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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 IκB 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 IκB, 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 IκB, 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, IκB, 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 tub^2, tub^3 and $tub^{R5.6}$ mutations, as well as the *tube* deficiency *Df(3R)XM3* and the *pelle* deficiency *Df(3R)IR16*, have been described previously (Letsou et al., 1993; Hecht and Anderson, 1993; Shelton and Wasserman, 1993). The *Toll* null genotype was $Df(3R)Tl^{9QRX}/Df(3R)ro^{XB3}$ (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 Lindsley 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 $MgCl_2$/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% KPBS (137 mM NaCl/ 2.7 mM KCl/1.5 mM K_2HPO_4/8 mM NaH_2PO_4, pH 7.3) and twice in KPBS. Embryos were blocked 4 hour at RT or overnight at 4°C in blocking buffer (1% BSA/0.1% Triton X-100 in KPBS). 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 KPBS. 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 Axiophot microscope. Embryos were illuminated with an argon laser and were visualized through a 40× Zeiss Plan NeoFluar 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*, $lacI^qZ\Delta M15$, Tn*10* (tet^r)]) (Stratagene, Incorporated) was used as the bacterial host. The yeast strain EGY48 (*MATα, 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 pJK103 reporter described previously (Gyuris et al., 1993; R. Brent, personal communication). A modified activation domain plasmid, pDE028, was constructed by inserting the *Eco*RI, *Bam*HI and *Sal*I sites from pEG202 into the pJG4-5 plasmid. *SNF1* was subcloned into pEG202 as an *Eco*RI/*Sal*I fragment; *SNF4* was subcloned into pDE028 as a *Bam*HI fragment.

Full-length *pll* and *tub* ORFs, as well as the intracellular portion of *Toll* (intTl, amino acids 829-1097), were generated by PCR with Vent polymerase (New England Biolabs) from available cDNAs. The *pelle*, *tube*, and *Toll*(829-1097) PCR products were inserted into the plasmid pEG202 as *Sal*I, *Eco*RI and *Bam*HI restriction fragments, respectively. The *pll*(1-209) and *pll*(210-501) PCR products were subcloned as *Eco*RI/*Sal*I restriction fragments. The truncated genes *tub*(1-258) and *tub*(257-462) were derived as subclones from pEG202(*tube*). Constructs for tub^2 and tub^3 were subcloned into pEG202(*tube*) from *Nar*I/*Hin*dIII cDNA fragments (Letsou et al., 1993). All constructs retain the stop codon from the wild-type ORF except *pll*(1-209) and *tub*(1-258), each of which is followed by amino acids encoded by vector sequences: VDLQPS and CSQANSGRISYDL, respectively.

Two-hybrid interaction and transcriptional repression assays

The reporter, DNA-binding domain fusion, and activation domain

fusion plasmids were transformed into EGY48 sequentially. Transformed 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-nitro-phenyl galactoside as the substrate and normalized to total protein concentration, as obtained using a modified BCA assay (Hill and Straka, 1988). Activity is expressed in Miller units (Miller, 1972).

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 transcriptional repression assay, as described by Brent and Ptashne (1984).

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 pSELECT (pS601; Shelton and Wasserman, 1993) were changed to a G and C respectively, thereby introducing a *Bst*EII site starting at position 652. The cDNA sequences corresponding to the first 455 amino acids of *torso* and *torso*^{4021} were obtained by cleavage of plasmids pBtor and pB4021 (Sprenger and Nusslein-Volhard, 1992) with *Hin*dIII and *Bst*EII and then inserted independently into the modified pS601. The resulting chimeric proteins contain the extracellular and transmembrane domains of *torso* or *torso*^{4021} 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 *Hin*dIII and *Sac*II 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 *Bst*EII site 5′ to the initiator ATG and an *Eco*RI site 3′ to the translational stop site. This amplified fragment was then used to replace the pelle coding region in the tor-pll construct described above.

RNA microinjection

Embryo collection, RNA microinjection and cuticle preparations were carried out as described previously (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 visualized by confocal microscopy.

During the early-cleavage stage of development, tube is distributed 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 fluorescently labeled Concanavalin 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 interphase 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 activation 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

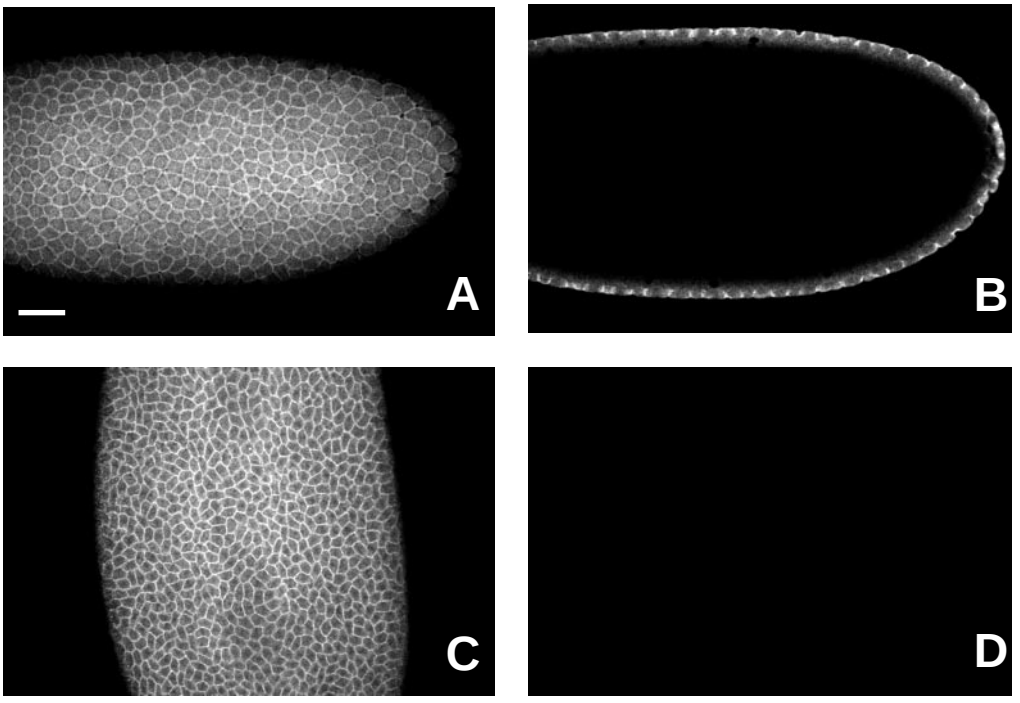

Fig. 1. Membrane association of tube protein in wild-type embryos. Embryos from wild-type females (A-C) or *tube* null (*tub*$^{R5.6}$/*Df(3R)XM3*) females (D) were labeled with anti-tube serum and a Cy3-conjugated secondary antibody and were analyzed by confocal immunofluorescence microscopy. (A) Surface 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.

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

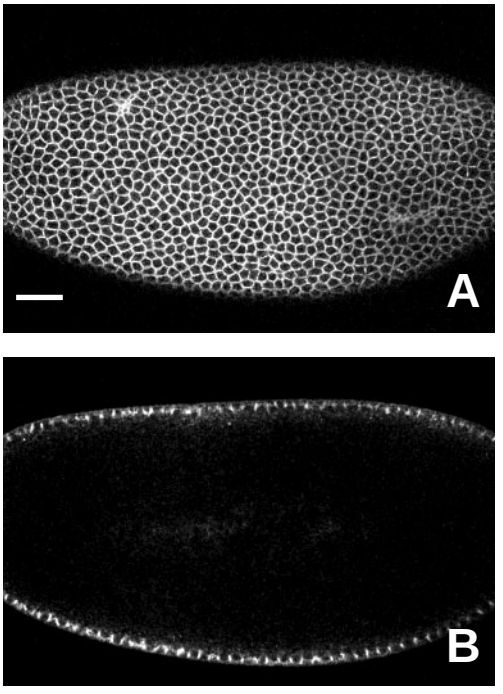

Fig. 2. Membrane localization of Toll protein in wild-type embryos. Embryos from wild-type females were labeled with anti-Toll antibodies and a Cy3-conjugated secondary antibody and were analyzed by confocal immunofluorescence microscopy. (A,B) Surface and longitudinal optical section, respectively,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

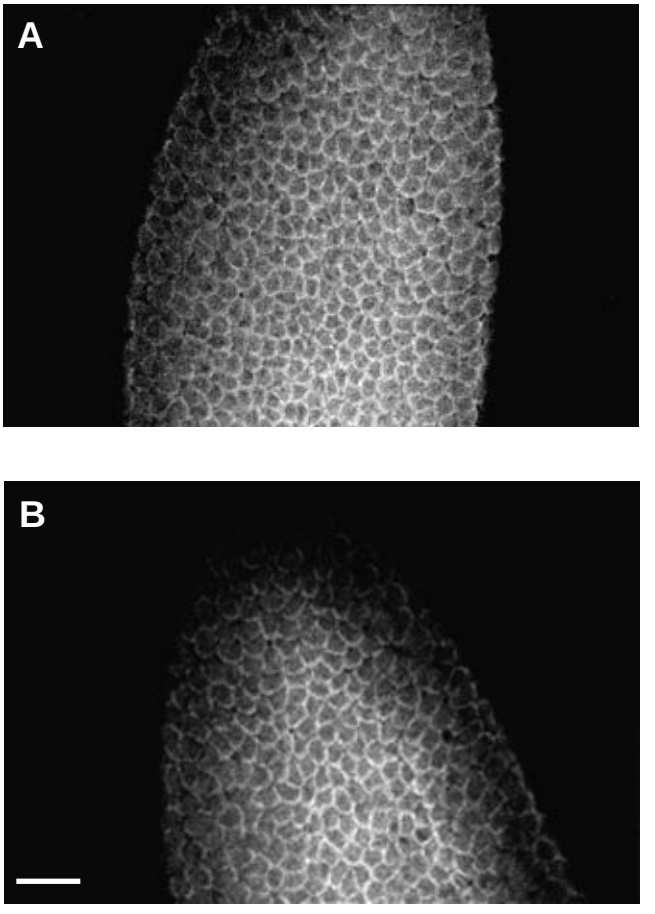

Fig. 3. Membrane localization of tube in Tl^{null} mutant embryos. Syncytial embryos from (A) wild-type or (B) Tl^{null} ($Df(3R)Tl^{9QRX}/Df(3R)ro^{XB3}$) females were labeled with anti-tube serum and a Cy3-conjugated secondary antibody and were analyzed by confocal immunofluorescence microscopy. Scale bar, 50 μm.

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

	LexA fusion	B42 fusion	Mean activity	Range
1	SNF1	SNF4	200	100-410
2	SNF1	tube	1	0.8-1.5
3	SNF1	pelle	2	1.1-2.7
4	tube	–	2500	2100-2800
5	pelle	SNF4	3	2.4-4.8
6	pelle	tube	320	160-510
7	intTl	SNF4	1	0.8-1.8
8	intTl	tube	3	1.9-4.9
9	intTl	pelle	2	1.6-4.5
10	pelle	tubNterm	550	110-1300
11	pelle	tubCterm	2	1.7-2.5
12	pelle	tub^2	3	1.4-3.8
13	pelle	tub^3	3	0.8-3.7
14	pllNterm	tube	790	570-1100
15	pllCterm	tube	1	0.5-2.3
16	pllNterm	tubNterm	1290	720-1800
17	pllCterm	tubCterm	1	0.6-1.8

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; pllNterm: pelle(1-209); pllCterm: pelle(210-501); intTl: 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 was 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-intTl construct reduced activity to 90 (±20) units. Thus, in yeast, the LexA-intTl 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, tub^2 and tub^3, 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 tub^2 or tub^3 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 (tor^{4021}, Sprenger and Nüsslein-Volhard, 1992; Fig. 4). Similar tor^{4021} 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 tor^{4021}-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 tor^{4021}-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; Lohs-Schardin et al., 1979). The tor^{4021}-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-

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

Injected RNA	Conc. (µg/µl)	Recipient genotype	No. of cuticles scored	Mutant recipients		Wild-type recipients	
				% Dorsalized cuticle	% Filzkörper ± vent. dent.	% Wild-type cuticle	% Ventralized cuticle
None	–	*pll*	50	100	0	–	–
	–	*tub*	50	100	0	–	–
	–	*gd*	50	100	0	–	–
tor^{4021}-pll	2.0	*pll*	30	17	83	–	–
	0.67	*pll*	39	10	90	–	–
	1.5	wt	17	–	–	6	94
tor-pll	2.0	*pll*	48	17	83	–	–
	0.67	*pll*	50	16	84	–	–
	2.0	wt	31	–	–	3	97
	2.0	*gd*	29	17	83	–	–
	2.0	*tub*	28	14	86	–	–
tor-pll^{K240R}	1.0	*pll*	50	100	0	–	–
	1.0	wt	50	100	–	100	0
tor^{4021}-tub	0.8	*tub*	62	5	95	–	–
	0.8	wt	45	–	–	36	64
	0.8	*gd*	62	13	87	–	–
	0.8	*pll*	34	100	0	–	–

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*^{385}/*Df(3R)IR16*; *tub* = *tub*$^{R5.6}$/*Df(3R)XM3*; *gd* = *gd*^{2}/*gd*^{2}; wt = Oregon R (for pelle constructs) or *w*$^{-}$; P[*w*$^{+}$, *twist-lacZ*] (for tube construct).

spanning segment, albeit in a manner distinct from the wild-type enzyme.

The activity of the tor^{4021}-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 tor^{4021} 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 extracellular 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

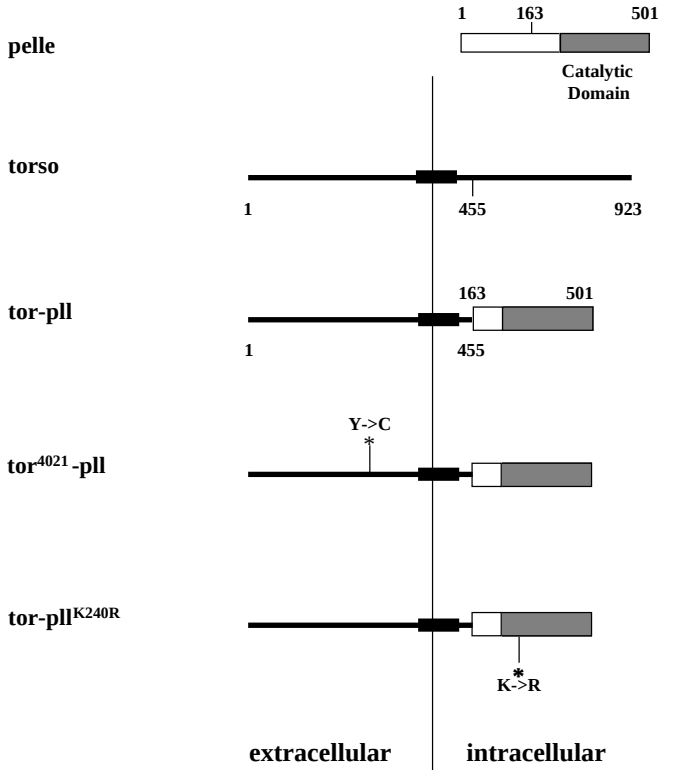

Fig. 4. Schematic representation of pelle chimeric protein constructs. For pelle, the amino-terminal domain is drawn as an open box; the carboxy-terminal catalytic domain is shaded. For torso, a thick bar representing the membrane-spanning segment separates the extracellular and intracellular domains. In the four chimeric proteins, amino acids 160-501 of pelle are fused to amino acids 1-455 of torso. The activating *torso* mutation, *tor*^{4021}, results in the substitution of a cysteine residue for the tyrosine at position 327. The inactivating *pelle* mutation, *pll*^{K240R}, results in the substitution of an arginine residue for the lysine at position 240.

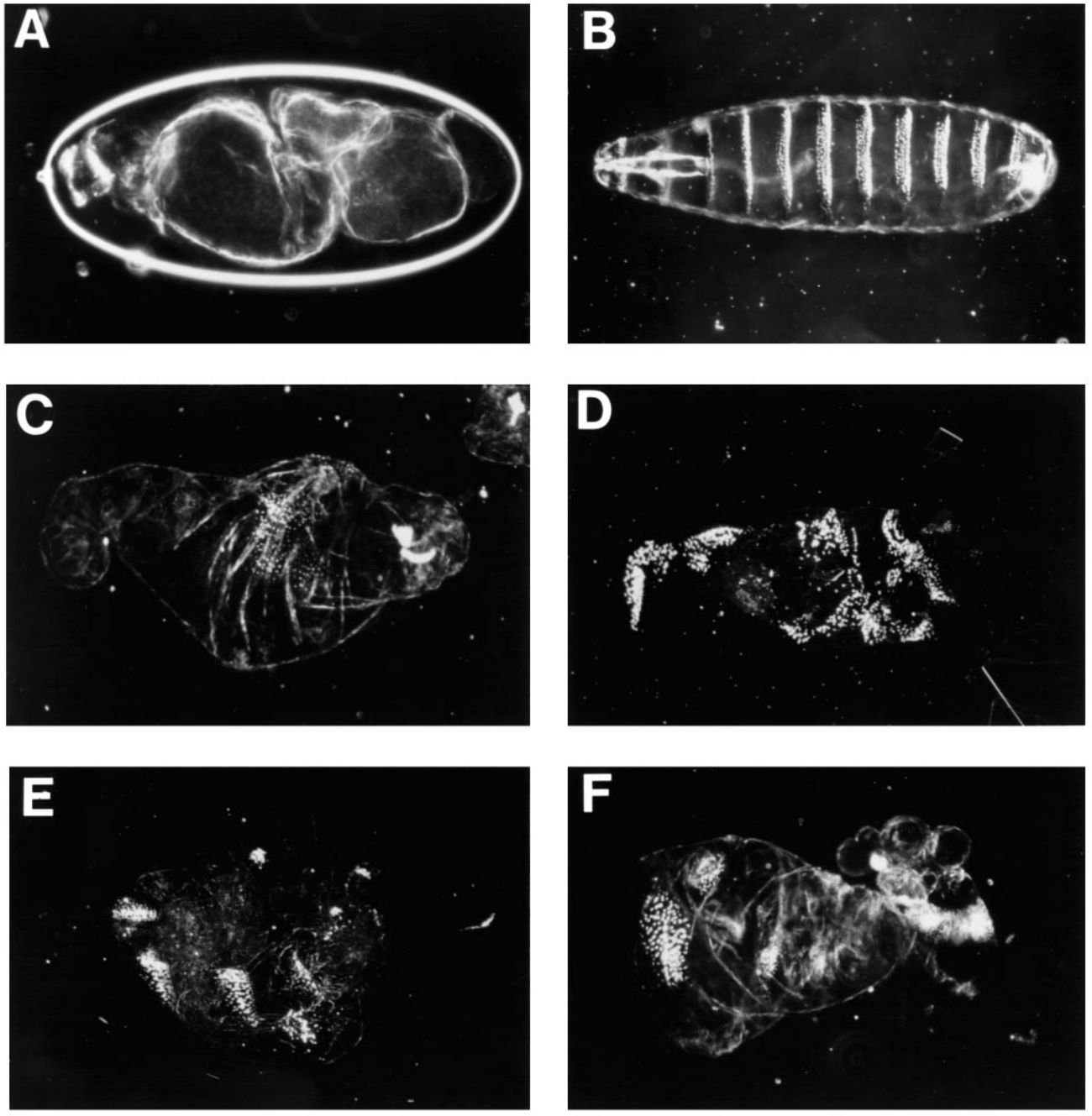

Fig. 5. Dominant activity of a *tor-pll* fusion construct. (A) Embryo from tub^{2}/tub^{3} female, encased within its vitelline membrane, showing the dorsalized phenotype characteristic of loss-of-function mutations in *Toll*, *tube*, *pelle*, and *dorsal*. (B) Ventral view of hatched, wild-type larvae. Three belts of thoracic ventral denticles and eight belts of abdominal ventral denticles are evident, as are a pair of refractile bodies, the filzkörper, at the posterior (right) end. (C-F) Embryos injected with 2 μg/μl tor-pll RNA. Maternal genotypes are indicated. (C) $pll^{385}/Df(3R)IR16$. (D) wild-type. (E) *tube* null ($tub^{R5.6}/Df(3R)XM3$). (F) gd^{2}/gd^{2}.

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 extracellu-

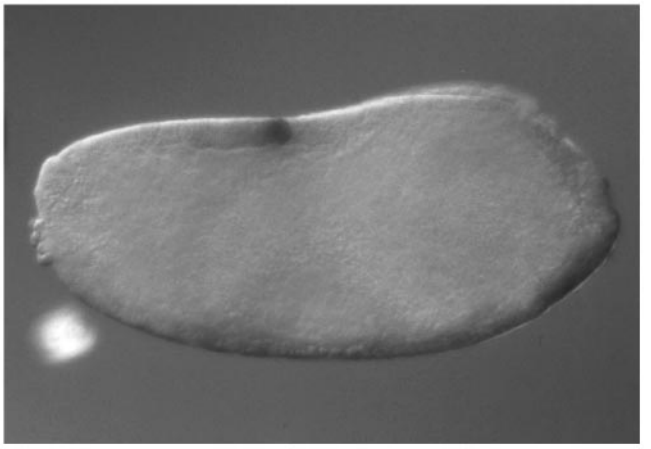

Fig. 6. Definition of ventral cell fate by tor-pll fusion protein. Embryo from gd^2/gd^2 mutant female injected dorsally with tor-pll RNA and then stained for expression of *β-galactosidase* gene driven by the promoter for the *twist* gene, a marker for ventral cell fates.

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 females crossed 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 surrounding 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 introduced 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 bacterially expressed pelle fusion protein (Shelton and Wasserman, 1993; C. Shelton and S. A. W., unpublished data). Tor-pll^{K240R} RNA had no activity when injected into wild-type or *pll* 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-independent 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 extracellular and transmembrane portions of the tor^{4021} protein. Injection of tor^{4021}-tub RNA had effects equivalent to those of tor-pll RNA in wild-type, *gd* and *tub* null embryos, i.e. the torso-tube chimera was constitutively active (Table 2). However, unlike tor-pll RNA, tor^{4021}-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

Localized activation of the Toll receptor initiates a signal transduction cascade that stimulates the nuclear import of the rel-related transcription factor dorsal. Transduction of the intracellular 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 of 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 transmembrane 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 transduction.

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 information 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 transduction. 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 conformation 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 interphase could facilitate its rapid interaction with Toll or other membrane-localized signaling components.

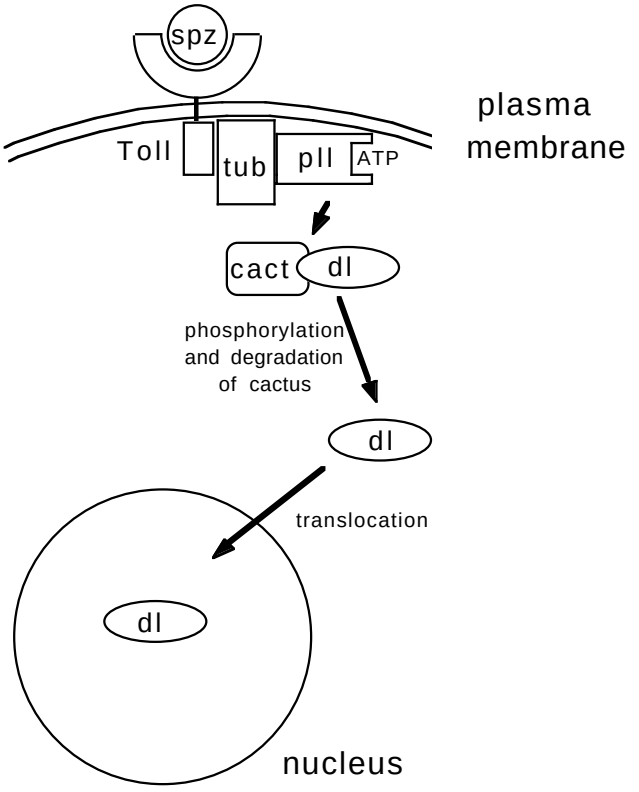

Fig. 7. Model of the signal transduction pathway for the regulated nuclear import of the dorsal protein. During interphase, signal transduction is triggered on the ventral side of the *Drosophila* embryo by binding of a processed form of spätzle (spz) to the Toll receptor (Stein et al., 1991; Morisato and Anderson, 1994; Schneider et al., 1994). The pelle kinase is then recruited to the plasma membrane through its association with tube. Subsequent signal-dependent phosphorylation of cactus by pelle leads to cactus degradation and dorsal nuclear import.

Fusion of the pelle catalytic domain to the torso transmembrane and extracellular domains not only activates 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).

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