Development 128, 4729-4736 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV5944

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

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Accepted 13 September 2001

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 relocalization 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 relocalization. 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 Dorsal nuclear localization. High, medium, and low levels of nuclear Dorsal lead to differential regulation of downstream genes, thus defining embryonic polarity.

The Toll signal transduction pathway is not limited in function to establishment of the embryonic dorsoventral axis. Rather, the pathway is reused during larval development and in adults, where it acts in innate immunity (Imler and Hoffmann, 2000). A homologous pathway mediates an innate immune response in mammals (Beutler, 2000), with bacterial challenge activating signaling by the mammalian Toll-like receptors Tlr2 and Tlr4. Signal transduction triggered by these Tlr proteins directs nuclear import of the Dorsal homologues p50 and p65, which then activate transcription of innate immunity loci.

Not only are the receptors and transcription factors conserved in innate immunity, but also proteins mediating signaling between these pathway components (Wasserman, 2000). In both vertebrates and invertebrates, members of the IκB family of inhibitors block Rel protein nuclear translocation in the absence of signaling. Furthermore, signaling to the complexes formed by Rel and IκB proteins involves the protein kinase Pelle in flies, and its homologue IRAK (interleukin 1 receptor-associated kinase) in mammals. Amino-terminal death domains present in both IRAK and Pelle interact directly with death domain-containing adaptor proteins: MyD88 in the case of IRAK and Tube in the case of Pelle (Medzhitov et al., 1998; Xiao et al., 1999). Thus, the mammalian and insect Toll pathways have common components and a largely, but not wholly, conserved mode of action.

Both Pelle and Tube are required to transduce the Toll-mediated signal to the Dorsal/Cactus complex (Hecht and Anderson, 1993; Anderson and Nusslein-Volhard, 1984). Epistasis analysis with constitutively active, chimeric proteins demonstrated that Tube requires Pelle to signal to Dorsal, whereas Pelle can signal to Dorsal in the absence of Tube (Grosshans et al., 1994; Galindo et al., 1995). These results suggest that Pelle acts downstream of Tube and hence Toll. However, Pelle can phosphorylate Tube and can bind to Toll in a phosphorylation-dependent manner (Grosshans et al., 1994; Shen and Manley, 1998). Additionally, the interaction of Tube and Pelle is modulated through phosphorylation, with

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 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. We further show that Pelle must bind to Tube and be catalytically active to downregulate 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 pll^{161}, a gift from Dave Stein, have been described previously (Hecht and Anderson, 1993). Characterization of the *tube* alleles tub^2 and tub^4 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 antiserum has been described previously (Letsou et al., 1993). The polyclonal Toll antiserum, 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.

Immunoblotting and kinase assays of *pelle* mutations

Zero- to four-hour old embryos were collected from mothers that were transheterozygous for the mutant *pll* 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, 10% 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 $MnCl_2$, 2 mM $MgCl_2$, 5% glycerol) and suspended in 30 μl kinase buffer. The kinase reaction was started by adding 100 ng of bacterially expressed and renatured Tube-His_6 protein and 10 μCi of [γ-^{32}P]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.

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 Tub^2 protein in embryos. The tub^2 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 Tub^2 protein concentrates strongly in a broad band down the presumptive ventral midline (Fig. 1D). The Tube localization gradient is thus greatly enhanced in tub^2 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 tub^4 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

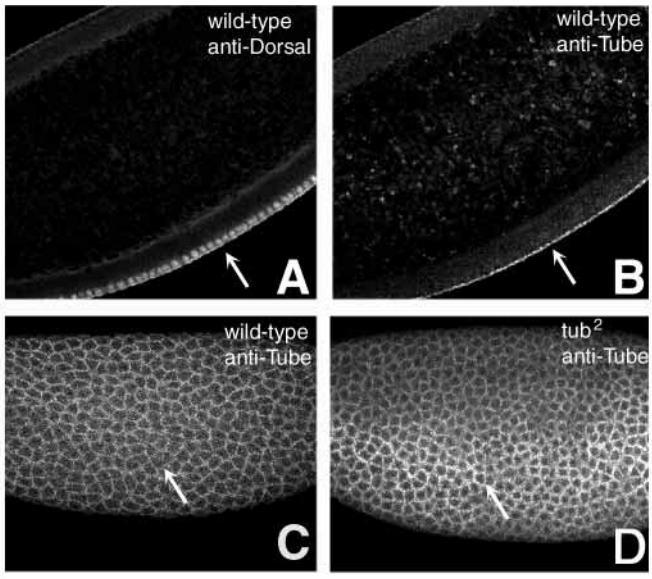

Fig. 1. The Tube localization gradient is enhanced in *tub*^{2} mutant embryos. (A,B) Paired images from a wild-type embryo, showing a longitudinal optical section in the plane of the dorsoventral axis. The embryo is stained with fluorescein-conjugated antibodies to Dorsal (A) and with antibodies to 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.

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,

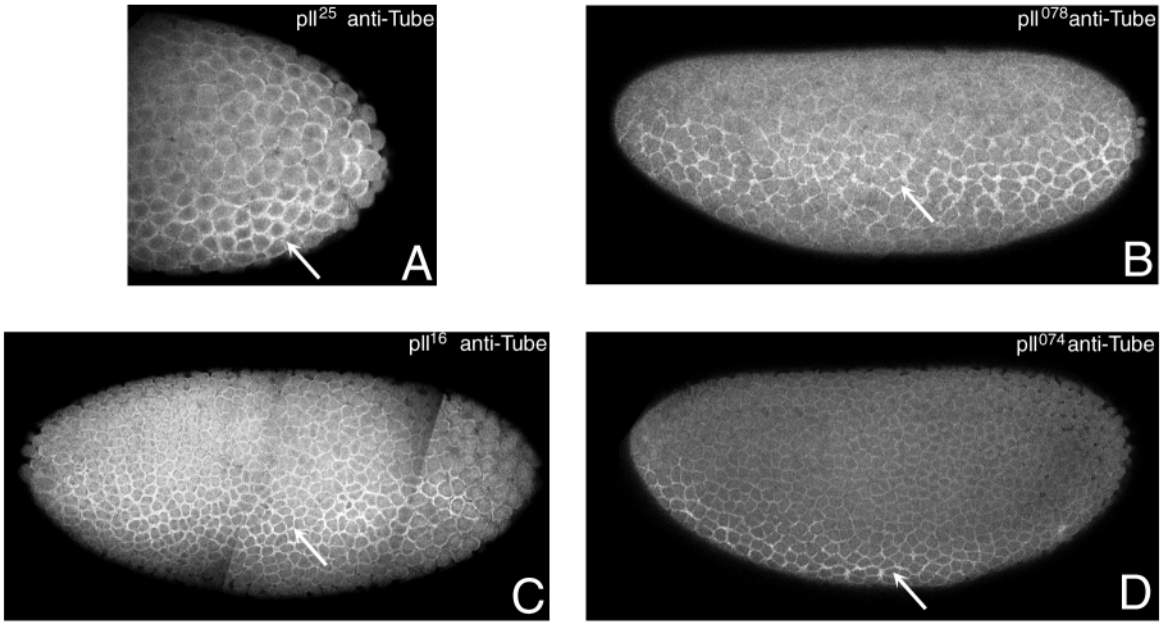

Fig. 2. The Tube localization gradient is enhanced in *pelle* mutant embryos. All images are surface views of embryos stained with antibodies to Tube detected with a Cy3-conjugated secondary antibody. (A) *pll*^{25,} lateral view of the posterior pole. (B) *pll*^{078}; (C) *pll*^{16}; (D) *pll*^{074}, composite images, lateral aspect. In each image, an arrow indicates the approximate position of the ventral midline.

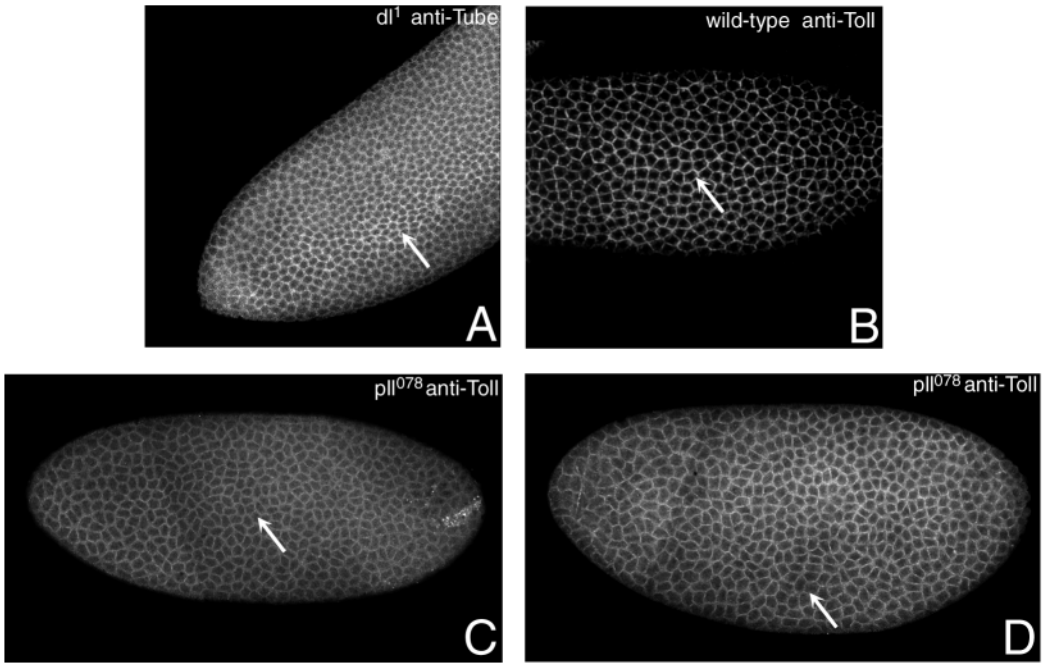

Fig. 3. Tube gradient formation is regulated at the level of the Tube/Pelle interaction. (A) *dl*^{1} embryo stained with antibodies to Tube detected with a Cy3-conjugated secondary antibody. Surface view of the anterior pole, lateral aspect. (B) Wild-type embryo stained with antibodies to Toll detected with a Cy3-conjugated secondary antibody. Surface view of the ventral aspect. (C,D) *pll*^{078} embryos stained with antibodies to Toll as above; composite images, surface view of the lateral aspect. In each image, an arrow indicates the approximate position of the ventral midline.

1998). We therefore examined Toll localization in wild-type embryos and in *pll*^{078} 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 *pll*^{078} 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 *pll*^{rm8} (Fig. 4) nor *pll*^{25} (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 *pll*^{078} mutation, which disrupts the ATP binding site, is representative of this class. As shown in Fig. 2, Tube clustering is enhanced as greatly by *pll*^{078} as by *pll*^{25}, a null mutation. The *pll*^{019} 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

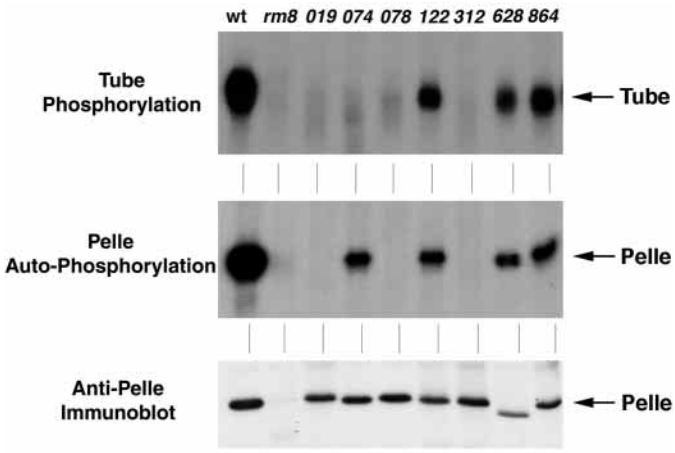

Fig. 4. Biochemical characterization of selected *pelle* mutations. The *pelle* alleles that were assayed in this experiment are indicated across the top. wt, wild type. (Top panel) Phosphorylation of Tube by Pelle mutant proteins. (Middle panel) Autophosphorylation of Pelle mutant proteins. (Bottom panel) Pelle protein levels in the indicated mutant backgrounds, as assayed by immunoblotting.

Table 1. EMS-induced alterations in the *pelle* coding region

Allele	Mutation	Phenotype	Antimorph?	Domain
25	Q93 STOP	D0	N	Death Domain
21	N228 STOP	D0	N	I
17	W232 STOP	D0	N	I/II
23	Q359 STOP	D0	N	VI/VII
rm8	Q422 STOP	D0	N	IX/X
12	Q443 STOP	D0	N	X
628	S197F	D1-WT	Y	Reg
14	S197F	D1-WT	Y	Reg
019	*G220E	D0-D1	N	I
078	*G225E	D0	N	I
864	E255K	D1-D2	N	II/III
16	L277F	D0	Y	IV
24	*G279E	D0	N	IV
122	C288Y	D0-D2	Y	IV/V
161	R320W	D3-WT	n.d.	VIA
074	Y332N	D0	N	VIA
385	I362F	D0	Y	VII
22	G363R	D0	Y	VII
13	G366S	D0	Y	VII
312	P392L	D0	Y	VII
15	C477Y	D3-WT	N	X/XI

Mutations are reported as follows: wild-type amino acid, position, and, except for stop codons, the substitution resulting from the mutation. Asterisks indicate residues known to have a conserved role in catalytic activity of the kinase domain. With the exception of pll^{161}, *pelle* allele designations and phenotypic characterizations have been described previously (Hecht and Anderson, 1993). D0 embryos are completely dorsalized; D1 moderately dorsalized; D2 weakly dorsalized; D3 very weakly dorsalized; and WT is wild-type. Whether listed alleles have an antimorphic character is indicated with a Y (Yes) or an N (No). Roman numerals indicate positions relative to the 11 catalytic subdomains conserved among protein kinases (Hanks and Hunter, 1995). n.d., not determined; Reg, regulatory domain.

activity (Fig. 4). Thus, the phosphotransferase activity of Pelle is required to downregulate Tube clustering.

Our analysis of mutations in the *pelle* protein kinase domain allows us to conclude that Pelle must phosphorylate a substrate other than itself in effecting signal transduction. This conclusion is based on a comparison of the properties of pll^{122}, pll^{864} and pll^{074}, three mutations that alter nonconserved residues in the catalytic domain (Table 1; Fig. 5). All three alleles produce a Pelle protein that catalyzes autophosphorylation (Fig. 4). However, the Pelle protein in pll^{122} and pll^{864} embryos, but not pll^{074} embryos, can phosphorylate Tube in vitro. This difference in catalytic activity correlates with a marked difference in the ability to signal to Dorsal. The pll^{122} and pll^{864} mutations have only a weak effect on signal transduction to Dorsal, whereas pll^{074} acts as a null (Hecht and Anderson, 1993). Thus Pelle's phosphorylation of another protein is apparently essential for efficient signaling.

Eight of the *pelle* alleles are antimorphic in that they can have a dominant negative effect on signal transduction in embryos partially impaired for Pelle function (Hecht and Anderson, 1993). Our molecular and biochemical analyses fail to reveal any simple explanation for this behavior. For example, in pll^{312}, all kinase activity of the encoded protein is lost (Fig. 4), similar to the effects of pll^{078}, which is not antimorphic. Therefore, although an antimorphic *pelle* allele may have lost phosphotransferase activity, loss of this activity is not sufficient to generate an antimorphic form of the protein.

Some, but not all, of the antimorphic alleles (pll^{22}, pll^{13}, pll^{312}, pll^{385}) 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 (pll^{14}, pll^{16}, pll^{122}, pll^{628}) alter Pelle residues in regions of ill-defined function. We tested pll^{16}, the strongest of these alleles in terms of both its phenotypic consequences and antimorphic activity, for effects on the Tube gradient, and found that pll^{16} 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 pll^{628} 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 pll^{628} 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 pll^{074} mutation, which blocks Tube phosphorylation but not Pelle autophosphoryation, 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 tub^2 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,

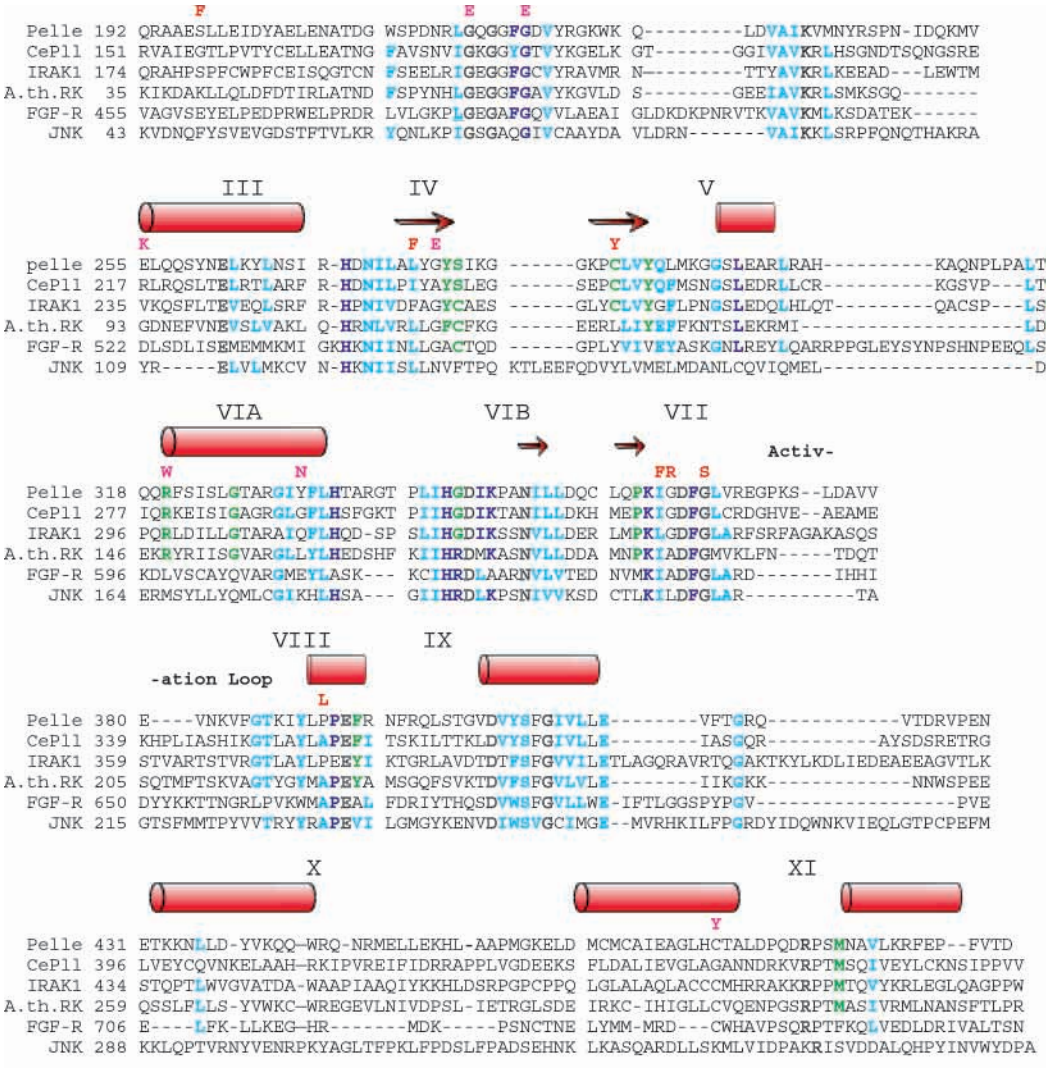

Fig. 5. Sequence alignment of Pelle with related protein kinase catalytic domains. Listed are the catalytic domains of *D. melanogaster* Pelle (gi|158046), *Caenorhabditis elegans* Pelle (gi|7505619), mouse IRAK-1 (sp|Q62406), *Arabidopsis thaliana* receptor kinase (gi|7488290), human FGF receptor 1 (gi|3114385, 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).

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). The term antimorphic indicates that the presence of this allele is more detrimental to development than a complete absence of the functional gene. Our analysis reveals that most of the *pelle* alleles classified as strong antimorphs alter residues in or near a part of the kinase structure known as the activation loop. Such activation loops often contain residues that are targets for upstream, activating kinases (Morgan and De Bondt, 1994). In the case of the protein kinase ERK2, for example, phosphorylation of residues in the activation loop alters the conformation of the active site cleft, affecting ERK2's ability to bind ATP and changing the conformation of the substrate-recognition region (Canagarajah et al., 1997). By analogy, the antimorphic effect of several *pelle* mutations may be due to their binding and sequestering either an upstream activating kinase for Pelle or a Pelle substrate.

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 βγ subunits of transducin (Gaudet et al., 1999). In the case of Tube and Pelle, such a change in phosphorylation state could be catalyzed by

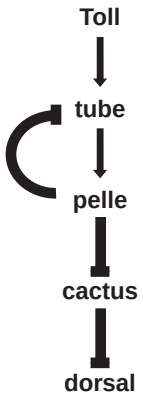

Fig. 6. Model for Pelle-mediated feedback in the dorsoventral signal transduction pathway. Control is shown as passing from each protein in the cascade in a linear fashion, although it is probable that all components are present in a large signaling complex (Edwards et al., 1997; Shen and Manley, 1998; 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).

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.

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.

We thank Sarah Johnson and Jason Baker for technical assistance, Tammie Stone for help with artwork, and Kathryn Anderson and Dave Stein for providing fly stocks used in this study. We also express special thanks to Christiane Nüsslein-Volhard, in whose laboratory some of the experiments were conducted. Larry Goldstein generously provided user time on the confocal microscope. Cynthia Lavoie supplied the anti-Toll serum, and suggested that the release of Tube from complexes may be used for gradient shaping as opposed to being a negative feedback mechanism. Ethan Bier and an anonymous reviewer provided critical and helpful comments on the manuscript. This work was supported by NIH grant GM50545 to S. A. W. and an NSF fellowship to P. T.

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