Interaction of the pelle kinase with the membrane-associated protein tube is required for transduction of the dorsoventral signal in Drosophila embryos

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SUMMARY

Within the Drosophila embryo, tube and the protein kinase pelle transduce an intracellular signal generated by the transmembrane receptor Toll. This signal directs import of the rel-related protein dorsal into ventral and ventrolateral nuclei, thereby establishing dorsoventral polarity. We show by immunolocalization that tube protein associates with the plasma membrane during interphase. We also find that tube sequences required for signaling interact with pelle in a yeast two-hybrid assay. We demonstrate that fusion of the pelle catalytic domain to the transmembrane receptor torso is sufficient to induce ventral fates; this activity is independent of Toll or tube. Lastly, we find that fusion of the tube protein to torso also induces ventral fates, but only in the presence of functional pelle. We propose a model wherein tube activates pelle by recruiting it to the plasma membrane, thereby propagating the axis-determining signal.

Key words: signal transduction, rel protein, two-hybrid, torso, Toll, pelle kinase, tube, Drosophila

INTRODUCTION

Cell fates along the dorsoventral axis of the Drosophila embryo are defined by a nuclear concentration gradient of the transcription factor dorsal (reviewed in St. Johnston and Nüsslein-Volhard, 1992). Prior to formation of the blastoderm, dorsal protein is distributed evenly throughout the syncytial cytoplasm of the embryo, where it is retained through an interaction with an inhibitory protein, cactus. Upon localized activation of the transmembrane receptor Toll, a signal is transduced in ventral and ventrolateral regions of the embryo that frees dorsal from cactus inhibition. Dorsal protein then enters nuclei, where it mediates activation of ventral-specific genes and repression of dorsal-specific genes.

Transmission of the signal from Toll to the dorsal/cactus complex is known to require two genes, pelle and tube. In the absence of maternal pelle or tube function, dorsal remains cytoplasmic in ventral as well as dorsal regions of the embryo, regardless of the activation state of Toll (Roth et al., 1989; Hecht and Anderson, 1993). Homozygous females carrying loss-of-function mutations in pelle, tube, Toll or dorsal produce embryos that die as twisted tubes of dorsal epidermis. In contrast, loss-of-function cactus mutations or gain-of-function Toll mutations cause dorsal to translocate into nuclei along the entire dorsoventral circumference, resulting in a ventralized phenotype (Roth et al., 1989, 1991; Steward, 1989; Schüpbach and Wieschaus, 1989).

Mammalian counterparts have been identified for three of the components of the intracellular dorsoventral signaling pathway (reviewed in Wasserman, 1993). Toll and the interleukin-1 (IL-1) receptor share 40% similarity over 130 amino acids of their intracellular domains. Dorsal displays a high degree of sequence similarity to the DNA-binding protein encoded by the proto-oncogene c-rel (80% similarity over the first 340 amino acids) and to the p50 and p65 subunits of the transcription factor NF-κB. Lastly, cactus and IkB are both members of the family of ankyrin-repeat containing proteins and share 36% similarity in their repeat-containing domains.

The IL-1 receptor, NF-κB, and IkB, like their Drosophila counterparts, constitute elements of a signal transduction pathway. Activation of the IL-1 receptor in lymphocytes frees NF-κB from inhibition by IkB, allowing NF-κB to translocate into nuclei. Thus, there exists molecular and functional conservation between the Drosophila dorsoventral and mammalian IL-1 signaling pathways.

Phosphorylation has been implicated in signaling initiated by both Toll and the IL-1 receptor, but only for the Drosophila pathway has a relevant protein kinase been definitively identified. The catalytic activity of this kinase, pelle, is required for signaling and is predicted to be specific for serine or threonine residues (Shelton and Wasserman, 1993). Although the substrate or substrates for pelle in vivo are unknown, both dorsal and cactus are potential targets. The dorsal protein undergoes signal-dependent phosphorylation in Drosophila embryos, but there is no evidence that this modification is catalyzed directly by pelle (Whalen and Steward, 1993; Gillespie and Wasserman, 1994). It is not yet known whether cactus is also modified in the course of signal transduction, but
its mammalian counterpart, IkB, is phosphorylated in vivo in response to IL-1 (Beg et al., 1993). Two additional questions regarding pelle remain unanswered. First, it is unclear whether pelle activity is regulated and, if so, how this regulation is achieved. Second, the relative position of pelle in the signaling pathway has not been determined.

Tube remains the least well understood of the five Drosophila proteins known to be involved in the intracellular transmission of the ventralizing signal. Tube shares no sequence similarity with any previously described protein (Letsou et al., 1991). Deletion analysis, together with an evolutionary comparison of tube genes within the Drosophila genus, has defined two domains in the tube protein (Letsou et al., 1993). The amino-terminal domain is well-conserved and is sufficient to rescue tube null embryos, though with an efficiency less than that of the full-length protein. All characterized inactivating mutations map to this domain. The carboxy terminal domain is notable for the presence of five copies of an evolutionarily conserved, eight-amino-acid motif.

Here, we further explore the roles of pelle and tube in dorsoventral signaling, beginning with tube immunolocalization studies. We demonstrate that tube protein localizes primarily to the membrane of interphase syncytial blastoderm embryos. We use the yeast two-hybrid system to show that pelle associates directly with the tube protein. We further find that pelle is activated by fusion to the extracellular and transmembrane regions of the receptor tyrosine kinase torso. This fusion creates an activated pelle kinase that specifies ventral fates in the absence of tube function or Toll activation. When fused to the same regions of torso, tube can also specify ventral fates in the absence of Toll activation. However, this activity is pelle-dependent, placing pelle downstream of tube in the signal transduction pathway. These results lead us to consider a model wherein tube recruits pelle to the plasma membrane, thereby activating the pelle kinase and triggering dorsal nuclear import.

MATERIALS AND METHODS

Fly stocks

Drosophila melanogaster stocks were maintained on standard cornmeal-yeast-agar medium (Ashburner, 1989) at 18°C, 22°C or 25°C. Oregon R was used as the wild-type stock. The mutations tub2, tub3 and tubR5.6 mutations, as well as the tube deficiency Df(3R)XM3 and the pelle deficiency Df(3R)JR16, have been described previously (Letsou et al., 1993; Hecht and Anderson, 1993; Shelton and Wasserman, 1993). The Toll null genotype was Df(3R)T908X4 Df(3R)RorR3 (Hashimoto et al., 1991). The 1.4 kb twist-lacZ construct has been described by Thisse and coworkers (Thisse et al., 1991). All other mutations and balancers are described in Lindley and Zimm (1992).

Immunocytochemistry

Aged embryos were dechorionated and fixed and their vitelline membranes removed by a modified version of a technique first described by Mitchison and Sedat (1983). All manipulations were carried out at room temperature (RT). Dechorionated embryos were fixed in glass vials by shaking for 20-45 minutes in a 1:1 mixture of heptane and fixative (3.5% formaldehyde (EM Sciences) in 100 mM Pipes/1 mM MgCl2/1 mM EGTA, pH 6.9). To devitellinize fixed embryos, fixative (bottom phase) was replaced with 5-10 ml of 90% methanol/50 mM EGTA and vials were vigorously shaken for 5 minutes. Devitellinized embryos sank to the bottom, where they were recovered and transferred to a microcentrifuge tube. Embryos were rinsed three times with methanol and were stored in methanol at 4°C.

Fixed embryos were rehydrated by washing twice in 50% methanol/50% KBPS (137 mM NaCl/2.7 mM KCl/1.5 mM KH2PO4/8 mM Na2HPO4, pH 7.3) and twice in KBPS. Embryos were blocked 4 hour at RT or overnight at 4°C in blocking buffer (1% BSA/0.1% Triton X-100 in KBPS). Embryos were incubated overnight at 4°C in primary antibody diluted in blocking buffer. Crude rabbit anti-tube serum (Letsou et al., 1993) was used at a concentration of 1:2000. Affinity-purified rabbit anti-Toll antibodies (a gift of C. Hashimoto) were used at a concentration of 1:50. All subsequent steps were carried out at RT. Embryos were washed six times in blocking buffer over a period of 1.5 to 4 hours. The secondary antibody, Cy3-conjugated goat anti-rabbit IgG (Jackson Immunochemicals), was diluted to 1 µg/ml in blocking buffer and was applied for 1 hour. Next, embryos were washed six times over 1.5 hours in either blocking buffer or KBPS. Embryos were mounted on slides in Fluoromount G.

Microscopy and digital image processing

Laser scanning confocal microscopy was employed to analyze whole-mount embryos labeled with fluorescent antibodies. Images were collected with an MRC-600 laser scanning confocal unit (Bio-Rad) attached to a Zeiss Axiopt photomicroscope. Embryos were illuminated with an argon laser and were visualized through a 40× Zeiss Plan Neofluor lens (NA 1.3).

Using the Bio-Rad COMOS software, the pixel intensity ranges of the raw confocal images were rescaled to the full range. Confocal images were imported into Photoshop (Adobe), where final adjustments were made to the black levels. Digital images were printed with a Tektronix Phaser IISDX dye-sublimation printer.

Bacteria and yeast

XL1 Blue (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F' proAB, lacZAM15, Tn10 (tet)]]) (Stratagene, Incorporated) was used as the bacterial host. The yeast strain EGY48 (MATa, trp1, ura3, his3, LEU2::pLexAop6-LEU2) was a gift of R. Brent (Gyuris et al., 1993). Yeast transformations were by electroporation or by a one-step alkali-ion method (Chen et al., 1992).

Two-hybrid plasmid construction

The DNA-binding domain plasmid, pEG202, and the activation domain plasmid, pJG4-5, were provided by R. Brent, as was the lacZ reporter plasmid, pSH18-34. This reporter plasmid, constructed by S. Hanes, has eight LexA operators and is more sensitive than the reporter plasmid, pSH18-34. This reporter plasmid, constructed by S.

Two-hybrid interaction and transcriptional repression assays

The reporter, DNA-binding domain fusion, and activation domain plasmid, pDE028, was constructed by inserting the DNA-binding domain plasmid, pEG202, and the activation domain plasmid, pJG4-5, into the pJG4-5 plasmid. The reporter, DNA-binding domain fusion, and activation domain plasmid, pDE028, was constructed by inserting the DNA-binding domain plasmid, pEG202, and the activation domain plasmid, pJG4-5, into the pJG4-5 plasmid. The DNA-binding domain plasmid, pEG202, and the activation domain plasmid, pJG4-5, were provided by R. Brent, as was the lacZ reporter plasmid, pSH18-34. This reporter plasmid, constructed by S. Hanes, has eight LexA operators and is more sensitive than the reporter plasmid, pSH18-34. This reporter plasmid, constructed by S.

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fusion plasmids were transformed into EGY48 sequentially. Transform-
imported yeast were grown to stationary phase in complete minimal
liquid media containing glucose. Samples were diluted 1:60 in 2% 
galactose/1% raffinose complete minimal liquid media and allowed
to grow to late-log phase. β-galactosidase activity was assayed using
ortho-nitrophenyl galactoside as the substrate and normalized to total
protein concentration, as obtained using a modified BCA assay (Hill
In cases where no interactions were detected for a DNA-binding
domain fusion protein, we verified that the fusion protein was
expressed and localized to the yeast nucleus by carrying out a tran-
scriptional repression assay, as described by Brent and Ptashne

Tor-pll and Tor-tub DNA constructs
For the tor-pll construct, site-directed mutagenesis of pelle was
performed with the Altered Sites system and the pSELECT vector
(Promega). Positions 652 and 657 of the pelle cDNA sequence in pSE-
SELECT (pS601; Shelton and Wasserman, 1993) were changed to
a G and C respectively, thereby introducing a BsrEII site starting at
position 652. The cDNA sequences corresponding to the first 455
amino acids of torso and torso^6021 were obtained by cleavage of
plasmids pBtor and pB4021 (Sprenger and Nusslein-Volhard, 1992)
with HindIII and BsrEII and then inserted independently into the
modified pS601. The resulting chimeric proteins contain the extra-
cellular and transmembrane domains of torso or torso^6021 fused to
residues 163-501 of pelle. To introduce the K240R mutation into tor-
pll, the cDNA sequence of tor-pll encoding up to pelle residue 229
was obtained by cleavage with HindIII and SacII and was placed into
a K240R-containing-pS601 plasmid.
For the tor-tube construct, DNA encoding the entire tube ORF was
PCR amplified with primers introducing a BsrEII site 5’ to the initia-
torso 4021

b-tubulin or dIII and thereafter inserted into pS601. To introduce the
K240R mutation into tor-tube, DNA encoding the entire tube ORF was
PCR amplified with primers introducing a HindIII site 5’ to the initia-
torso 4021

was then used to replace the pelle coding region in the tor-pll construct described
above.

RNA microinjection
Embryo collection, RNA micro-injection and cuticle preparations
were carried out as described pre-
vously (Shelton and Wasserman,
1993). Embryos were injected at
stage 2 through the posterior end
and RNA was deposited in the
center of the embryo, except as
noted in the text.

RESULTS
Tube localization in syncytial blastoderm
embryos
The tube protein is an essential
link in the signaling pathway that
couples activation of the Toll
receptor to dorsal protein
nuclear translocation. To begin
to define tube’s biochemical
role in signaling, we examined
the distribution of tube in wild-
type embryos. Tube protein
was labeled in whole-mount
embryos with polyclonal anti-tube serum and a fluorophore-
conjugated secondary antibody. Tube protein was then visual-
ized by confocal microscopy.

During the early-cleavage stage of development, tube is dis-
tributed evenly throughout the embryo (data not shown). In the
majority of syncytial blastoderm embryos examined, tube
protein was concentrated in a conspicuous mesh-like array
organized at the surface of the embryo (Fig. 1A,C). A fraction
of the tube protein was dispersed within the cytoplasm.
The mesh-like tube surface array resembles that of the embryonic
plasma membrane visualized with fluororesently labeled Con-
canavalin A (Warn et al., 1984). Indeed, in cross-sectional
views of syncytial embryos, tube was associated with
membrane invaginations around the nuclei (Fig. 1B). These
membrane invaginations most likely represent the boundaries
of somatic buds (cytoplasmic protrusions overlying each inter-
phase nucleus), but might also be pseudocleavage furrows
(membrane invaginations that segregate neighboring spindles
during mitosis) (Foe and Alberts, 1983).

The membrane association of tube was readily apparent in
embryos in mitotic cycles 10 through 13. It is during these
stages that the dorsal nuclear localization gradient is most
apparent and the signaling pathway is most susceptible to acti-
vation with exogenous Toll ligand (Roth et al., 1989; Rushlow
et al., 1989; Steward, 1989; Stein et al., 1991). In slightly older
embryos, those beginning to undergo cellularization (mitotic
cycle 14), tube appeared to be entirely cytoplasmic; specific
membrane labeling was undetectable (data not shown). We also identified a small fraction of syncytial blastoderm

![Fig. 1. Membrane association of tube protein in wild-type embryos. Embryos from wild-type females (A-
C) or tube null (tube^625/Df(3R)X3M3) females (D) were labeled with anti-tube serum and a Cy3-conjugated
secondary antibody and were analyzed by confocal immunofluorescence microscopy. (A) Surface view
and (B) longitudinal optical section of an embryo in mitotic cycle 11. (C) Surface view of a cycle 12
embryo. (D) Surface view of an embryo from a tube null female. No staining was observed using the
same confocal scan parameters employed in the acquisition of A–C. However, when the gain was
increased five-fold, the faint image of an embryo became visible (data not shown). Scale bar, 50µm.](image-url)
embryos in which tube was distributed in a distinctively spotted pattern suggestive of nuclear localization. When embryos were additionally labeled with the DNA-binding dye, DAPI, the spotted distribution of tube was seen to colocalize exclusively with nuclei in prophase or prometaphase of mitosis (data not shown).

The localization of tube is independent of Toll

The Toll transmembrane protein resides in the embryonic plasma membrane (Hashimoto et al., 1991). Toll is distributed in the mesh-like pattern typical of membrane proteins, a localization that is clearly similar to that of tube (Fig. 2A,B, compare to Fig. 1A,B). Unlike tube, Toll localizes solely to the membrane; no cytoplasmic Toll protein was observed.

Since no dorsoventral asymmetry was evident in the localization of tube in wild-type embryos, the localization of tube did not appear to be signal-dependent. To assay whether the presence or activity of Toll was required for tube’s localization to the membrane, we examined the distribution of tube in embryos derived from females null for the Toll locus. In the absence of the Toll protein, tube remains membrane associated (Fig. 3).

Direct association of the tube and pelle proteins

To extend our investigation of tube’s role in dorsoventral signaling, we initiated studies to identify proteins with which tube associates. Genetic experiments suggest that pelle is one such protein. Though neither tube nor pelle is haploinsufficient, females heterozygous for specific alleles of both loci produce dorsalized embryos, indicating the possibility of a direct physical interaction (Hecht and Anderson, 1993). Our localization studies suggest that Toll might also associate with tube.

Interactions among tube, pelle and Toll were assayed using the yeast two-hybrid system (Fields and Song, 1989; Fields and Sternglanz, 1994). For these studies, the vectors developed by Brent and coworkers were used (Gyuris et al., 1993). The DNA-binding domain is derived from the LexA protein, the activation domain is an artificial construct, B42, and the lacZ reporter gene is controlled by lexA-binding sites from the ColE1 operator.

The two-hybrid experiments revealed a strong interaction between tube and pelle (Table 1). Cotransformation of the LexA-pelle and the B42-tube fusion constructs into yeast resulted in a greater than 100-fold increase in the activity of the reporter gene, β-galactosidase (Table 1, rows 2, 5 and 6). The tube and pelle proteins did not interact with the control proteins, SNF1 and SNF4, though these two yeast proteins interacted with one another (Table 1, row 1), as reported previously (Fields and Song, 1989).

Although tube and pelle interacted strongly, neither had a detectable interaction with the intracellular domain of Toll (intTl, Table 1). To confirm that the LexA-intTl fusion used in

Fig. 2. Membrane localization of Toll protein in wild-type embryos. Embryos from wild-type females were labeled with anti-Toll antibodies and a Cy3-conjugated secondary antibody and were analyzed by confocal immunofluorescence microscopy. (A,B) Surface and longitudinal optical section, respectively, of a cycle 12 embryo. Scale bar, 50 μm.

Fig. 3. Membrane localization of tube in Tlnull mutant embryos. Syncytial embryos from (A) wild-type or (B) Tlnull (Df[3R]Tl9QRX/Df[3R]roXB3) females were labeled with anti-tube serum and a Cy3-conjugated secondary antibody and were analyzed by confocal immunofluorescence microscopy. Scale bar, 50 μm.
Pelle interacts with tube or pelle. InterTl protein enters the nucleus and binds DNA, but does not reduce activity to 90 (±20) units. Thus, in yeast, the LexA-730 (±150) units; introduction of the LexA-interTl construct by a ColE1 site adjacent to the TATA box.

In vivo is necessary for signaling in embryos, then the pelle interaction domain should map to the amino-terminal domain of tube. This was indeed the case. The B42 activation domain was fused to either the amino-terminal tube domain (tubNterm) or the carboxy-terminal domain (tubCterm). When assayed in combination with the LexA-pelle construct, the amino-terminal tubNterm activated the reporter gene to an extent comparable to that seen with the full-length B42-tube construct. The B42 activation domain was fused to either the amino-terminal tube domain (tubNterm) or the carboxy-terminal domain (tubCterm). When assayed in combination with the LexA-pelle construct, the amino-terminal tubNterm activated the reporter gene to an extent comparable to that seen with the full-length B42-tube construct (Table 1, rows 12 and 13). This diminution in activity did not reflect differences in protein stability, since the mutant forms were detected at the same level as the wild-type fusion protein in immunoblot analysis (data not shown). Thus, two distinct tube point mutations that abolish dorsoventral patterning in the embryo block a specific association of the tube and pelle proteins in yeast.

**Table 1. The amino-terminal domains of tube and pelle interact in the yeast two-hybrid system**

<table>
<thead>
<tr>
<th>LexA fusion</th>
<th>B42 fusion</th>
<th>Mean activity</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SNF1</td>
<td>SNF4</td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td>SNF1</td>
<td>tube</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>SNF1</td>
<td>pelle</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>tube</td>
<td>–</td>
<td>2500</td>
</tr>
<tr>
<td>5</td>
<td>pelle</td>
<td>SNF4</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>pelle</td>
<td>tube</td>
<td>320</td>
</tr>
<tr>
<td>7</td>
<td>interTl</td>
<td>SNF4</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>interTl</td>
<td>tube</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>interTl</td>
<td>pelle</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>pelle</td>
<td>tubNterm</td>
<td>550</td>
</tr>
<tr>
<td>11</td>
<td>pelle</td>
<td>tubCterm</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>pelle</td>
<td>tub2</td>
<td>3</td>
</tr>
<tr>
<td>13</td>
<td>pelle</td>
<td>tub3</td>
<td>3</td>
</tr>
<tr>
<td>14</td>
<td>pellNterm</td>
<td>tube</td>
<td>790</td>
</tr>
<tr>
<td>15</td>
<td>pellCterm</td>
<td>tube</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>pellNterm</td>
<td>tubNterm</td>
<td>1290</td>
</tr>
<tr>
<td>17</td>
<td>pellCterm</td>
<td>tubCterm</td>
<td>1</td>
</tr>
</tbody>
</table>

For each pair of constructs, Mean activity and Range reflect the average and range of values for a minimum of four independent β-galactosidase assays involving two or more independent yeast transformations. All values were normalized to the level of activation seen with SNF1 and tube; means were rounded off to the nearest integer. The Drosophila portions of the fusion constructs are as follows: tube: full-length tube; tubNterm: tube(1-258); tubCterm: tube(257-462); pelle: full-length pelle; pellNterm: pelle(1-209); pellCterm: pelle(210-501); interTl: Toll intracellular domain (829-1097). A fusion of tube to the LexA DNA-binding domain activated transcription in the absence of a partner protein (row 4).

These assays were properly expressed and localized in yeast, we used a transcriptional repression assay devised by Brent and Ptashne (1984). In this assay, constitutive expression of a β-galactosidase reporter gene can be repressed by binding of a LexA fusion protein to a ColE1 site adjacent to the TATA box. β-galactosidase activity with the pJK101 reporter alone was 730 (±150) units; introduction of the LexA-interTl construct reduced activity to 90 (±20) units. Thus, in yeast, the LexA-interTl protein enters the nucleus and binds DNA, but does not interact with tube or pelle.

**Tube mutations that block signaling disrupt the interaction of tube with pelle**

We have previously demonstrated that the amino-terminal domain of tube is sufficient for signal transduction (Letsou et al., 1993). If the association of tube and pelle demonstrated in vivo is necessary for signaling in embryos, then the pelle interaction domain should map to the amino-terminal domain of tube. This was indeed the case. The B42 activation domain was fused to either the amino-terminal tube domain (tubNterm) or the carboxy-terminal domain (tubCterm). When assayed in combination with the LexA-pelle construct, the amino-terminal tube construct activated the reporter gene to an extent comparable to that seen with the full-length B42-tube construct (Table 1, rows 6 and 10). In contrast, the carboxy-terminal fusion protein did not detectably interact with pelle (Table 1, row 11), although a LexA-tubCterm construct entered the nucleus and bound DNA (β-galactosidase activity was repressed from 730 (±150) units to 50 (±40) units in the transcriptional repression assay).

We next assayed the association of pelle with mutant forms of the tube protein. Two inactivating mutations, tub2 and tub3, each alter a single residue within the conserved amino-terminal domain (Letsou et al., 1993). Females carrying either of these mutations produce dorsalized embryos. Fusion of either the tub2 or tub3 coding region to B42 resulted in 100-fold less activity in the two-hybrid assay than seen with wild-type B42-tube (Table 1, rows 12 and 13). This diminution in activity did not reflect differences in protein stability, since the mutant forms were detected at the same level as the wild-type fusion protein in immunoblot analysis (data not shown). Thus, two distinct tube point mutations that abolish dorsoventral patterning in the embryo block a specific association of the tube and pelle proteins in yeast.

**Pelle associates with tube through its putative regulatory domain**

The pelle protein sequence can be divided into a carboxy-terminal domain that provides catalytic function and an amino-terminal domain that, by analogy to other protein kinases, is predicted to play a regulatory role (Shelton and Wasserman, 1993). We generated fusion constructs for each of the two pelle domains and assayed their ability to interact with tube and tubNterm in the two-hybrid assay. In the presence of B42-tube or B42-tubNterm, the amino-terminal pelle construct provided substantial activation of the lacZ reporter gene, whereas the carboxy-terminal construct did not increase lacZ expression above background levels (Table 1, rows 14-17). Thus, the interaction of pelle with tube is mediated by the putative regulatory domain of pelle and not by the protein kinase catalytic domain.

**The pelle catalytic domain is active in a chimeric transmembrane protein**

Given that Toll and tube localize to the plasma membrane and that tube and pelle physically interact, we postulated that pelle is recruited to the plasma membrane by tube as a necessary step in signaling. To determine whether pelle can function at the plasma membrane, we substituted the catalytic domain of pelle for the tyrosine kinase domain of a ligand-independent form of the Drosophila torso transmembrane receptor (tor4021, Sprenger and Nüsslein-Volhard, 1992; Fig. 4). Similar tor4021 fusions, which are localized to the plasma membrane, have been shown to activate the catalytic domains of both tyrosine and serine/threonine specific protein kinases in Drosophila (Dickson et al., 1992a,b).

To assay the activity of the tor4021-PLL fusion construct, we carried out an RNA microinjection assay. Whereas uninjected embryos from pelle mutant females (hereafter, pll embryos) develop only dorsal epidermis, pll embryos injected with wild-type pelle RNA develop a wild-type cuticle pattern and frequently hatch (Shelton and Wasserman, 1993). When the tor4021-PLL construct was transcribed in vitro and the transcripts injected into pll embryos, we observed the production of both filzkörper and ventral denticles, representative of dorsolateral and ventral ectodermal fates, respectively (Table 2; Lohsh-Scharin et al., 1979). The tor4021-PLL RNA did not, however, restore a wild-type cuticle pattern. The number and width of the ventral denticle belts was aberrant and holes were sometimes present, as described for dominant ventralizing mutations of Toll (Erdélyi and Szabad, 1989; Schneider et al., 1991). Thus, the pelle catalytic domain can function in signal transduction when anchored to the cell surface by a membrane-
spanning segment, albeit in a manner distinct from the wild-type enzyme.

The activity of the tor4021-pll construct in pll mutant embryos was not dependent on the activating mutation in the torso sequences. When RNA transcripts were generated from a fusion of pelle to wild-type torso sequences (Fig. 4) and injected into pll mutant embryos, dorsolateral and ventral fates were restored to a degree comparable to that seen with the tor4021 fusion construct (Table 2). We observed the induction of both filzkörper and ventral denticles, with the latter frequently generated in the center of the embryo, surrounding the site of RNA deposition (Fig. 5C).

RNA microinjection experiments have demonstrated that the restriction of the torso ligand to the poles of tor+ embryos prevents torso activation between the poles (Sprenger and Nüsslein-Volhard, 1992). Since injection of tor-pll RNA into the center of pll mutant embryos induced ventral denticle formation, our results indicate that the activity of the tor-pll construct is not dependent on ligand activation of the extra-cellular torso domain. Tor-pll protein that bound the torso ligand at either pole should not induce ventral fates between the poles, because Toll, which acts through pelle, produces only a localized response in injection experiments (Anderson et al., 1985). The activity of the tor-pll construct is also not the result of deletion of the first 162 amino acids in pelle. We have shown that a deletion of residues 4-160 inactivates, rather than activates, pelle in the RNA injection assay (C. Shelton and S. A. W., unpublished data). These experiments indicate that

Table 2. Signal-independent determination of ventral fates by torso-pelle and torso-tube fusion constructs

<table>
<thead>
<tr>
<th>Injected RNA</th>
<th>Conc. (µg/µl)</th>
<th>Recipient genotype</th>
<th>No. of cuticles scored</th>
<th>% Dorsalized cuticle</th>
<th>% Filzkörper ± vent. dent.</th>
<th>% Wild-type cuticle</th>
<th>% Ventralized cuticle</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>pll</td>
<td>50</td>
<td>100</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>tub</td>
<td>50</td>
<td>100</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>gd</td>
<td>50</td>
<td>100</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>tor4021-pll</td>
<td>2.0</td>
<td>pll</td>
<td>30</td>
<td>17</td>
<td>83</td>
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<tr>
<td></td>
<td>0.67</td>
<td>pll</td>
<td>39</td>
<td>10</td>
<td>90</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>wt</td>
<td>17</td>
<td>–</td>
<td>–</td>
<td>6</td>
<td>94</td>
</tr>
<tr>
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<td>pll</td>
<td>48</td>
<td>17</td>
<td>83</td>
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<td>0.67</td>
<td>pll</td>
<td>50</td>
<td>16</td>
<td>84</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>31</td>
<td>–</td>
<td>–</td>
<td>3</td>
<td>97</td>
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<tr>
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<td>–</td>
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<tr>
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<td>tub</td>
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<td>14</td>
<td>86</td>
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<td>–</td>
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<td>tor-pllK240R</td>
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<td>pll</td>
<td>50</td>
<td>100</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>wt</td>
<td>50</td>
<td>100</td>
<td>–</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>tor4021-tub</td>
<td>0.8</td>
<td>tub</td>
<td>62</td>
<td>5</td>
<td>95</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
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<td>45</td>
<td>–</td>
<td>–</td>
<td>36</td>
<td>64</td>
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<td>0.8</td>
<td>gd</td>
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<td>13</td>
<td>87</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>pll</td>
<td>34</td>
<td>100</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

RNA transcripts were produced and injected at the indicated concentrations. Embryos from mutant females were scored either as having only dorsal hairs or as having filzkörper and/or ventral denticles. Embryos from wild-type females were scored either as having a wild-type pattern (whether hatched or not) or as having a ventralized cuticle containing expanded belts of ventral denticles (see Fig. 5D). Cuticles lacking dorsal hairs, filzkörper and ventral denticles were not scored. Recipient maternal genotypes: pll = pll(1)Df(3R)IR16; tub = tub[R5.6]Df(3R)XM3; gd = gd2/gd2; wt = Oregon R (for pelle constructs) or w; P[w+, twist-lacZ] (for tube construct).

Fig. 4. Schematic representation of pelle chimeric protein constructs. For pelle, the amino-terminal domain is drawn as an open box; the carboxy-terminal catalytic domain is shaded. For torso, a thick bar representing the membrane-spanning segment separates the extracellular and intracellular domains. In the four chimeric proteins, amino acids 160-501 of pelle are fused to amino acids 1-455 of torso. The activating torso mutation, tor4021, results in the substitution of a cysteine residue for the tyrosine at position 327. The inactivating pelle mutation, pllK240R, results in the substitution of an arginine residue for the lysine at position 240.
membrane localization via the tor transmembrane domain triggers pelle activation in the chimeric protein.

**Signal-independent function of pelle fused to torso**

When tor^{4021}-pll or tor-pll RNA was injected into wild-type embryos, we consistently observed ectopic ventral denticles (Table 2; Fig. 5D). Only the dorsoventral axis appeared affected, since these denticles appeared in belts reflecting segmentation along the anteroposterior axis. These results suggest that, when membrane associated, the pelle catalytic domain is able to direct dorsal nuclear import independent of Toll function.

To examine directly whether the tor-pll construct is active in the absence of Toll activation, we injected tor-pll RNA into embryos from females mutant for the gastrulation defective (gd) locus. Such mutations block production of the extracelli-
lar Toll ligand and, hence, signal transduction. As shown in
Table 2 and Fig. 5F, injection of tor-pll RNA into these gd
embryos resulted in the appearance of both filzkörper and
ventral denticles. The tor-pll construct thus has an activity that
is independent of the ligand-mediated activation of Toll.

Given the signal-independent nature of the tor-pll activity,
we expected that this fusion protein might define dorsoventral
polarity in injected embryos. To test this hypothesis, we
injected tor-pll RNA into embryos derived from gd mutant
cellularized to males carrying a twist-lacZ transgene, a
reporter for ventral cell fates (Thisse et al., 1991). Transgene
expression, as assayed by staining for β-galactosidase activity,
was absent in uninjected embryos. Upon injection of tor-pll
RNA, lacZ expression was detected in a localized region sur-
rounding the site of injection (Fig. 6).

To confirm that pelle catalytic function is responsible for the
generation of ventral cuticle in the injected embryos, we intro-
duced an inactivating mutation into the catalytic domain (Fig.
4). The K240R mutation destroys a putative ATP-binding site
conserved among serine/threonine-specific protein kinases. In
vivo, it abolishes the rescuing activity of full-length pelle RNA
transcripts and in vitro it blocks autophosphorylation of a bac-
terially expressed pelle fusion protein (Shelton and
Tor-pllK240R RNA had no activity when injected into wild-type
or null mutant embryos (Table 2), demonstrating that pelle
catalytic function is responsible for the activity of the tor-pll
fusion protein.

Pelle acts downstream of tube in the signal transduction pathway

The finding that the tor-pll construct exhibits signal-indepen-
dent pelle activity afforded us the opportunity to determine the
order of function of tube and pelle by epistasis analysis. We
injected tor-pll RNA into embryos from tube null females and
examined the cuticular phenotype. As with pll and gd embryos,
we observed both filzkörper and ventral denticles (Fig. 5E,
Table 2). Thus, pelle can function in signal transduction in the
absence of tube and cannot therefore act upstream of tube in
the signaling pathway.

Based on the observations that tube is membrane associated,
that tube and pelle can interact, and that pelle is activated when
fused to torso, we assayed the activity of a torso-tube construct.
The entire tube ORF was fused to DNA encoding the extra-
cellular and transmembrane portions of the tor4021 protein.
Injection of tor4021-tub RNA had effects equivalent to those of
tor-pll RNA in wild-type, gd and tub null embryos, i.e. the
intra-membrane chimera was constitutively active (Table 2).
However, unlike tor-pll RNA, tor4021-tub RNA did not rescue
lateral or ventral fates in embryos derived from pll mutant
females. We therefore conclude that pelle must act downstream
of tube in the signaling pathway.

DISCUSSION

Localised activation of the Toll receptor initiates a signal trans-
duction cascade that stimulates the nuclear import of the rel-
related transcription factor dorsal. Transduction of the intra-
cellular signal generated by Toll requires the tube protein and
the pelle protein kinase. We have used immunolocalization
studies, a yeast two-hybrid assay, and a molecular genetic
analysis of protein chimeras to explore the functions of the tube
and pelle proteins in dorsoventral patterning. Specifically, we
have shown that (1) tube is associated with the plasma
membrane during interphase, (2) the amino-terminal domain
described pelle interacts with tube sequences that are strictly required
for signal transduction, (3) pelle is constitutively activated
when localized to the plasma membrane by fusion to a trans-
membrane protein, (4) membrane-localized pelle induces
signal transduction in the absence of tube function and (5) a
tor-tube fusion requires pelle function to induce signal trans-
duction.

Transduction of the ventral signal in interphase
embryos

The epistatic relationship between tube and pelle places pelle
downstream of tube in the signaling pathway and makes clear the
basic architecture of the dorsoventral signaling pathway (Fig.
7). The Toll protein is activated upon binding its ligand,
a processed form of the extracellular protein spätzle (Morisato
and Anderson, 1994; Schneider et al., 1994). The ligand-bound
form of Toll acts through tube to stimulate the pelle protein
kinase, most probably at the plasma membrane. Pelle likely
then phosphorylates one or more target sites in the dorsal-
cactus complex, triggering production of free dorsal protein,
which is then transported into nuclei.

Because the intracellular domain of Toll does not contain an
obvious catalytic domain, it is not clear how spatial informa-
tion is transferred from Toll to tube and/or pelle. Tube’s
membrane localization suggests the possibility that Toll
directly associates with tube as the first step in signal trans-
duction. Although Toll and tube failed to interact in the two-
hybrid assay, this result might simply reflect the inability of
the intracellular fragment of Toll to adopt an active confor-
mation or oligomerization state when expressed in yeast. It is also
possible that Toll communicates with tube in embryos through
another, as yet unidentified, protein (see discussion below). In
either case, tube’s membrane association throughout inter-
phase could facilitate its rapid interaction with Toll or other
membrane-localized signaling components.

Fig. 6. Definition of ventral cell fate by tor-pll fusion protein.
Embryo from gd/gd2 mutant female injected dorsally with tor-pll
RNA and then stained for expression of β-galactosidase gene driven
by the promoter for the twist gene, a marker for ventral cell fates.
that raf kinase undergoes a similar membrane-mediated activation is unknown. However, it has recently been demonstrated that, in wild-type embryos, tube recruits pelle to the membrane and extracellular domains not only activate pelle, but also transforms it into a kinase that is no longer dependent on Toll. Furthermore, this activity is not restricted to those regions of the embryo in which torso itself is active. Membrane localization is therefore apparently sufficient to activate the pelle kinase. Tube’s membrane localization and the strong interaction between tube and pelle in the two-hybrid assay suggests that, in wild-type embryos, tube recruits pelle to the membrane in response to Toll activation. This suggestion is strongly supported by the fact that two-point mutations in tube that block signal transduction also prohibit interaction between tube and pelle in the two-hybrid assay.

Toll may activate a preformed complex of tube and pelle by bringing it into close contact with membrane proteins or with membrane lipids. Alternatively, Toll might free the tube amino-terminal domain from an inhibitory interaction, allowing membrane localized tube to interact with pelle. Either mechanism would be consistent with the signal-independent association of tube and pelle seen in the yeast two-hybrid system.

The biochemical basis for membrane-mediated pelle activation is unknown. However, it has recently been demonstrated that raf kinase undergoes a similar membrane-mediated activation (Leevers et al., 1994; Stokoe et al., 1994). The lipid-rich environment of the membrane might alter the conformation of the regulatory or catalytic domains of pelle, thereby activating the enzyme. Alternatively, exposure of pelle to another membrane protein might stimulate its kinase activity.

The transduction of positional information requires the pelle-catalyzed phosphorylation of one or more proteins in the dorsoventral pathway (Shelton and Wasserman, 1993). Since activated pelle drives dorsal nuclear import in a tube null background, tube cannot be the critical substrate. Given the parallels between the dorsal and IL-1 pathways, cactus is a likely target. IκB, the mammalian counterpart of cactus, is rapidly phosphorylated upon treatment of cells with IL-1 (Kerr et al., 1991). Immediately thereafter, IκB is degraded and active NF-κB appears in the nucleus. By examining the levels of cactus protein in different genetic backgrounds, we have found that cactus, like IκB, is degraded in response to signal transduction (P. Towb, J. Allen and S. A. W., personal communication). It may be difficult, therefore, to detect cactus modification in the absence of methods for synchronizing signaling and stabilizing cactus.

**Additional components in the dorsoventral pathway?**

The cellular portion of the *Drosophila* dorsoventral pathway is defined genetically by five genes: *Toll, tube, pelle, cactus* and *dorsal*. Other proteins may participate in transduction of the dorsoventral signal or may interact with the five intracellular dorsoventral proteins. These include membrane proteins that may couple Toll with tube or pelle, a nuclear tube-binding protein, proteins that regulate tube’s change in subcellular localization and, by analogy to the IL-1 pathway, a protease involved in the degradation of cactus after its release from dorsal. However, our finding that the function of tube and pelle can be linearly arranged, that both proteins are active when localized to the plasma membrane and that these two proteins interact with one another indicates that current models for dorsoventral signal transduction may require refinement but are unlikely to need reconfiguration.

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Note: While this manuscript was under review, Großhans and colleagues reported similar findings on the activation of pelle by tube (Nature 372, 563; 1994).

**REFERENCES**


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