is often involved in activation of receptors that, like Toll, have a single transmembrane domain (Guo et al., 1995). Furthermore, Toll and Tube interact in vitro (Shen and Manley, 1995).

**Fig. 1.** The Tube localization gradient is enhanced in tub^{2} mutant embryos. (A,B) Paired images from a wild-type embryo, showing a longitudinal optical section in the plane of the dorsoventral axis. The embryo is stained with fluorescein-conjugated antibodies to Dorsal (A) and with antibodies to Tube detected with a Cy3-conjugated secondary antibody (B). (C,D) Surface images of (C) a wild-type embryo and (D) a tub^{2} embryo, stained with antibodies to Tube, ventrolateral aspect. In each image, an arrow indicates the approximate position of the ventral midline.

**Fig. 2.** The Tube localization gradient is enhanced in pelle mutant embryos. All images are surface views of embryos stained with antibodies to Tube detected with a Cy3-conjugated secondary antibody. (A) pll^{25}, lateral view of the posterior pole. (B) pll^{078}; (C) pll^{16}; (D) pll^{074}, composite images, lateral aspect. In each image, an arrow indicates the approximate position of the ventral midline.
We therefore examined Toll localization in wild-type embryos and in pll078 embryos, which exhibit an enhanced Tube localization gradient (Fig. 2B). In wild-type embryos, Toll distribution was uniform across the dorsoventral axis (Fig. 3B), as reported previously (Hashimoto et al., 1991). The distribution of Toll in pll078 embryos appeared identical (Fig. 3C,D, compare with 3B). Thus, there is no detectable aggregation of Toll concomitant with activation or with disruption of downstream signaling events.

**Pelle acts as a protein kinase in downregulating Tube clustering**

Experiments employing the yeast two-hybrid system have shown that the interaction of Tube and Pelle is modulated by Pelle catalytic function (Edwards et al., 1997). In particular, expression of a reporter for the Tube-Pelle interaction is enhanced more than 30-fold when Tube is paired with a form of Pelle carrying an inactivating mutation in the kinase catalytic domain. We were therefore interested in determining whether elimination of Pelle catalytic activity is sufficient to generate the enhanced Tube gradient. To identify mutations specifically affecting Pelle catalytic function, we sequenced a large collection of EMS-induced pelle mutations. We then assayed representative pelle alleles biochemically, to determine whether the allele made a protein product and, if so, whether the Pelle produced was active as a kinase and whether it could phosphorylate Tube (Fig. 4).

In 21 of the 22 pelle mutants sequenced we detected a single nonsense or missense mutation in the coding region (Table 1). Each of these 21 mutations involves a single base pair change; more than three-quarters are the C → T or G → A alterations most frequently generated by EMS mutagenesis. Surprisingly, all but three of the 21 mutants map to the Pelle kinase catalytic domain.

We found that our molecular and biochemical analysis of pelle mutations correlated well with previously published phenotypic analysis of these mutations (Hecht and Anderson, 1993). For example, six of the strongest alleles, according to phenotypic criteria, are due to stop codons that truncate the protein (Table 1). We examined a subset of these alleles for protein expression, and found that neither pll068 (Fig. 4) nor pll25 (data not shown) produced a stable protein.

Several of the pelle mutants alter residues that are highly conserved in protein kinases and are required for catalytic activity (Table 1; Fig. 5). The pll078 mutation, which disrupts the ATP binding site, is representative of this class. As shown in Fig. 2, Tube clustering is enhanced as greatly by pll078 as by pll25, a null mutation. The pll019 mutation also alters a residue required for ATP binding and results in a similar increase in Tube clustering (data not shown). These two alleles each produce a full-length protein that lacks protein kinase activity.
Some, but not all, of the antimorphic alleles (pll122, pll13, pll132, pll1322) are in or near the “activation loop”, a region that in many kinases is the site of regulatory modifications (Table 1; Fig. 5). The remaining antimorphic alleles (pll14, pll16, pll122, pll1222, pll1228) alter Pelle residues in regions of ill-defined function. We tested pll16, the strongest of these alleles in terms of both its phenotypic consequences and antimorphic activity, for effects on the Tube gradient, and found that pll16 enhanced the gradient as strongly as the other pelle alleles (Fig. 3C).

Our biochemical analysis of Pelle protein from mutant backgrounds also provides evidence for Pelle phosphorylation in vivo. The Pelle protein from pll1228 embryos has an increased mobility relative to that of both wild-type Pelle and Pelle from other missense mutants (Fig. 4). Moreover, sequence analysis reveals that the pll1228 mutation converts the serine residue at position 197 to a phenylalanine. Since the loss of a phosphorylation site typically results in an increase in mobility in SDS-PAGE, the data strongly suggest that serine 197 is modified by phosphorylation in embryos and that this modification is required for wild-type Pelle activity.

### DISCUSSION

**Pelle modulation of Tube signal-dependent clustering**

Tube and Pelle translocate to the plasma membrane upon activation of the Toll receptor (Towb et al., 1998). We find that an activity of Pelle, possibly direct phosphorylation of Tube, is required to disaggregate membrane-associated Tube clusters (Fig. 6). In pelle mutant embryos, Tube clustering along the ventral midline is greatly enhanced over that seen in wild-type embryos. Elimination of either of two wild-type activities of Pelle – Tube binding or protein kinase function – is sufficient for this enhancement.

Pelle can phosphorylate Tube in vitro (Grosshans et al., 1994; Shen and Manley, 1998) (Fig. 4). Furthermore, the pll1074 mutation, which blocks Tube phosphorylation but not Pelle autophosphorylation, enhances Tube clustering. We suggest, therefore, that in the wild-type embryo Pelle-mediated phosphorylation of Tube causes dissociation of multiprotein complexes containing Tube. The weak gradient of Tube seen in the wild type would thus represent a balance between Tube recruitment to clusters and Tube release from clusters upon Pelle-catalyzed phosphorylation.

Although we favor the idea that Pelle directly phosphorylates Tube to disrupt Tube-containing complexes, alternative mechanisms for regulating complex stability are possible. For example, Pelle might phosphorylate and activate an unidentified downstream target that would then disaggregate the Tube-containing complex. Distinguishing between these possibilities is made difficult by the fact that all known mutations that prevent the association of Tube and Pelle also block Pelle activation and hence downstream function. For instance, the tub2 mutation, which prevents interaction with Pelle, completely blocks signaling to Dorsal. Similarly, mutations in Pelle that do not affect the catalytic domain, but that alter the Tube binding domain, disrupt Pelle function (Xiao et al., 1999).

Nearly all the Pelle missense mutations we have sequenced here map to the kinase domain rather than the death domain,
the site of interaction with Tube. A possible explanation for this bias became apparent in our structural analysis of the interaction between Tube and Pelle (Xiao et al., 1999). A combination of crystallographic studies and mutational analyses revealed that the Tube death domain can productively interact with two different sets of surface residues in the Pelle death domain. One would therefore predict that most Tube binding sites in the Pelle death domain would be redundant in function and that such sites would not mutate to a loss-of-function phenotype. Thus, mutations that block signaling would most frequently map to the death domain of Tube, but outside the death domain in Pelle, as is in fact the case.

Antimorphic alleles of pelle

Extensive genetic characterization of pelle mutations led to the classification of several alleles as recessive antimorphs (Hecht and Anderson, 1993). A combination of crystallographic studies and mutational analyses revealed that the Tube death domain can productively interact with two different sets of surface residues in the Pelle death domain. One would therefore predict that most Tube binding sites in the Pelle death domain would be redundant in function and that such sites would not mutate to a loss-of-function phenotype. Thus, mutations that block signaling would most frequently map to the death domain of Tube, but outside the death domain in Pelle, as is in fact the case.

Feedback in dorsoventral patterning

Regulation of protein-protein interaction through phosphorylation has been widely documented in signal transduction pathways. In visual signal transduction, for example, modulation of the phosphorylation state of phosducin regulates the interaction of phosducin and the $\beta$ subunits of transducin (Gaudet et al., 1999). In the case of Tube and Pelle, such a change in phosphorylation state could be catalyzed by

Fig. 5. Sequence alignment of Pelle with related protein kinase catalytic domains. Listed are the catalytic domains of D. melanogaster Pelle (gi|158046), Caenorhabditis elegans Pelle (gi|7505619), mouse IRAK-1 (sp|Q62406), Arabidopsis thaliana receptor kinase (gi|7488290), human FGF receptor 1 (gi|3114358, PDB: 1FGI) and human C-Jun N-terminal kinase (gi|5542282, PDB: 1JNK). Roman numerals indicate the positions of the 12 conserved subdomains found in all eukaryotic protein kinases. Red cylinders indicate alpha helices found in both the FGF-R and the JNK structure; red arrows indicate beta sheets present in both structures. Amino acids shown above the alignment indicate the substitutions found in pelle mutant alleles; pink indicates a loss-of-function allele, while red indicates a loss-of-function allele with antimorphic activity. In the alignment, residues in bold type are present in greater than 95% of kinases, residues in dark blue are present in greater than 70% of kinases, and residues in light blue are similar in greater than 50% of kinases. Residues in green are conserved in the Pelle family, but are uncommon (present in less than 15% of a broad sampling) in other eukaryotic protein kinases (Hanks and Hunter, 1995).
Pelle itself. This could explain why a catalytically inactive form of Pelle interacts more strongly with Tube in the yeast two-hybrid system than does the wild-type Pelle.

Phosphorylation of Tube by Pelle could act negatively on the pathway, breaking apart Tube-containing complexes, as well as weakening the Tube/Pelle interaction. Since Pelle acts downstream of Tube (Galindo et al., 1995; Grosshans et al., 1994) and requires Tube-mediated targeting to the membrane for activation (Towb et al., 1998), both of these feedback mechanisms could act to turn Pelle activity down and decrease downstream signaling. This regulation may serve to increase the fidelity of downstream signal transduction. High, medium, and low nuclear levels of the Dorsal transcription factor control the transcription of different sets of genes in highly circumscribed ventral-to-dorsal territories in the early embryo (Jiang and Levine, 1993). Feedback mechanisms in the signal transduction pathway downstream of Toll may have evolved to ensure that proper nuclear Dorsal levels are maintained in the correct spatial regions of the embryo.

In considering negative regulation of Toll signaling, it is necessary to take into account that signal transduction is taking place during the highly rapid nuclear division cycles of early Drosophila embryogenesis. The Dorsal nuclear localization gradient does not persist during mitosis, but rather reforms after every nuclear division. Furthermore, Tube becomes associated with nuclei during each mitosis (S. Gillespie and S. A. W., unpublished results). Negative feedback may therefore be required to return the signaling cascade downstream of Toll to a starting state, setting the stage for reformation of the Dorsal gradient in the next interphase. In this regard, we note that the activation of both Spätzle and Easter, which act upstream of Toll, appears to be subject to a distinct form of negative feedback regulation (Misra et al., 1998; Morisato and Anderson, 1994).

Although the phenomenon of Tube gradient enhancement could, as outlined above, reflect a negative feedback mechanism, it is also possible that Tube release from clusters and Tube/Pelle complex dissociation are mechanisms that act to promote or spatially regulate downstream signaling. Activated Tube molecules might need to diffuse away from a signaling complex, or other components might need to cycle through steps of activation and deactivation, in order for downstream signaling to occur. Alternatively, if the Toll receptor is only activated in a narrow ventral stripe, the diffusion of activated Tube and Pelle could serve to establish a gradient of signaling away from the ventral midline, and thus fine tune the gradient of Dorsal nuclear translocation.

**Fig. 6.** Model for Pelle-mediated feedback in the dorsoventral signal transduction pathway. Control is shown as passing from each protein in the cascade in a linear fashion, although it is probable that all components are present in a large signaling complex (Edwards et al., 1997; Shen and Manley, 1998; Yang and Steward, 1997). Not all components that may be acting in this cascade have been included in this diagram (Grosshans et al., 1999; Zapata et al., 2000).

**Feedback regulation in mammalian Rel pathways**

The mechanism that we have proposed for downregulation of the dorsoventral pathway signal may function in mammals. The adaptor protein, MyD88, has been reported to bind more tightly to IRAK when the IRAK kinase domain has been inactivated, similar to the situation with Tube and Pelle (Edwards et al., 1997; Wesche et al., 1997). Therefore, upon activation, IRAK may phosphorylate MyD88 and cause the signaling complex to dissociate. This mechanism of regulating death domain interactions through phosphorylation, would thus be another aspect of the host defense system inherited from the common ancestor of flies and humans.

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