# An activity-dependent network of interactions links the Rel protein Dorsal with its cytoplasmic regulators

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## SUMMARY

A signaling pathway active on the ventral side of the *Drosophila* embryo defines dorsoventral polarity. A cell surface signal relayed by Toll, Tube and Pelle releases the Rel-related protein Dorsal from its cytoplasmic inhibitor Cactus; free Dorsal translocates into nuclei and directs expression of ventral fates. Using the yeast two-hybrid system and immunoprecipitation experiments, we define scaffolding and anchoring interactions among the pathway components. We show that Dorsal binds specifically to Tube, Pelle and Cactus, and that the protein kinase activity of Pelle differentially regulates its interactions with Dorsal and Tube. We also identify *Drosophila* Filamin as a potential adaptor linking the interaction network, via Tube, to the transmembrane receptor Toll.

Key words: *Drosophila*, Toll, Tube, Pelle, Cactus, Dorsal, dorsoventral polarity, cell surface signal, cytoplasmic regulation

## INTRODUCTION

Cellular responses to a variety of external stimuli are mediated by the activation of transcription factors via their translocation from the cytoplasm to the nucleus (see, e.g. Rao, 1995; Darnell, 1996). Members of the Rel protein family constitute some of the best studied examples of such transcription factors. Rel proteins are active in vertebrate and invertebrate acute phase immune responses, as well as the maternally directed dorsoventral patterning of the *Drosophila* embryo (Govind and Steward, 1991; Hultmark, 1994; Verma et al., 1995; Belvin and Anderson, 1996).

Rel proteins such as NF-κB are held in the cytoplasm by inhibitory interactions that mask their nuclear localization signals. Upon activation of a cell surface receptor, signal transduction results in phosphorylation of the inhibitor, an ankyrin repeat-containing protein such as IκBα. This signal-dependent phosphorylation triggers ubiquitin- and proteasome-mediated degradation of the inhibitor, freeing the Rel protein for translocation into the nucleus. Receptors have been identified for many of the signals regulating Rel protein activity. However, the pathways linking these receptors to the transcription factors that they regulate remain ill-defined.

In *Drosophila*, a nuclear concentration gradient of the Rel protein Dorsal establishes the embryonic dorsoventral axis (reviewed in Chasan and Anderson, 1993). Prior to axis formation, Dorsal and its inhibitor Cactus are distributed throughout the embryonic cytoplasm. Upon binding the extracellular ligand Spätzle, the transmembrane receptor Toll initiates signal transduction in the ventral and lateral portions of the embryo, freeing Dorsal via Cactus degradation. Free Dorsal translocates into nuclei, where it activates ventral-specific genes and represses dorsal-specific genes.

Two proteins, Tube and Pelle, are known to mediate signal transduction from Toll to the Dorsal-Cactus complex (Letsou et al., 1991; Hecht and Anderson, 1993; Shelton and Wasserman, 1993). Tube has no known homolog in other species; Pelle is a protein kinase with a recently described mammalian counterpart, IRAK (Cao et al., 1996). Although Pelle is capable of phosphorylating Tube in vitro, the in vivo significance of this activity is unclear, since epistatic analysis places Pelle downstream of Tube in the genetic hierarchy (Großhans et al., 1994; Galindo et al., 1995) (Fig. 1A).

Previous characterization of this pathway revealed two interactions among pathway components. The Dorsal-Cactus complex can be immunoprecipitated from embryonic extracts with antisera directed against either protein (Kidd, 1992; Whalen and Steward, 1993; Gillespie and Wasserman, 1994). In addition, yeast two-hybrid studies and affinity chromatography with heterologously expressed proteins demonstrated that Tube and Pelle bind to one another (Großhans et al., 1994; Galindo et al., 1995). However, no interactions have been identified that link Tube or Pelle to either Toll or the Dorsal-Cactus complex.

Using both molecular genetic and biochemical approaches, we have discovered a network of interactions among the four pathway proteins downstream of Toll. We have also found evidence for activity-dependent modulation of the interaction network and for a new pathway component linking this network to Toll.

## MATERIALS AND METHODS

### Two-hybrid plasmid construction

The LexA (DNA-binding) fusion plasmid pEG202 and the B42 (activation domain) fusion plasmid pJG4-5 were provided by R. Brent and coworkers, as was the reporter plasmid pSH18-34 (Gyuris et al.,

1993). Modifications to these plasmids were limited to insertion of new polylinkers. Full-length inserts of *Drosophila* proteins for chimera construction were amplified from available cDNAs using PCR and Vent Polymerase (New England Biolabs). Deletion derivatives were generated by restriction enzyme digestion of the full-length PCR clone or PCR from the available cDNA. Point mutations were introduced into fusion proteins with restriction fragments from previously generated mutant cDNAs.

## Pairwise two-hybrid analysis

The yeast strain EGY48 was serially transformed with the reporter plasmid, LexA-fusion, and B42-fusion by a modified alkali ion method (Schiestl and Gietz, 1989). Three or four colonies were picked from the selective transformation plate, mixed and stabbed into selective media at pH 7 containing 40 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside), 1% raffinose and 2% galactose; interacting clones led to growth of a blue patch of yeast. For each pair of proteins, we transformed yeast with two fusion constructs, in which one *Drosophila* protein was fused to the LexA DNA-binding domain and the other to the B42 transcriptional activation domain (Gyuris et al., 1993). A fusion of the yeast SNF4 gene to the B42 domain served as a positive control when combined with its interaction partner SNF1 (Fields and Song, 1989) and as a negative control when paired with each of the *Drosophila* LexA fusion proteins. Immunoblot analysis of yeast extracts with appropriate antibodies was used to confirm expression of fusion proteins that failed to interact with any other clones (Gillespie and Wasserman, 1994); nuclear localization was confirmed by the transcriptional repression assay described by Brent and Ptashne (1984).

## β-galactosidase reporter assay

Three or four colonies of a transformed yeast strain containing fusions of interest were transferred into 3 ml of selective media containing 2% glucose and grown to stationary phase. The culture was then diluted 1:60 in 3 ml of selective media containing 1% raffinose and 2% galactose and again grown to late log or stationary phase. Cells were recovered, washed and lysed in the presence of 0.17 mM PMSF (phenylmethylsulfonyl fluoride). Ortho-nitro-phenyl-galactoside was used as the substrate for β-galactosidase activity; activity was normalized to protein concentration (Miller, 1972; Rose and Botstein, 1983), obtained by a Bradford assay (Bio-Rad). For each LexA-*Drosophila* protein fusion, baseline activity was assayed in the presence of the yeast protein fusion B42-Snf4p.

## Antibodies

The anti-Dorsal, anti-Cactus, anti-Tube and anti-Pelle sera have been described previously (Letsou et al., 1993; Gillespie and Wasserman, 1994; Großhans et al., 1994; Reach et al., 1996). In order to directly conjugate IgG to alkaline phosphatase, the IgG fraction from anti-Dorsal or anti-Cactus sera was purified away from other serum proteins by passing diluted whole sera over a Protein A-Sepharose column (Pharmacia). The column was washed extensively using 10 mM Tris, pH 8.0 and also 10 mM Tris, 500 mM NaCl, pH 8.0. Bound proteins were eluted using 100 mM glycine, pH 2.5 into a tube containing 1/10 volume 1M Tris, pH 8.0 to immediately neutralize the eluate. The purified IgG was then concentrated to 7 mg/ml in 100 mM NaHCO$_3$/Na$_2$CO$_3$, pH 9.0 (buffer exchange was accomplished while concentrating) and coupled to activated alkaline phosphatase (Boehringer Mannheim) as per the manufacturer's instructions.

## *Drosophila* strains

The wild-type strain used was Oregon R. All mutants and deficiency chromosomes are described in FlyBase (http://flybase.bio.indiana.edu).

## Coimmunoprecipitation of interacting proteins from embryos

Dechorionated, frozen 0-3 hour *Drosophila* embryos were homogen-

ized in Lysis Buffer (10 mM Tris pH 8.0, 140 mM NaCl, 0.025% NaN$_3$, 1% Triton X-100, 1% bovine hemoglobin, 5 mM iodoacetamide, 0.3 µM aprotinin, 1 mM PMSF, 10 µM leupeptin, 1 µM pepstatin A) and a 13,000 *g* supernatant obtained. The supernatant was adjusted to a concentration of 10 mg/ml total embryonic protein. To 500 µl supernatant, 5 µl of the appropriate sera was added and the mixture rocked overnight at 4°C. To recover immunoprecipitated complexes, 50 µl Protein A-Sepharose (Sigma) preswelled in Lysis Buffer was added and incubation at 4°C continued for 4 hours. Protein A-Sepharose beads were then spun down, washed twice in dilution buffer (10 mM Tris pH 8.0, 140 mM NaCl, 0.025% NaN$_3$, 0.1% Triton X-100, 0.1% bovine hemoglobin), once in TSA buffer (10 mM Tris pH 8.0, 140 mM NaCl, 0.025% NaN$_3$), and once in 50 mM Tris, pH 6.8. Precipitated proteins were eluted by adding 50 µl SDS-PAGE sample buffer (12.5 mM Tris, pH 6.8, 5% glycerol, 0.4% SDS, 2.9 mM 2-mercaptoethanol, 0.02% bromophenol blue) and heating to 100°C for 5 minutes. Immunoprecipitates were analyzed by SDS-PAGE in 8% gels, electroblotting to PVDF membranes (Immobilon-P) and immunoblot analysis (Gillespie and Wasserman, 1994), using IgG directly conjugated to alkaline phosphatase.

## Two-hybrid screens

Yeast strains containing the reporter plasmid and a Toll LexA-fusion construct were transformed with cesium gradient purified library DNA by a modified large-scale alkali ion protocol (Schiestl and Gietz, 1989). The libraries used were ovarian and 0-12 hour embryonic libraries generated by Brent and coworkers (Finley et al., 1996) and an ovarian library generated by J. Großhans. For each library, a minimum of 10$^6$ independent transformants were pooled and a minimum of 5×10$^6$ colony-forming units from each pool were subjected to leucine selection on galactose/raffinose media. Colonies answering leucine selection were assayed on X-gal-containing media for β-galactosidase activity.

Inserts from candidate library clones were amplified from total yeast DNA by PCR with Taq Polymerase (Boehringer Mannheim). Following restriction enzyme analysis, representative plasmids were recovered by electroporation of KC8 bacteria with selection for tryptophan prototrophy (Gyuris et al., 1993), then transformed back into the library screening strain and a panel of negative control strains to check specificity. Insert fusions were sequenced with an automated sequencer (Applied Biosystems Inc.).

# RESULTS

## The Rel protein Dorsal interacts specifically with Tube and Pelle

Using the yeast two-hybrid system (Fields and Song, 1989), we assayed for all possible pairwise interactions among Tube, Pelle, Cactus, Dorsal and Toll[829-1097], the intracellular domain of Toll. For each pair of proteins, we transformed yeast with two fusion constructs, in which one *Drosophila* protein was fused to a DNA-binding domain (LexA) and the other to a transcriptional activation domain (B42) (Gyuris et al., 1993). We then used an X-gal assay to identify colonies expressing a *lacZ* reporter gene. In this manner, we confirmed the interactions between Tube and Pelle and between Dorsal and Cactus (Kidd, 1992; Whalen and Steward, 1993; Gillespie and Wasserman, 1994; Großhans et al., 1994; Isoda and Nusslein-Volhard, 1994; Galindo et al., 1995; Tatei and Levine, 1995). In addition, we detected interactions between Dorsal and Pelle and between Dorsal and Tube, neither of which had been reported previously or had been expected on the basis of genetic studies. These results are summarized in Fig. 1B.
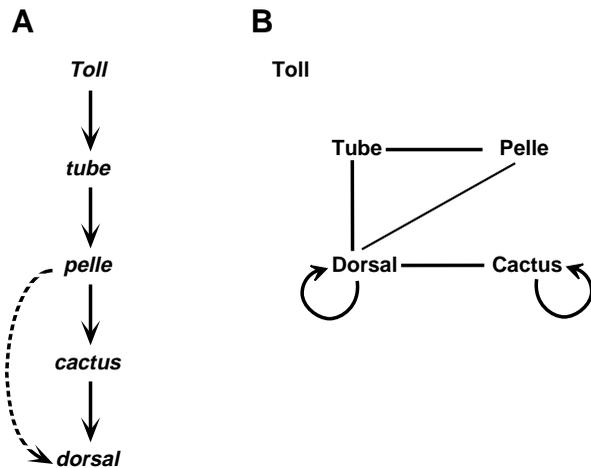
**A**                    **B**



**Fig. 1.** Genetic hierarchy and protein interaction network among components of the dorsoventral signaling pathway. (A) Model for intracellular signal transduction based on the epistatic relationships among the five genetically defined pathway components. Tube and Pelle are required downstream of Toll (Hecht and Anderson, 1993), but upstream of Dorsal and upstream of or in parallel to Cactus (Roth et al., 1991). Experiments with gain-of-function alleles of Pelle and Tube indicate that Pelle is required downstream of Tube (Großhans et al., 1994; Galindo et al., 1995). The dotted line illustrates one possible explanation for the observation that there is residual dorsoventral polarity in an embryo null for Cactus (Bergmann et al., 1996). (B) Diagram of specific protein-protein interactions detected in the yeast two-hybrid system. The presence of a heterodimeric interaction is indicated by a bar and of a homodimeric interaction by an arrow. All pairs were tested except Tube-Tube (see text).

To quantitate reporter gene expression in the pairwise experiments, we assayed DNA-binding domain fusions for Dorsal, Pelle and Cactus; a Tube DNA-binding domain fusion could not be used since it activates transcription in the absence of a pairing partner (Galindo et al., 1995). Pairwise combinations with a Dorsal DNA-binding domain fusion led to a mean elevation in reporter gene expression of 8-fold for a Pelle activation domain construct and 30-fold for a comparable Tube construct (Table 1, compare rows 3 and 4 with row 2). When Pelle was used as the DNA-binding domain fusion, the elevation in reporter gene expression averaged 10-fold with Tube (Table 1, compare rows 6 and 7) and 600-fold with Dorsal (Table 1, compare rows 6 and 9). The results for Pelle and Dorsal were comparable to those seen for the known interaction partners Dorsal and Cactus (Table 1, compare rows 5 and 13 to rows 2 and 10, respectively).

Several lines of evidence indicate that the pairwise interactions of Dorsal with Pelle and with Tube are quite specific. First, fusion constructs for these proteins failed to interact with a battery of negative controls, including the *Drosophila* proteins Bicoid, Diaphanous and Bag-of-marbles; representative results with the yeast protein Snf4p are shown in Table 1. Second, using a Pelle DNA-binding domain fusion in a two-hybrid screen of $10^7$ transformants from ovarian and embryonic libraries, we found that Dorsal represented 39 of 159 interacting plasmids (D. N. E. and S. A. W., unpublished results). Third, the interaction of Tube with Dorsal requires a

**Table 1. Two-hybrid interactions among components of the intracellular dorsoventral signaling pathway**

| | DNA-binding domain fusion | Activation domain fusion | Mean activity | Range |
|---|---|---|---|---|
| 1 | Snf1p | Snf4p | 260 | 200-300 |
| 2 | Dorsal$^{1-598}$ | Snf4p | 10 | 9-13 |
| 3 | Dorsal$^{1-598}$ | Tube | 320 | 250-400 |
| 4 | Dorsal$^{1-598}$ | Pelle | 80 | 60-90 |
| 5 | Dorsal$^{1-598}$ | Cactus | 240 | 190-250 |
| 6 | Pelle | Snf4p | 2 | 1-4 |
| 7 | Pelle | Tube | 20 | 10-30 |
| 8 | Pelle | Cactus | 1 | 1-2 |
| 9 | Pelle | Dorsal | 1300 | 800-1800 |
| 10 | Cactus | Snf4p | 1 | 0-2 |
| 11 | Cactus | Tube | 2 | 1-3 |
| 12 | Cactus | Pelle | 1 | 0-2 |
| 13 | Cactus | Dorsal | 1400 | 640-2100 |

For each pair of constructs, mean activity and range reflect the average and range of values in Miller Units (Miller, 1972) normalized to the Cactus-Snf4p value. Full-length Tube could only be assayed as an activation domain fusion, since DNA-binding domain fusions to Tube activate transcription in the absence of an interaction partner (Galindo et al., 1995). A similar problem with Dorsal was surmounted by deleting 80 amino acids from the carboxy terminus, thereby removing its intrinsic activation domain. A minimum of three independent β-galactosidase assays was performed for each pair, each involving three or more independent yeast transformants.

distinct region of Tube from that which mediates binding to Pelle (see below).

For four of the pathway components, we also assayed for self-association. We found that Dorsal and Cactus, but not Pelle or Toll$^{829-1097}$, form homodimers in the two-hybrid assay (Fig. 1B). Homodimers of Dorsal and Cactus have been detected previously in both molecular genetic and biochemical experiments (Govind et al., 1992; Isoda and Nusslein-Volhard, 1994).

## Mapping domains of protein-protein interaction

We used the two-hybrid system to characterize further the pairwise interactions, carrying out deletion mapping studies to define specific binding domains in each protein (Fig. 2).

Amino acids 47-345 of Dorsal were sufficient for interaction with both Tube and Pelle. This same region, the Rel homology domain, is also required for dimerization, for DNA binding and for interaction with Cactus (Kidd, 1992; Govind et al., 1996). Furthermore, the Dorsal Rel domain is both necessary and sufficient for generation of a dorsoventral nuclear concentration gradient (Govind et al., 1996).

Pelle and Dorsal interact with two separable domains of Tube previously defined on the basis of sequence conservation and function in embryonic signal transduction (Letsou et al., 1993). Pelle binds to the amino-terminal region of Tube that spans residues 25 to 173. This region is highly conserved (71% identity) between the *D. melanogaster* and *D. virilis* Tube proteins. Furthermore, residues 1 to 173 are sufficient to mediate significant levels of signal transduction in *tube* null embryos. In contrast, Dorsal binds a carboxy-terminal domain of Tube, amino acids 257 to 462. This region contains five copies of an evolutionarily conserved, 8-amino-acid repeat and is required for full Tube function. Thus the two halves of Tube mediate distinct heteromeric interactions.

Interaction with Dorsal required full-length Pelle. In contrast, only about 100 amino acids of Pelle (residues 26 to
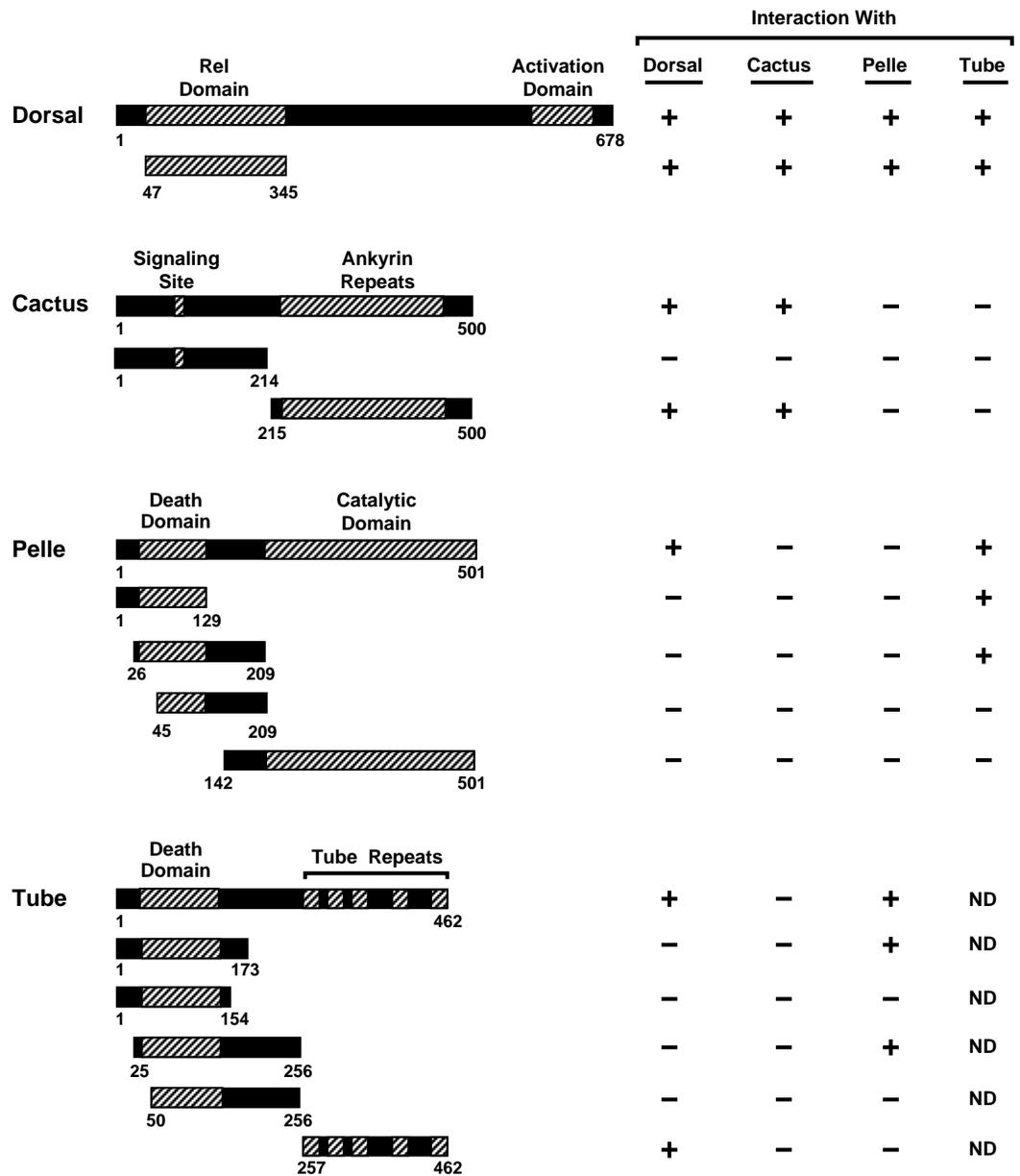
**Fig. 2.** Interaction domain mapping. (Left) Diagram of the full-length and deletion derivatives of the four proteins used in the domain mapping experiments. Scale drawings highlight structural and functional features of each protein (hatched regions); numbering in each case is based on the full-length sequence. (Right) The four columns indicate the presence (+) or absence (−) of a detectable two-hybrid interaction between each of the listed proteins and the domain or domains diagrammed to the left. ND, not determined.

129) were necessary and, most likely, sufficient for interaction with Tube (Fig. 2). This suggests either that the binding of Dorsal to Pelle involves an interaction surface spanning both the carboxy-terminal and amino-terminal halves of Pelle or that neither half of Pelle is sufficient to provide an activity, e.g. autophosphorylation, required for binding to Dorsal.

## Pelle catalytic activity modulates its interactions with Dorsal and Tube

To investigate whether Pelle catalytic activity is in fact required for interaction with Dorsal, we took advantage of two site-directed mutations, *pll*$^{K240R}$ and *pll*$^{D346A}$, previously shown to inactivate the Pelle catalytic domain (Shelton and Wasserman, 1993). Substitution of either Pelle$^{K240R}$ or Pelle$^{D346A}$ for wild-type Pelle in the DNA-binding domain fusion virtually eliminated the interaction between Pelle and Dorsal (Table 2, rows 1-6).

**Table 2. Modulation of two-hybrid interactions by Pelle catalytic activity**

|   | DNA-binding domain fusion | Activation domain fusion | Mean activity |
|---|---|---|---|
| 1 | Pelle | Dorsal | 1300 |
| 2 | Pelle$^{K240R}$ | Dorsal | 6 |
| 3 | Pelle$^{D346A}$ | Dorsal | 20 |
| 4 | Dorsal$^{1-598}$ | Pelle | 80 |
| 5 | Dorsal$^{1-598}$ | Pelle$^{K240R}$ | 14 |
| 6 | Dorsal$^{1-598}$ | Pelle$^{D346A}$ | 13 |
| 7 | Pelle | Tube | 20 |
| 8 | Pelle$^{K240R}$ | Tube | 480 |
| 9 | Pelle$^{D346A}$ | Tube | 740 |

Each pair of constructs was measured and normalized identically, and is therefore comparable, to those presented in Table 1. Mutant Pelle fusions appear similar to wild-type Pelle fusions with regard to size and expression by SDS-PAGE and immunoblot analysis(data not shown).

The failure of Pelle[K240R] and Pelle[D346A] to interact with Dorsal can be explained in two ways. Dorsal might only bind catalytically active Pelle. Alternatively, Pelle-dependent phosphorylation of Dorsal or autophosphorylation of Pelle might be required for the two proteins to interact. In this regard, we note that coexpression of a wild-type Pelle fusion protein did not rescue the two-hybrid interaction between Dorsal and catalytically inactive Pelle (D. N. E., unpublished results). Wild-type Pelle is phosphorylated in yeast, whereas phosphorylation of Pelle[K240R] and Pelle[D346A] is undetectable (D. N. E., unpublished results).

We also assayed the interaction of Tube with Pelle[K240R] and Pelle[D346A]. Both isoforms interacted with Tube. Indeed, the pairing of Tube with catalytically inactive Pelle resulted in reporter gene activity ~30-fold greater than that observed with Tube and wild-type Pelle (Table 2, rows 7-9). These findings are consistent with the finding that Tube can be a direct substrate for Pelle (Großhans et al., 1994) and with the idea that the interaction of a protein kinase and its substrate can be stabilized by catalytic inactivation of the enzyme.

In summary, mutations that block Pelle catalytic function affect both known heterodimeric interactions of Pelle, stabilizing the binding to Tube and disrupting binding with Dorsal.

## Coimmunoprecipitation of Dorsal with other pathway components

We next carried out immunoprecipitation experiments to examine the interactions among pathway components in *Drosophila* embryos. Polyclonal antisera to Tube, Pelle, Cactus and Dorsal were used to immunoprecipitate complexes of pathway proteins in extracts prepared from 0-3 hour wild-type embryos; such embryos actively transduce the ventral fate determining signal (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989; Stein et al., 1991; Gillespie and Wasserman, 1994; Reach et al., 1996). We then assayed the composition of the immunoprecipitates by immunoblot analysis.

We observed coimmunoprecipitation of Dorsal with both anti-Cactus and anti-Tube sera (Fig. 3A, lanes 3 and 4). The anti-Tube serum was less efficient in coprecipitating Dorsal than the anti-Cactus serum, but was quite specific: no Dorsal appeared in anti-Tube immunoprecipitates generated from embryos lacking Tube (Fig. 3B). In reciprocal experiments, neither the anti-Dorsal nor anti-Cactus sera coprecipitated Tube (data not shown). This suggests that Tube-Dorsal complexes are unstable or represent only a small fraction of the Dorsal protein in developing embryos.

The immunoprecipitation of low levels of Cactus with anti-Tube sera (Fig. 3A, lower panel, lane 4) indicates that Dorsal can form a complex that contains both Cactus and Tube. Thus an interaction between Tube and Dorsal in embryos occurs prior to Cactus degradation and Dorsal nuclear import.

As shown in Fig. 4 (lane 1), a small amount of Dorsal coimmunoprecipitated with wild-type Pelle. To test whether the association of Dorsal and Pelle in embryos requires that Pelle be catalytically active, we also carried out immunoprecipitations using embryos generated by *pll[078]* females; the Pelle protein in these embryos is full-length, but lacks kinase activity (Bergmann, 1996). Little if any Dorsal was immunoprecipitated from the *pll[078]* extracts (Fig. 4, lane 2), although Pelle levels were comparable for the wild-type and *pll[078]* samples (Fig. 4, lower panel). Thus, in both the immunoprecipitation
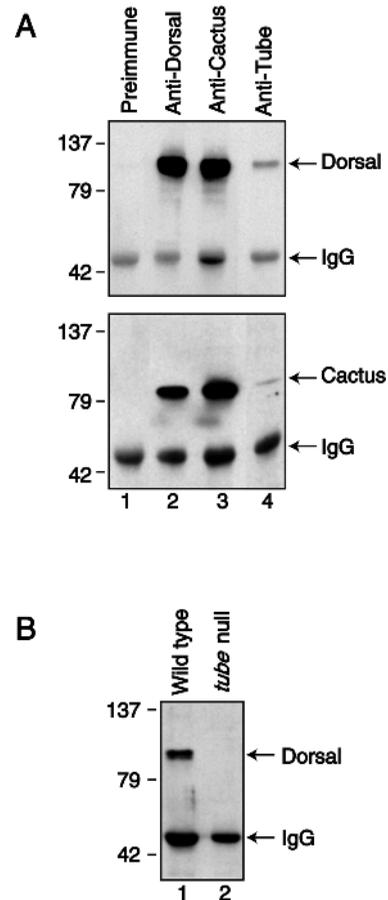


**Fig. 3.** Coimmunoprecipitation of Dorsal, Cactus and Tube from embryonic extracts. (A) Immunoprecipitates were prepared from wild-type embryos using the sera listed. The precipitated complexes were analyzed by SDS-PAGE and immunoblotting using alkaline-phosphatase-conjugated anti-Dorsal IgG (top panel) or anti-Cactus IgG (bottom panel). The positions of molecular weight markers are listed to the left; the positions of Dorsal, Cactus and the IgG heavy chain are indicated to the right. (B) Immunoprecipitates were prepared from either wild-type or *tube* null (*tub[R5.6]/Df(3R)XM3*) embryos using anti-Tube sera. The precipitated complexes were then analyzed by SDS-PAGE and immunoblotting using anti-Dorsal IgG conjugated to alkaline phosphatase. Positions of molecular weight markers are indicated to the left; positions of Dorsal and the IgG heavy chain indicated to the right.
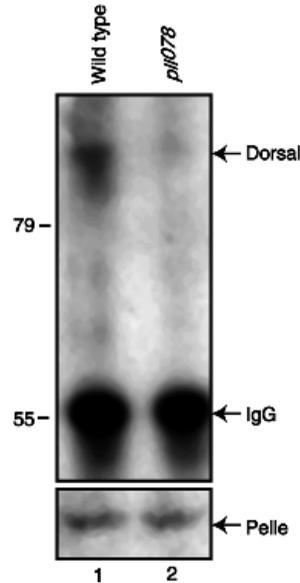
and two-hybrid experiments Dorsal fails to interact with catalytically inactive Pelle.

We were unable to detect immunoprecipitation of Pelle with anti-Tube sera, or of Tube with anti-Pelle sera, in either wild-type or mutant backgrounds (data not shown).

## *Drosophila* Filamin interacts specifically with both Toll and Tube

We have previously demonstrated that Toll[829-1097] interacts with neither Tube nor Pelle in the yeast two-hybrid system (Galindo et al., 1995). Experiments with Dorsal and Cactus also failed to detect a two-hybrid interaction with Toll (data not shown). The absence of detectable interactions with the Toll[829-1097] construct was not due to a failure in expression or nuclear localization, since protein expression was detected in

**Fig. 4.** Coimmunoprecipitation of Dorsal and Pelle from embryonic extracts. (Top panel) Immunoprecipitates were prepared from wild-type or $pll^{078}$ ($pll^{078}/Df(3R)IR16$) embryos using anti-Pelle sera. The precipitated complexes were analyzed by SDS-PAGE and immunoblotting using anti-Dorsal IgG conjugated to alkaline phosphatase. Positions of molecular weight markers are indicated to the left; the positions of Dorsal and the IgG heavy chain are shown to the right. (Bottom panel) Lysates used in the immunoprecipitation experiments shown in the top panel were analyzed by SDS-PAGE and immunoblotting using anti-Pelle sera.





**Fig. 5.** Filamin interacts specifically with the Toll IL-1R homology domain and with Tube. (Left) Scale drawings of the constructs used in pairwise two-hybrid experiments with *Drosophila* Filamin. Structural and functional features of the proteins are highlighted as hatched regions; numbering in each case is based on the full-length protein sequence. (Right) Tally of the presence (+) or absence (−) of a detectable two-hybrid interaction between the carboxy-terminal Filamin fragment and the indicated Toll or Tube fusion construct.

immunoblots of yeast extracts and a specific interaction of Toll$^{829-1097}$ with another pairing partner could be detected with the two-hybrid system (see below).

We carried out a two-hybrid screen with Toll$^{829-1097}$ to search for a link between the intracellular domain of Toll and other pathway components. Using Toll$^{829-1097}$ to screen ovarian and embryonic cDNA libraries, we did not recover any candidate clones that displayed a strong and specific interaction. It has been suggested, however, that the carboxy-terminal portion of this intracellular domain is inhibitory; removal of 61 residues from the Toll carboxy terminus increases reporter gene expression from two- to twelve-fold in transfection experiments (Norris and Manley, 1996). We therefore repeated the library screens using the truncated Toll$^{829-1036}$ construct. From a screen of $8\times10^6$ clones, we identified 17 positive clones, all derived from a single mRNA species. Sequencing from the 5′ ends of representative clones followed by BLAST searches of available databases (Altschul et al., 1990) revealed that the Toll$^{829-1036}$ interacting clones encode portions of a *Drosophila* homolog of ABP280 or Filamin, an actin-binding protein that localizes to the inner surface of the cell membrane (Gorlin et al., 1990; Field and Alberts, 1995).

The *Drosophila* Filamin two-hybrid clones all include the carboxy-terminal end of the proteins in addition to a variable number of the Filamin backbone repeats. In mammalian Filamin, this carboxy-terminal region has been shown to contain a homodimerization domain (Gorlin et al., 1990). The Filamin fragment identified in our screen is also competent for self-association, as demonstrated by a 25-fold increase in reporter gene expression upon cotransformation of DNA-binding domain and activation domain fusions for the *Drosophila* clone (data not shown).

The Toll/IL-1 receptor homology domain appears to be both necessary and sufficient for the interaction of Toll with Filamin: a carboxy-terminal deletion that extends 63 residues into the homology domain eliminated the Toll-Filamin interaction (Fig. 5). Similar deletions abolish Toll receptor activity in vivo (Norris and Manley, 1996). Furthermore, an amino terminal deletion into the IL-1R homology domain also elim-
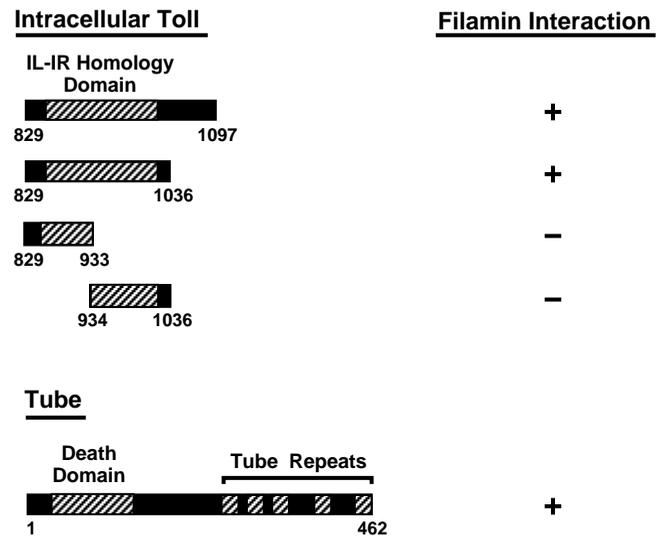
inated the interaction between Filamin and Toll (Fig. 5). Although we did not identify Filamin in our screens with the intact Toll intracellular domain, we found that Filamin will also interact with Toll$^{829-1097}$, albeit more weakly than with Toll$^{829-1036}$.

To investigate whether Filamin might serve to link Toll to other components of the intracellular pathway, we carried out pairwise interaction assays with each of the other four pathway components. The Filamin DNA-binding domain fusion failed to interact with activation domain fusions for Pelle, Dorsal and Cactus, but had a readily detectable interaction (five-fold above background) with a Tube activation domain fusion (Fig. 5; data not shown). This result indicates that Filamin may function between Tube and Toll in dorsoventral signaling.

## DISCUSSION

### A network of interactions among components of the dorsoventral signaling pathway

Our experiments reveal a network of interactions involving the *Drosophila* Rel protein Dorsal and additional components of the dorsoventral signaling pathway. Based on these findings, as well as the results of previous genetic and molecular studies, we propose a model for transduction of the ventral fate-determining signal in the *Drosophila* syncytial blastoderm (Fig. 6).

Of the five genetically defined pathway components, only Pelle is not enriched at the cell surface of the embryo prior to Toll activation (Hashimoto et al., 1991; Galindo et al., 1995; Bergmann et al., 1996; Reach et al., 1996; P. T., unpublished results). As shown in Fig. 6A, we envision that Tube associates with the submembranous cytoskeleton via its interaction with Filamin. This interaction would bring Tube in proximity
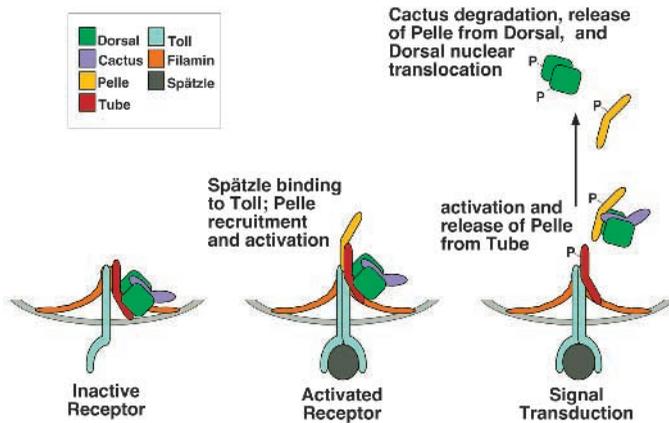
**Fig. 6.** Model for the activation and release of the dorsoventral signaling complex at the membrane. (Left) Prior to Toll activation, Tube, Dorsal and Cactus are enriched at the plasma membrane, presumably through the interaction of Tube with both the Dorsal-Cactus complex and Filamin. Toll also associates with Filamin, bringing the receptor and the intracellular complex into proximity at the cell surface. (Center) The binding of Spätzle to Toll induces the oligomerization of Toll or the association of Toll with a coreceptor (see text). The change in Toll quaternary structure exposes the Tube death domain, allowing recruitment of inactive Pelle to the membrane. Membrane localization activates the Pelle phosphotransferase activity (Galindo et al., 1995; Großhans et al., 1994). (Right) Within the signaling complex, activated Pelle phosphorylates Tube, decreasing the affinity of the two molecules for each other and binds Dorsal. Following Pelle-mediated phosphorylation of Cactus or a Cactus kinase (and perhaps Dorsal), Cactus is degraded, Pelle is released, and Dorsal moves into the nucleus; Tube remains membrane associated.

to Filamin-bound Toll and, we propose, mask the Tube amino-terminus. In addition, some fraction of the Dorsal-Cactus complex in the embryo would interact with Tube via the Dorsal Rel domain and become cortically localized.

An extracellular protease cascade triggered on the ventral side of the embryo is believed to catalyze Spätzle cleavage, generating an active Toll ligand (for review, see Chasan and Anderson, 1993). Based on studies of mammalian cytokine receptors, we propose that ligand binding leads to the association of two or more Toll monomers or of Toll and a coreceptor analogous to the IL-1R accessory protein (Kishimoto et al., 1994; Greenfeder et al., 1995; Wesche et al., 1997). Within the embryo, this association would expose the Tube amino-terminus, providing a binding site for the efficient recruitment of Pelle to the cell surface. Activation of Pelle at the membrane could then occur in close proximity to a potential substrate, such as Dorsal, Cactus or a Cactus kinase.

Evidence for activation of Pelle at the plasma membrane comes from experiments with Torso-Pelle and Torso-Tube fusion proteins (Galindo et al., 1995; Großhans et al., 1994). Although we hypothesize that Pelle becomes activated when bound to Tube, we do not detect a stable complex of Tube and Pelle. Furthermore, the association of Pelle with Tube in the yeast two-hybrid system is relatively weak when Pelle is catalytically active. Our evidence thus suggests that the association of Pelle and Tube is transient.

Once activated, Pelle directly or indirectly mediates phosphorylation of sites in the Cactus amino-terminus, triggering

ubiquitin-dependent Cactus degradation. Cactus proteolysis in turn unmasks the nuclear localization sequence in Dorsal, leading to the efficient translocation of Dorsal into nuclei. Since the pathway is activated in a graded fashion, the result is a nuclear concentration gradient of Dorsal.

By our model, the interaction of Pelle and Dorsal is limited to the time period between Toll activation and Dorsal nuclear localization, such that only a small fraction of the Dorsal protein in embryos can be coimmunoprecipitated with Pelle. The association of Pelle and Dorsal could enhance signaling by colocalizing Pelle with a potential substrate, such as Cactus or a Cactus kinase. This association cannot, however, be strictly required for signaling, since Toll activation can trigger the degradation of Cactus in the absence of Dorsal (Belvin et al., 1995; Bergmann et al., 1996). Alternatively, Dorsal might be a direct substrate for Pelle (see below).

The model illustrated in Fig. 6 provides a potential explanation for the observed activity of a truncated form of Tube (Tube[1-256]). This protein, which lacks the carboxy-terminal, Dorsal interaction domain, provides substantial, but not wild-type, Tube function (Letsou et al., 1993). We envision that Tube[1-256] mediates wild-type Pelle activation, but cannot aid in the recruitment of the Dorsal-Cactus complex to the cell surface.

## Tube and Dorsal as scaffolding and anchoring proteins

Studies in other systems indicate that proteins that engage in multiple interactions with other pathway components serve to increase the efficiency or specificity of signal transduction. Anchoring proteins, such as AKAPs (A-Kinase Anchoring Proteins) and RACKs (Receptors for Activated C Kinase) (reviewed in Mochly-Rosen, 1995), recruit pathway proteins to a specific subcellular location, whereas scaffolding proteins, such as Ste5p, colocalize enzymes and their substrates (Faux and Scott, 1996).

There are substantial similarities between the activities of Tube and those of Ste5p, a protein in the yeast pheromone response pathway. Ste5p, like Tube, interacts through differing domains with multiple pathway components. In addition, Ste5p, through its interaction with Bem1p, associates with the actin cytoskeleton (Leeuw et al., 1995). It is thought that Ste5p serves, at least in part, to segregate the pheromone response pathway from other yeast MAP kinase cascades (Choi et al., 1994; Marcus et al., 1994; Printen and Sprague, 1994).

We believe that the interactions of Dorsal and Tube with Filamin, Pelle, Cactus and each other enhance signal transduction by localizing multiple pathway components to the site of receptor activation. In support of this hypothesis, signal-dependent degradation of Cactus is restricted to the cortical cytoplasm of the *Drosophila* embryo (Bergmann et al., 1996).

Enhancement of signaling by membrane localization of a pathway substrate occurs in other systems. For example, membrane localization of IRS-1, the major substrate for the insulin receptor, is thought to be mediated by a pleckstrin homology (PH) domain (Pawson, 1995). Deletion of this domain decreases tyrosine phosphorylation of IRS-1 by the insulin receptor and attenuates downstream signaling in CHO, COS-7 and 32D[IR] cells (Myers et al., 1995; Voliovitch et al., 1995; Yenush et al., 1996).

Our data indicate that the Dorsal morphogen is capable of

assembling into a complex three of the intracellular components required for its regulated nuclear localization. The interactions of Pelle with Dorsal and Cactus might enhance signaling by colocalizing Pelle with a potential substrate, such as Cactus or a Cactus kinase. In this way, Dorsal would function both as effector and scaffold for the signaling pathway.

## Downstream functions for Tube or Pelle?

Working with transfected *Drosophila* Schneider (SL2) cells, Norris and Manley demonstrated localization of Tube to the nucleus upon coexpression of Dorsal (Norris and Manley, 1995). Our two-hybrid mapping studies indicate that this recruitment of Tube into SL2 nuclei likely resulted from the binding of the Dorsal Rel homology domain to the carboxy-terminal, repeat-containing domain of Tube. It is possible that Dorsal-bound Tube also functions as a transcriptional coactivator in embryonic nuclei. If so, the weakly dorsalized phenotype of embryos expressing only the Tube amino terminus (Letsou et al., 1993) might reflect a requirement for such coactivation in wild-type dorsoventral patterning. We note, however, that Cactus, as well as Dorsal, complexes with Tube in embryos, indicating that at least some of the interaction of Tube and Dorsal occurs prior to Dorsal nuclear translocation.

Although evidence outlined earlier strongly suggests that Pelle must phosphorylate Cactus or a Cactus kinase to effect Dorsal nuclear localization, Dorsal might also be a direct substrate for Pelle. Dorsal, a phosphoprotein in embryos (Kidd, 1992; Whalen and Steward, 1993), undergoes hyperphosphorylation in response to either signal transduction, which causes Cactus degradation, or mutational inactivation of Cactus (Gillespie and Wasserman, 1994). If Pelle phosphorylates free, but not Cactus-bound, Dorsal, the correlation between the elimination of active Cactus and the increased phosphorylation of Dorsal is explained. Pelle-mediated phosphorylation of Dorsal might regulate the rate or efficiency of Dorsal nuclear localization and thereby help shape the Dorsal gradient. Alternatively, phosphorylation might modify the activity of Dorsal as a transcriptional regulator.

## A role for death domains in the Toll and IL-1R signaling pathways

The studies reported in this paper have defined minimal interaction domains for Pelle (residues 26-129) and Tube (residues 25-173) that correspond closely to the regions with similarity to a consensus death domain (Feinstein et al., 1995; Hofmann and Tschopp, 1995). Death domains have been identified in pathways regulating apoptosis (Tartaglia et al., 1993; Chinnaiyan et al., 1995; Hsu et al., 1995; Stanger et al., 1995; Baker and Reddy, 1996; Chen et al., 1996), but their participation in the dorsoventral signaling cascade suggests a more general role in protein interactions mediating signal transduction.

The death domain of Pelle exhibits 72% identity with *D. virilis* Pelle (Z. Cao and V. Baichwal, personal communication) and 22% amino acid identity with the Interleukin-1 Receptor Associated Kinase (IRAK), a human homolog of Pelle (Cao et al., 1996). IRAK forms a complex with the IL-1 type I receptor, a Toll counterpart, only after stimulation with IL-1. Our results suggest that this recruitment of IRAK into a complex at the cell surface is likely to be mediated by an as yet unidentified mammalian Tube counterpart or other death-domain-containing protein.

## The role of *Drosophila* Filamin in dorsoventral signal transduction

Filamin is an actin crosslinking protein localized predominantly in the submembranous skeleton. *Drosophila* Filamin has been identified previously by Field and Alberts in actin affinity chromatography experiments (Field and Alberts, 1995), but no mutations have been described. In mammalian cells, Filamin is a dimer of two 280 kDa subunits. Each subunit contains an amino terminal actin-binding site, as well as carboxy terminal homo- and heterodimerization domains (Gorlin et al., 1990; Meyer et al., 1997). Filamin is known to interact with and be required for the proper function of a number of receptors in mammalian systems. Both signal-independent and signal-dependent interactions have been described.

The glycoprotein Ib-IX complex (GPIb-IX), the platelet von Willebrand factor receptor, engages in a signal-independent interaction with Filamin (Fox, 1985). Upon Thrombin activation of platelets, the relocation of the GP1b-IX/Filamin complex from the membrane skeleton to the activated cytoskeleton depends on this interaction (Kovacsovics and Hartwig, 1996). The interaction between Toll and Filamin might also be a signal-independent phenomenon related to the morphogenetic changes that occur in the ventral furrow. GPIb-IX, like Toll, promotes cellular adhesion and is involved in cellular migrations (Keith and Gay, 1990; Kovacsovics and Hartwig, 1996). Furthermore, the extracellular domains of these two proteins bear substantial sequence similarity (Hashimoto et al., 1988).

Unlike GPIb-IX, the immunoglobulin G Fc receptor I (FcγRI) in leukocytes engages in a signal-regulated interaction with Filamin. FcγRI, like Toll, has a relatively short cytoplasmic domain for which Filamin is the only known protein interaction partner. In the case of FcγRI, it is known that binding of ligand disrupts the highly specific interaction with Filamin (Ohta et al., 1991).

Our mapping studies indicate that the same intracellular region of Toll required for signal transduction, the IL-1R homology domain (Schneider et al., 1991; Norris and Manley, 1995), is necessary for the interaction of Toll with Filamin. Furthermore, deletion of amino acids from the putative inhibitory domain at the Toll carboxy terminus enhances the Toll-Filamin interaction. These correlations, together with the two-hybrid interaction between Tube and Filamin, implicate Filamin in Toll signaling.

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