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Mechanisms of Development 81 (1999) 127–138

Oligomerisation of Tube and Pelle leads to nuclear localisation of Dorsal

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Received 28 September 1998; accepted 4 December 1998

Abstract

In the *Drosophila* embryo the nuclear localisation of Dorsal, a member of the Rel family, is regulated by an extracellular signal, which is transmitted to the interior of the egg cell by a cascade of proteins involving the novel protein Tube and the protein kinase Pelle. Here we analyse the activation mechanism of Tube and Pelle and the interaction between these two components. We show that both proteins, although having different biochemical activities, are activated by the same mechanism. Membrane association alone is not sufficient, but oligomerisation is required for full activation of Tube and Pelle. By deletion analysis we determined the domains of Tube and Pelle mediating the physical interaction and the signalling to downstream components. In order to investigate the link between Pelle and the target of the signalling cascade, the Dorsal/Cactus complex, we isolated and characterised the novel, but evolutionary conserved protein Pellino, which associates with the kinase domain of Pelle.

Keywords: *Drosophila*; Development; Pattern formation; Gradient; Nuclear transport; Signal transduction; Nuclear localisation; Signalling cascade; Axis formation; Axis specification; Dorsoventral; Oligomerisation; Dimerisation; Membrane association; Membrane localisation; Kinase; Protein complex; Phosphorylation; Two-hybrid; Fusion protein; Hybrid protein; Pelle; Tube; Dorsal; Cactus; Toll; Snake; Torso ; Pellino; NF-kB; IRAK; Rel

1. Introduction

Patterning of the dorsoventral axis of *Drosophila* embryos is determined by a nuclear localisation gradient of the transcription factor Dorsal. The protein Cactus is a negative regulator of Dorsal, in that it forms a tight complex that prevents Dorsal from going into the nuclei and from binding to DNA (Geisler et al., 1993; Kidd, 1993). Dorsal is also regulated in a positive way by a signalling cascade transmitting a signal for nuclear localisation from the outside to the interior of the ventral side of the egg (reviewed in Morisato and Anderson, 1995). The intracellular part of the signalling cascade consists of Toll, Tube, and Pelle. They act via an unknown mechanism on the Dorsal-Cactus complex and induce the degradation of Cactus (Belvin and Anderson, 1996). The molecular mechanism of the dorsoventral signal transduction and the regulation of the Dorsal-Cactus complex is poorly understood. However, it is known, that specific regions in the N-terminal part of Cactus are required to receive the signal, which releases Dorsal from Cactus (Bergmann et al., 1996; Reach et al., 1996). In addition, there must be a further factor regulating Dorsal that is independent of Cactus, as albeit not as steep, Dorsal still forms a localisation gradient in embryos with a deletion of the *cactus* gene (Bergmann et al., 1996).

The functional order of the known components within the dorsoventral cascade is well established and has been determined mainly in genetic experiments (Roth et al., 1991; Hecht and Anderson, 1993; Großhans et al., 1994; Galindo et al., 1995; Großhans, 1996). The membrane receptor Toll acts upstream of the novel protein Tube and the serine/threonine protein kinase Pelle. The target of this cascade is the Dorsal-Cactus complex, whereby Cactus is genetically upstream of Dorsal. Toll is a membrane protein with unknown biochemical activities in its small intracellular part (Hashimoto et al., 1988). The biochemical activity of the novel protein Tube is also unknown and no homologous

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0925-4773/99/$ - see front matter
PII: S0925-4773(98)00236-6

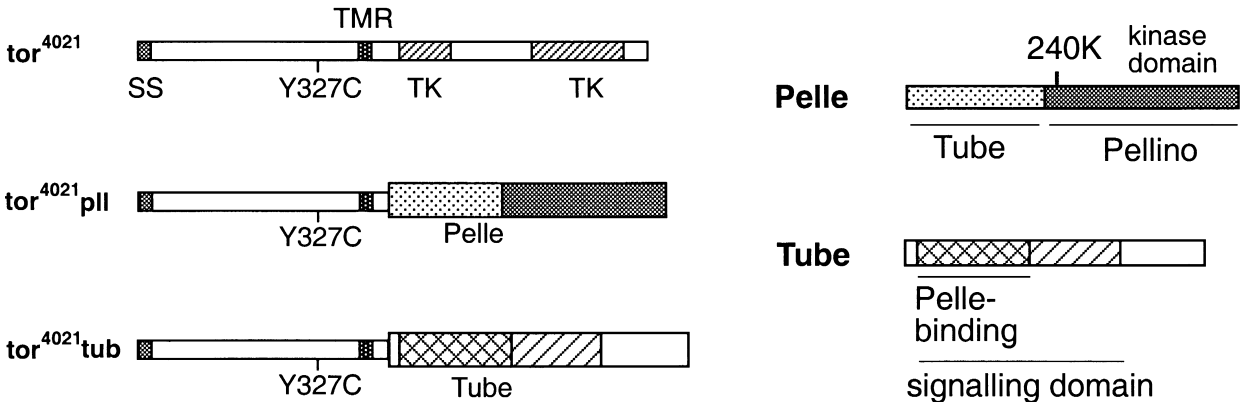

Fig. 1. Structure of the Torso fusion proteins and Tube and Pelle. The indicated domains in Torso are the signal sequence (SS), the membrane spanning region (TMR) and the splitted tyrosine kinase domain (TK). Tyrosine 327 is mutated to a cystein in the Torso^{4021} allele (Sprenger and Nüsslein-Volhard, 1992). In the Torso fusion proteins the intracellular part containing the tyrosine kinase domain was exchanged with Tube, Pelle, or the respective deletion alleles of them. Pelle consists of two domains: the N-terminal part that binds to Tube and the C-terminal part with the serine/threonine kinase domain that binds to Pellino. Lysine 240 lays in the ATP binding pocket. Mutation of lysine 240 to argine prevents binding of ATP.

proteins have as yet been identified (Letsou et al., 1991). The serine/threonine protein kinase Pelle (Shelton and Wasserman, 1993) does not appear to phosphorylate Cactus directly in vitro. However, it phosphorylates itself and its upstream regulator Tube (Großhans et al., 1994). Since Tube acts upstream of Pelle and also binds to it, Tube is a potential activator of Pelle. Recent experiments, however, suggest that the molecular mechanism of the signalling process might be more complicated than the linear genetic model implies, since physical interactions between Dorsal-Tube and Dorsal-Pelle have been observed (Großhans, 1996; Edwards et al., 1997; Yang and Steward, 1997).

Since the enzymatic activities of Tube and Toll are as yet not known, it is unclear how Tube is activated, and how it stimulates the kinase activity of Pelle. The link between Pelle and the Dorsal-Cactus complex also remains to be resolved. Based on studies with gain-of-function alleles of Tube and Pelle we have proposed that oligomerisation or induced membrane association might be involved in the activation of these components (Großhans et al., 1994). Tube and Pelle can be constitutively activated by fusion to the extracellular part and the membrane spanning region of a gain-of-function allele of the receptor tyrosine kinase Torso (Großhans et al., 1994; Galindo et al., 1995). Torso is a component of the terminal system and is not involved in the patterning of the dorsoventral axis.

We assume that the fusion proteins with the gain-of-function allele Torso^{4021} acquire two properties. First, they become fixed in the plasma membrane. They have a signal sequence and a membrane spanning region, and immunhistochemistry shows for Tube or Pelle fusion proteins a grid-like pattern characteristic for membrane proteins (data not shown). Secondly, we assume that they form oligomers. Normally the intracellular tyrosine kinase of Torso is activated by ligand induced oligomerisation, which is followed by intermolecular autophosphorylation (Ullrich and Schlessinger, 1990, Sprenger and Nüsslein-Volhard, 1993). In the Tor^{4021} allele the extracellular YC point mutation substitutes for the function of the ligand, leading to ligand-independent autophosphorylation and oligomerisation of the intracellular tyrosine kinase domain (Sprenger and Nüsslein-Volhard, 1992).

During early embryogenesis Tube is partly associated with the plasma membrane, displaying an asymmetric dis-

Table 1
Oligomerisation but not membrane localisation leads to full activation of Tube and Pelle

RNA (1 mg/ml)	Maternal genotype[b]	Cuticle phenotype[a]				Partial rescue (FK or VD; %)[c]
		D0	FK	FK + VD	VD	
tor^{4021}pll	*snake*	41	46	22	5	64
tor^{WT}pll	*snake*	18	0	0	0	0
tor^{WT}pll	*pelle*	5	3	6	2	70[d]
tor^{4021}tub	*snake*	18	15	29	49	83
tor^{WT}tub	*snake*	5	23	7	0	85

[a]FK, filzkörper or filzkörper material, VD ventral denticles, DO, egg shells containing significant amount of cuticle but neither filzkörper nor ventral denticles.
[b]Of the recipient eggs; amorphic allelic combinations of *pelle* and *snake* (pll^{078}/pll^{rm8}, snk^{073}/snk^{229}).
[c]No difference was observed between dorsally and ventrally injected embryos
[d]Injection was only on the ventral side of the embryo. Injection on the dorsal side gave a much weaker effect.

tribution along the dorsoventral axis. On the ventral side of the egg the membrane association is more prominent than on the dorsal side. However, in dorsalised eggs Tube is still partioned to the membrane, although to a lower degree than in ventralised eggs (Towb et al., 1998), implying that the ventralising signal is only partly responsible for the membrane portion. No membrane association has been observed for Pelle in wild-type or ventralised eggs. Only with artificially high levels of the ventralising signal in eggs, in which the RNA of a constitutively active allele of Toll (Tl^{10b}) has been targeted to the anterior pole (Huang et al., 1997), membrane enrichment in anterior regions is observed (Towb et al., 1998).

In this study we analysed how Tube and Pelle become activated and mapped the domains sufficient for the signalling activity as well as the domains that mediate physical interactions. With the two-hybrid system we isolated Pellino, a putative new component of the dorsoventral signalling cascade, that associates with the kinase domain of Pelle and is probably involved in the signalling events downstream of Pelle.

2. Results

2.1. Oligomerisation, and not membrane association, leads to full activation of Tube and Pelle

It has been shown previously that Tube and Pelle can be constitutively activated by combining them with the extracellular part and the membrane region of the gain-of-function allele Tor^{4021} of the receptor tyrosine kinase Torso (Fig. 1, Großhans et al., 1994; Galindo et al., 1995). These fusion proteins can induce nuclear localisation of Dorsal even in embryos in which the signalling cascade has been interrupted by maternal mutations in components upstream of the receptor Toll, for example *snake*. Embryos from *snake* females lack all ventral structures and develop instead only dorsal tissue. In order to determine the signalling levels and the degree of Dorsal activation, we determined the morphological structures that developed in the injected eggs. Since different levels of Dorsal nuclear localisation direct different developmental pathways, the observed morphological structures in the developing embryo are indicative of the nuclear concentrations of Dorsal in the blastoderm. For example, filzkörper is induced by very low nuclear concentrations of Dorsal, whereas formation of ventral denticles requires intermediate concentration levels. If Dorsal protein is completely localised in the nuclei, mesoderm is induced, which forms only internal tissue and no cuticle (Roth et al., 1989).

To decide whether Tube and Pelle are activated by membrane association or oligomerisation, we compared the effects of Torso wild-type (Tor^{WT}) and Tor^{4021} fusion proteins, when expressed in eggs with a block in the signalling cascade upstream of the Toll receptor (eggs from *snake* females). We observed a striking difference between the Pelle fusion proteins Tor^{WT}pll and Tor^{4021}pll (Table 1). As

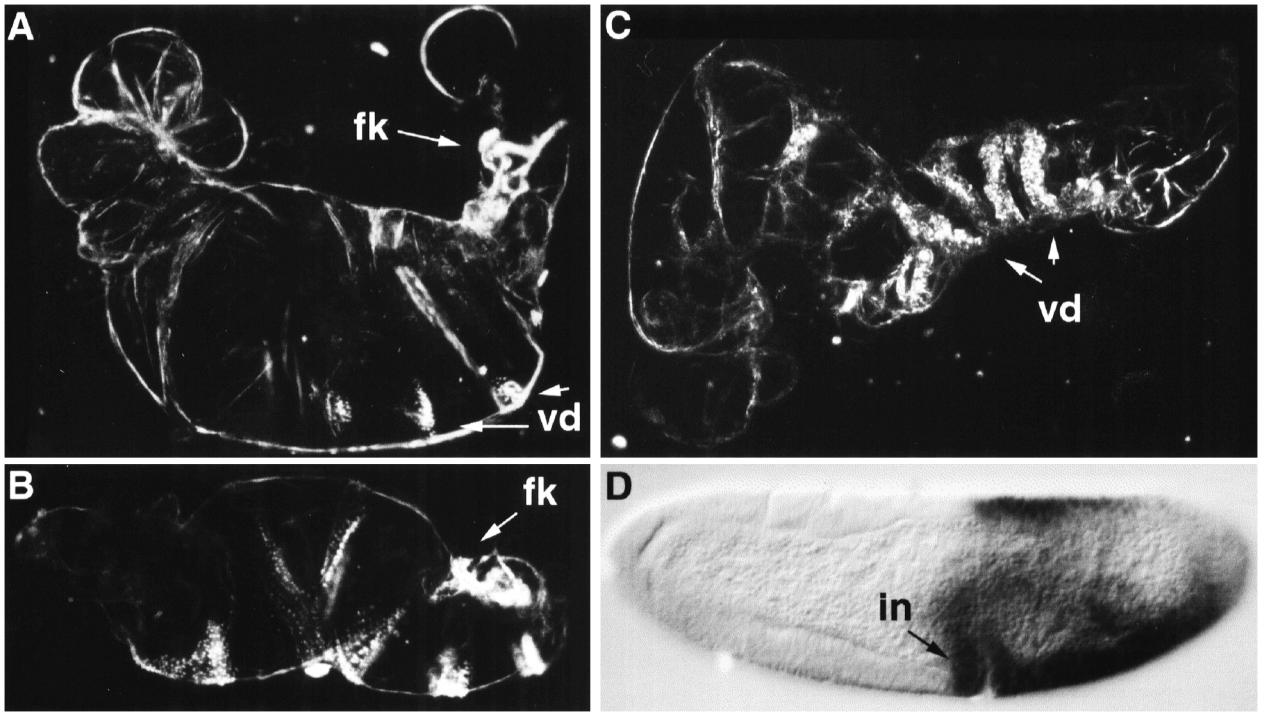

Fig. 2. Constitutive activation of Tube and Pelle in Torso fusion proteins. Cuticles (A–C) and Twist expression (D) in embryos that were injected with synthetic RNA (1 mg/ml) on the dorsal or ventral side at 30% egg length, fk, Filzkörper, vd, ventral denticles, in, local invagination of Twist expressing cells. The type of RNA and the genotypes of the females were as follows: (A) tor^{4021}pll RNA, $tub^{R5.6}$/Df(3R)XM3; (B) tor^{4021}tub RNA, snk^{073}/snk^{229}; (C,D) tor^{4021}pll3 RNA, $tub^{R5.6}$/Df(3R)XM3.

previously described (Großhans et al., 1994), tor^{4021}pll RNA injection induces filzkörper and ventral denticles (Fig. 2A). In contrast, the Tor^{WT}pll fusion protein did neither induce filzkörper nor ventral denticles, although it has wild-type *pelle* function. Injection of tor^{WT}pll RNA into eggs that lack functional Pelle (eggs from *pelle* females) restored development of the filzkörper and ventral denticles (Table 1). These experiments show that functional Pelle protein was expressed and that therefore membrane association alone is not sufficient to activate Pelle.

We tested the activities of the fusion proteins at higher expression levels. With the 3′UTR of the *pelle* cDNA (tor^{4021}pll3) included, which does not change the coding sequence, the RNA gained a greater potency presumably caused by more efficient translation and higher protein concentrations. Tor^{4021}pll3 RNA induced the strongest signalling response (Fig. 2C,D). We observed cell invaginations during gastrulation, large Twist expression domains extending around the egg circumference, and reduced amounts of cuticle, indicating that large domains of mesoderm were induced. At these higher expression levels the tor^{wt}pll3 RNA now also induced filzkörper and ventral denticles, comparable to the activity of the tor^{4021}pll RNA. The activity, however, was much weaker than the activity after Tor^{4021}pll3 RNA injection, since neither cell invaginations nor reduced amounts of cuticle were induced (data not shown).

The Torso fusion proteins with Tube showed a comparable behaviour (Table 1). Both, Tor^{WT}tub and Tor^{4021}tub induced filzkörper and ventral denticles, but Tor^{4021}tub has a much higher activity (Fig. 2B, Table 1). A large proportion of the injected embryos (49 of 111) contained only denticle belts with no filzkörper, whereas in embryos injected with Tor^{WT}tub, none had only denticle belts, while the majority (23 of 35) contained only filzkörper. The filzkörper is indicative of very low nuclear concentrations of Dorsal.

2.2. Signalling domains in Tube and Pelle

To map the domains of Tube and Pelle activating the downstream components of the cascade, we constructed a series of Tube and Pelle deletion mutants fused to Tor^{4021} and used RNA injection to assay their activity (Table 2). The C-terminal kinase domain of Pelle (pllΔN183) is sufficient to activate Dorsal, whereas the N-terminal regulatory domain (pllΔC215) as well as a Pelle allele with a defective

Table 2

Signalling domains in Tube and Pelle

injected RNA (1mg/ml)#	cuticle phenotype				partial rescue
	D0	FK	FK+VD	VD	(FK or VD)
tor^{4021}pll	41	46	22	5	64%
tor^{4021}pllΔN184	32	22	1	0	42%
tor^{4021}pllDC215	18	0	0	0	0%
tor^{4021}pllK240R	18	0	0	0	0%
tor^{4021}tub	18	15	29	49	84%
tor^{4021}tubΔC366	9	7	11	12	75%
tor^{4021}tubΔC331	18	14	18	14	72%
tor^{4021}tubΔC313	13	14	17	13	77%
tor^{4021}tubΔC252	33	0	0	0	0%
tor^{4021}tubΔC145	25	0	0	0	0%
tor^{4021}tubΔN19	9	10	9	14	78%
tor^{4021}tubΔN45	12	0	0	0	0%
tor^{4021}tubDN72	27	0	0	0	0%
tor^{4021}tubΔN202	13	0	0	0	0%

kinase domain (pllK240R), had no signalling activity. This observation suggests that the kinase activity is necessary for the subsequent signalling steps, which may be phosphorylation of the following cascade components or phosphorylation of Pelle itself.

Tube has no obvious structural domains except for several small repeats in the C-terminal half (Letsou et al., 1991). With a series of C- and N-terminal truncations we mapped the downstream signalling domain to residues 19 to 313 of Tube (Fig. 1, Table 2). This region contains the entire signalling activity of Tube, since the deletion alleles are as efficient as the full length Tube protein in our injection assay. The mapping data of the signalling domains in Tube and Pelle suggest that the molecular interaction between Tube and Pelle is conveyed by the N-terminal part of Tube and the N-terminal part of Pelle. The propagation of the signal to the components forming the link between Pelle and the Dorsal/Cactus complex, however, is mediated via Pelle's kinase domain.

2.3. The conserved protein Pellino binds to Pelle

In an attempt to identify downstream components linking Pelle to the Dorsal/Cactus complex we identified Pellino (Pli) in a two-hybrid screen in yeast with Pelle as the given binding partner. The five clones that activated the reporter gene strongest contained *pellino* cDNAs encoding a protein with 424 amino acid residues and an estimated molecular weight of 47 kDa (Table 3; Genbank accession number AF091624). The interaction of Pelle and Pellino in yeast is specific, since the lex-pll fusion protein does not interact with any out of a set of other proteins (Großhans et al., 1994), and the JG-Pli fusion protein does not associate with any of the other lex fusion proteins that we have tested (lex-cactus, lex-exuperantia, lex-dl^{QD}, lex-Toll^{intra}, data not shown). In the sequence databases we only found identical sequences (*Drosophila*, LD10775 (5′)) and highly conserved sequences of unknown function with about 50% identity and 70% homology of *C. elegans* (cosmid U64842, gene product F25B4.2), *O. volvulus* (SWm L3CO1966), mouse (Z31105), and human (e.g. R28569). The *pli* gene of *Drosophila* maps to 95C and is deleted in the deficiency chromosome Df(3R)06624 (data not shown). We have not yet identified *pellino* mutations.

Table 3
Interaction of Pelle and Pellino

lex-	JG-	β-gal activity[a]	Relative factor
pll	–	0.9	0.5
pll	tub	180	100
pll	Pli70[b]	1750	970
pll	Pli323[b]	1660	920
pll	Pli408[b]	1780	990
pllK240R[c]	tub	826	460
pllK240R	Pli70	15	8
Pli408	Pli70	106	
Pli408	tub	8	

[a] β-Galactosidase activity of permeabilised yeast cells containing the lacZ reporter plasmid pSH18-34 and the indicated plasmids encoding the lex and JG fusion proteins.
[b] Pli70 (residues 33–424), Pli323 (residues 32–424), and Pli408 (additional six residues at N-terminus encoded by the *pellino* cDNA) are two-hybrid isolates of Pellino.
[c] Mutant allele of Pelle with no kinase activity.

The *pellino* gene is expressed during all stages of development as a 3 kb transcript. In addition, there is a transcript of 1.9 kb which is found only in females and in eggs that are zero to 2 h old (Fig. 3A). A polyclonal rabbit antibody specific for Pellino detects two bands with the expected molecular weight (Fig. 3B). The protein is present during all stages of embryonic development at comparable levels (data not shown). To check whether Pellino is post-translationally modified by the ventral signal we analysed protein extracts with an immuno blot (Fig. 3B). We did not observe a difference in the banding pattern when using extracts from dorsalised, ventralised, and eggs without Pelle protein in comparison to wild-type extracts (Fig. 3B). Staining of fixed wild-type eggs with Pellino specific antibody was uniform with no obvious asymmetry along the dorsoventral axis (data not shown).

2.4. Binding of Pellino to Pelle requires Pelle kinase activity

The specific complex of Pelle and Pellino can be reconstituted *in vitro* with recombinant Pellino and radioactively labelled Pelle (Fig. 5A). In this assay, Tube did not significantly bind to GST-Pli, and vice versa, labelled Pellino did not associate with GST-Tube (Fig. 5B). In addition to binding to Pelle, Pellino is able to form oligomers in yeast cells (Table 3) and in vitro (Fig. 5A). None of the labelled protein bound to recombinant GST, indicating that the Pll-Pli and Pli-Pli complexes are specific (Fig. 5A).

Tube is the only protein known so far to be phosphorylated by Pelle (Großhans et al., 1994). To test whether Pellino is a substrate of Pelle, we investigated the interaction of Pellino with the PllK240R mutation, which has no kinase activity due to a mutation of a conserved lysine to an argine residue within the ATP binding site (Shelton and Wasserman, 1993). Such a mutation strengthens the interaction between the kinase and its substrates, since the substrate cannot be phosphorylated and therefore remains bound to the enzyme. An example for such a behaviour is observed with Tube and Pelle. The reporter gene activation in the two-hybrid assay with the pair PllK240R and Tube is about 5-fold increased when compared with the pair Pelle and Tube (Table 3; Edwards et al., 1997). In contrast, in the pair PllK240R and Pellino the reporter gene activity is reduced to about 1% (Table 3), suggesting that Pellino does not bind to a catalytically inactive form of Pelle. Consistent with the binding experiments is our finding that in contrast to Tube, recombinant GST-Pellino protein is not

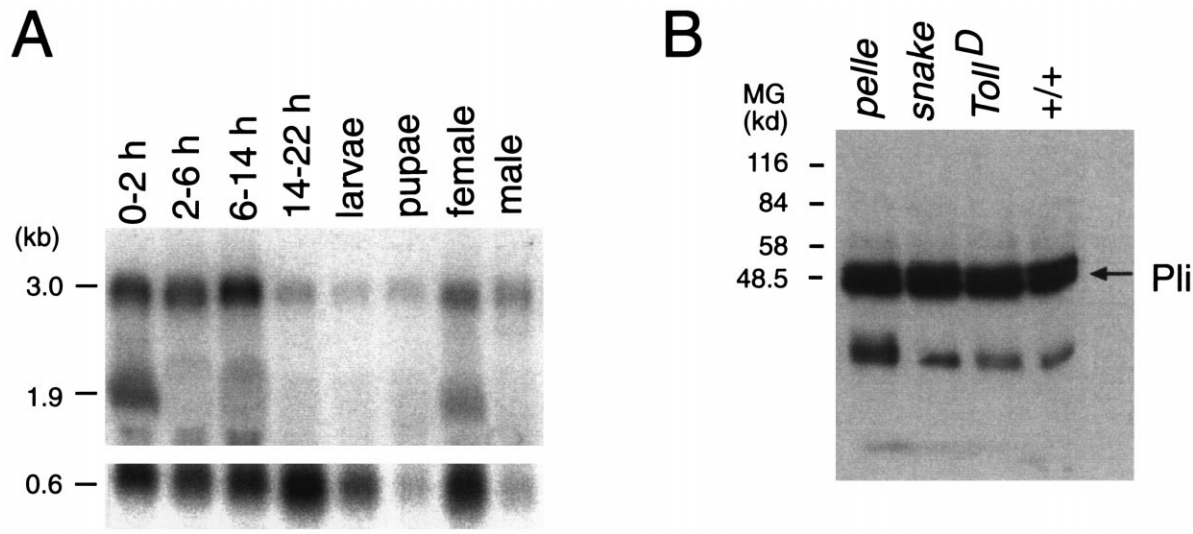

Fig. 3. RNA and protein expression of *pellino*. *pellino* RNA (A, northern blot with polyA RNA of the indicated developmental stages or sex) of 3 kb is present throughout development. A smaller transcript of 1.9 kb is present only in 0–2 h old eggs and females. The RP49 RNA of 0.6 kb serves as a loading control. With a polyclonal antibody specific for Pellino two bands at 48.5 kDa are detected in an immuno blot with extracts of 0–4 h eggs from females of the indicated genotypes (B, *pelle*, pll^{rm8}/Df(3R)D605, *snake*, snk^{073}/snk^{229}, $Toll^{D}$, Tl^{10b}/+, +/+, wild-type).

phosphorylated by Pelle in vitro (Fig. 4). These observations indicate, that Pellino, unlike Tube, is not a phosphorylation substrate of Pelle, but that it probably only binds to the autophosphorylated form of Pelle. Other possibilities are that Pellino directly binds to the ATP binding site or more unlikely, that Pellino has to be phosporylated by Pelle to efficiently bind to Pelle.

2.5. Tube and Pellino bind to different domains of Pelle

As mentioned before, Pelle consists of a C-terminal kinase and a N-terminal domain which is not required for downstream signalling (Fig. 1, Table 2). With our binding assay we determined the parts of Pelle that bind Tube or Pellino. We found that the N-terminal domain of Pelle (pllΔC215) associates with GST-Tube, but not with GST-Pli. The kinase domain (pllΔN183) shows the reverse behaviour. It binds to GST-Pli and not to GST-Tube (Fig. 5B).

No proteins which are homologous to Tube are known. In order to further analyse the signalling mechanism between Tube and Pelle we identified the part of Tube which is sufficient for binding to Pelle with the two-hybrid assay. To this end we constructed a library of random fragments of the *tube* cDNA and screened for Pelle-interacting clones. All fragments that we isolated and analysed encoded N-terminal parts of Tube (Fig. 6A). This experiment defines the Pelle binding domain to be the amino acid residues 26 to 172 (Fig. 1, Edwards et al., 1997).

In a second experiment we screened a library of randomly mutagenised *tube* cDNA to isolate Tube alleles that bind to Pelle with higher affinity. Each of the four clones that we have analysed has a single point mutation, which leads to a C-terminal truncation of Tube, again defining the N-terminal part as the Pelle binding domain (Fig. 6B). These Tube alleles activate the reporter genes four to eight times stronger than full-length Tube. This finding is reminiscent of our observation that the Tube-Pelle interaction is strengthened with the kinase-defective Pelle allele (PllK240R, Table 3). Together these observations suggest that the function of the C-terminal part of Tube is to destabilise the Tube-Pelle complex, after Pelle has been activated and autophosphory-

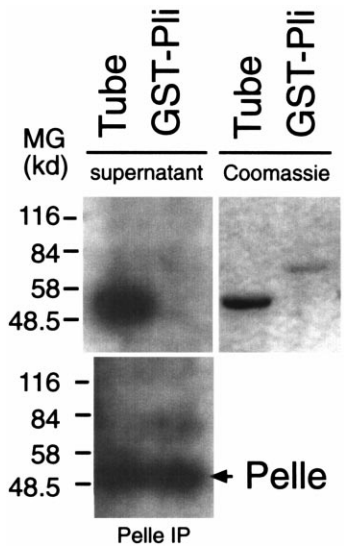

Fig. 4. Pellino is not phosphorylated by Pelle. Phosphorylation assay with recombinant His-Tube and GST-Pli with immuno-precipitated Pelle. The upper panels show the autoradiography and the Coomassie stain of the supernatant with the recombinant proteins. The lower panel shows the autoradiography of the pellet fraction containing the autophosphorylated Pelle.

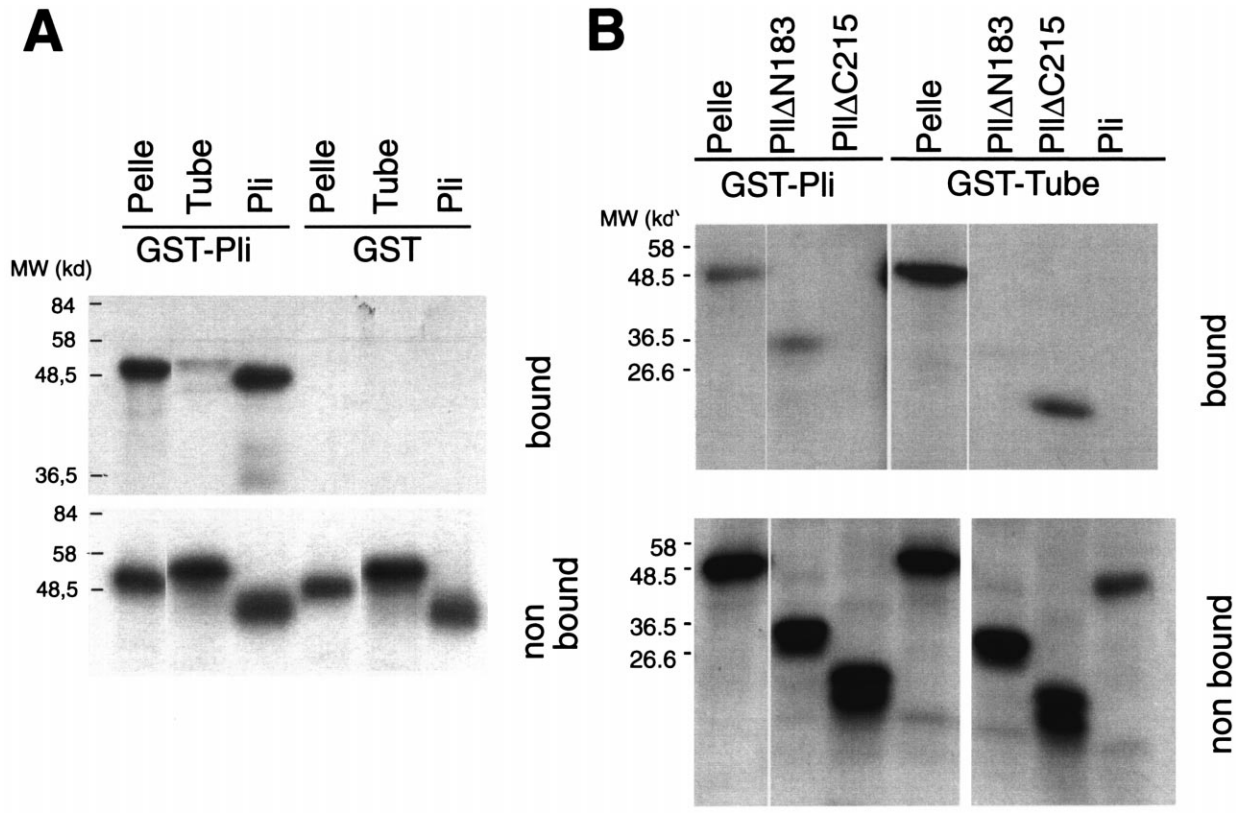

Fig. 5. Tube and Pellino bind to different domains of Pelle. Binding assay with the indicated GST proteins and [^{35}S]labelled protein synthesised in reticulocyte lysate. The upper panel (bound) shows the labelled proteins bound to the beads, the lower panel (unbound), the labelled proteins in the solution. The autoradiograph shown in the upper panel of (A) was exposed for a longer period of time. The autoradiographs in (B) showing the non-bound fractions and the bound fractions to GST-Tube were exposed for the same period of time than the autoradiograph in the lower panel. The autoradiograph showing the bound fractions to GST-Pli was exposed five times longer.

lated. An alternative explanation could be that the C-terminal domain of Tube may regulate Pelle kinase activity.

3. Discussion

In our previous study (Großhans et al., 1994) we described that Tube and Pelle can be constitutively activated by fusion to a gain-of-function allele of the receptor tyrosine kinase Torso. Here, we used this observation as a starting point to analyse further the mechanism of signal transduction between the membrane receptor Toll and the cytoplasmic Dorsal/Cactus complex.

3.1. Oligomerisation of Torso fusion proteins

Although we did not directly investigate the molecular properties of the Torso fusion proteins, we assume that they induce oligomerisation of their fusion partners for several reasons. As already mentioned in the introduction, the Tor^{4021} point mutation substitutes for the ligand-induced oligomerisation of the intracellular domain in the Torso pathway. Since Tor^{4021} fusion proteins can be used to constitutively activate unrelated proteins, like Tube, D-Raf, and the Dpp receptor subunits Punt and Thick vein, it is likely that the Tor^{4021} point mutation affects a general property of the fusion proteins, namely oligomerisation. Tor^{4021} fusion proteins with Punt and Thick vein lead to Dpp-independent activity only when both of them are present and not, when only one of the fusion proteins is expressed (Nellen et al., 1996). Normally, the ligand Dpp induces heterodimer formation of Punt and Thick vein. In the Tor^{4021} fusion proteins the Dpp pathway becomes activated, because instead of Dpp, the extracellular Tor^{4021} domains mediate the formation of heterooligomers between the Punt and Thick vein intracellular domains.

The studies with D-Raf and Sevenless fusion proteins (Dickson et al., 1992a,b) show in a different way that the activation effect of the fusion proteins is not a unique property of the Tor^{4021} point mutation. Activation is also obtained with the gain-of-function allele Tor^{Y9} and wild-type Torso. The strength of the activation effect corresponds to the phenotypic strength of the *tor*^{4021}, *tor*^{Y9}, and *tor*^{WT} alleles. This supports the view that the extracellular mutations do not affect a specific property of the intracellular part, but rather the normal activation mechanism, namely oligomerisation of the intracellular domains. These studies also show that even the Torso wild-type allele can mediate

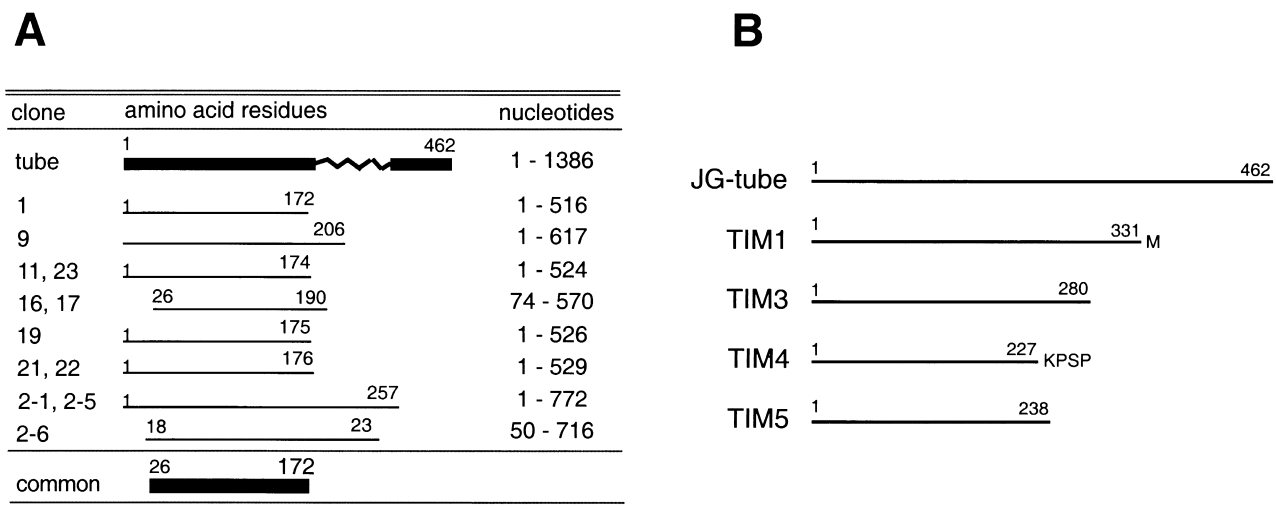

Fig. 6. Pelle binds to the N-terminal part of Tube. (A) Random fragments of Tube that bind to Pelle. Shown are the breakpoints of the fragments when compared to the coding sequence and the corresponding amino acid residues of Tube. Amino acid residues 26 to 172 were present in all of the clones that were analysed. Because of the cloning procedure that was applied, clones starting with codon 1 of the tube coding sequence are enriched. (B) Tube alleles binding more strongly to Pelle. All four clones analysed contain mutations creating stop codons or frame shifts with following stop codons. When expressed in reticulocyte lysate correspondingly smaller Tube proteins were detected (data not shown).

oligomerisation of the fusion proteins, when the expression is high enough. This is not surprising, since even in the absense of the Torso ligand wild-type Torso contains phospho-tyrosine, although less than the gain-of-function alleles (Sprenger and Nüsslein-Volhard, 1993).

3.2. Activation of Tube and Pelle by oligomerisation

To test whether membrane association or oligomerisation mainly contribute to the activation of Tube and Pelle, we compared the effects of fusion proteins with the Torso wild-type and a gain-of-function allele. We assume that the Tor^{4021} fusion proteins strongly oligomerise whereas the Tor^{WT} fusion proteins oligomerise only to a lower extent which is not sufficient for activation or, at higher concentrations, only sufficient for weak activation. Although Tor^{WT}pll has no constitutive activity at the same concentration at which Tor^{4021}pll is active, it can still rescue *pelle* mutants. At higher expression levels Tor^{WT}pll3 shows some constitutive activity, which is much weaker, however, than what we observed with the Tor^{4021}pll3 fusion protein. Therefore we conclude that restricting Pelle to the membrane does not inhibit its function and is not sufficient for activation, but that Pelle is mainly activated by oligomerisation. The same differential behaviour was observed between the Tor^{WT}tub and Tor^{4021}tub. Our data suggest that Tube and Pelle are activated by similar mechanism. This is surprising, since the molecular structures and biochemical functions of Tube and Pelle are not related.

The constitutive activity of the Tor^{WT} fusion proteins, which is weaker than the activity of the fusion proteins with Tor^{4021}, can be explained by the fact that in the embryo wild-type Torso is weakly autophosphorylated even in the absence of Torso ligand (Sprenger and Nüsslein-Volhard, 1993). This is presumably caused by residual oligomerisation, which is not sufficient for activation of the signalling cascade. Another explanation for our data could be that there are two ways to activate Tube and Pelle. At lower concentrations of the fusion proteins, oligomerisation would lead to activation of the intracellular domain, whereas at a higher concentration, membrane association would be sufficient to trigger the activation.

Towb et al. (1998) addressed related questions in their recent study. They used a fusion of Tube or Pelle with the myristylation signal of src (src^{90}) to achieve an assumed constitutive membrane association. This, however, activated Tube and Pelle only weakly. The effects that Towb *et al*. describe are comparable to the respective Tor^{WT} fusion proteins that were used in our study. Tor^{WT}tub and Tor^{WT}pll3 (injected at 1 mg/ml) have comparable activity with src^{90}tube and src^{90}pll (injected at 2 mg/ml). The weak constitutive activity of the Tor^{WT} and the src^{90} fusion proteins is presumably the result of their high concentration in the membrane. The threshold concentration for efficient oligomerisation is lower at the membrane than in the cytoplasm, since diffusion is limited to two dimensions in the membrane. By injection of tor^{WT}pll3 RNA or src^{90}pll RNA the threshold concentration could be reached, but not by injection of tor^{WT}pll RNA, which is expressed at a lower level.

Studies of the Tube and Pelle distribution in the egg (Galindo et al., 1995; Towb et al., 1998) support our observations that the main mechanism of Tube and Pelle activation is by oligomerisation and not by membrane association alone. First, Tube is also enriched at the membrane on the dorsal side of the egg and in dorsalised eggs, where Dorsal is not transported into the nuclei. The same observation is made for the ‘clusters’ of Tube staining, which have been

described by Towb et al. (1998), and are also found on the dorsal side of the egg. Second, Pelle was not detected at the membrane in wild-type eggs and eggs with a constitutively active Toll receptor, suggesting that Pelle only transiently associates with the membrane. Membrane association can therefore not be the mechanism that keeps Pelle in an activated state. Instead the activation of Pelle might be very similar to the autoactivation of the receptor tyrosine kinases. We suggest that the activated Toll receptor induces, directly or indirectly, oligomerisation of Tube and of Pelle, which is then followed by intermolecular autophosphorylation of Pelle.

3.3. The function of Tube

The function of Tube regarding the activation of Pelle and the function of the assumed activator of Tube can each be substituted by the fusion of either Tube or Pelle to the Tor^{4021} part. The Tube activity on a molecular level might be to mediate the oligomerisation of Pelle at the membrane. The not yet identified upstream component of Tube might have a similar activity for Tube. Having induced Pelle to oligomerise, Tube is not required any further in the subsequent signalling steps as shown by the observation that Tor^{4021}pll has the same activity in eggs lacking either Snake or Tube. Injection of Tor^{4021}pll3′ RNA into eggs with no Tube protein resulted in highest signalling levels. In such eggs, similar to the ones lacking Snake, local cell invaginations and large regions of Twist expression were induced (Fig. 2C,D). In contradiction to our conclusion, that Tube has only a function for the activation of Pelle and no function for the signalling events downstream of Pelle, are studies proposing a function of Tube in the modulation of the transcriptional activity of Dorsal (Norris and Manley, 1995, 1996; Edwards et al., 1997; Yang and Steward, 1997).

The only recognised structural motif of Tube are several short repeats in the C-terminal half (Letsou et al., 1991). The C-terminal part, however is dispensable for the function of Tube (Letsou et al., 1993). Both domains that we mapped, the signalling domain (residues 19 to 313) and the Pelle-binding domain (residues 26 to 172) reside in the N-terminal part of Tube (Fig. 1, Edwards et al., 1997). A clue to the function of the C-terminal part of Tube might come from the observation that C-terminal truncated alleles of Tube bind more strongly to Pelle than full length Tube. A similar stabilisation of the Tube-Pelle complex is observed with the catalytically inactive Pelle allele (PllK240R). The C-terminal part of Tube and the activated Pelle kinase may therefore be involved in dissociation of Tube from Pelle after Pelle has been transformed into an activated state.

Simple binding of Pelle to Tube seems not to be the only requirement for Pelle activation, since alleles of Tube which bind more strongly to Pelle in yeast (Fig. 6B) still have to be activated by the upstream components and have no signalling activity on their own, as tested with our RNA injection assay (data not shown). This notion is supported by our observation that the Pelle-binding domain is not sufficient to confer the signalling event. The part of the signalling domain of Tube that is not covered by the binding domain could mediate the oligomerisation of Pelle after having bound to Tube. Since the C-terminal part of Tube is not required for its function (Letsou et al., 1993), the Tube signalling domain might also receive the signal from the upstream components.

3.4. Function of the Pelle-associated protein Pellino

With the RNA injection experiments and the binding assays it was possible to dissect the functions of Pelle. The interaction with its upstream activator Tube is mediated by the N-terminal domain (Edwards et al., 1997), implying that this part of Pelle is responsible for receiving the ventralising signal, whereas the C-terminal kinase domain transmits the signal for nuclear localisation of Dorsal to the Dorsal/Cactus complex and binds Pellino. Pellino is a protein that was conserved during evolution of the metazoan. Structural homologous genes are present in the genomes of nematodes, mouse, and human. Since the homologous components of the dorsoventral signalling cascade are involved in the regulation of NF-kB in mammals (Belvin and Anderson, 1996; Medzhitow et al., 1997), it is very likely that the function of Pellino is also conserved in addition to its structural conservation. The mammalian Pellino is a good candidate for an associate protein of IRAK (Cao et al., 1996) or the Pelle-like kinase (Trofimova et al., 1996).

Pellino is probably involved in the signalling steps downstream of Pelle, since it only interacts with the Pelle kinase domain of a catalytically active form, but appears not to be phosphorylated by Pelle. It seems likely that Pellino associates only with the autophosphorylated and activated form of Pelle. Pellino could stabilise the activated state of Pelle after the dissociation from the Tube oligomers or could mediate the interaction to the downstream phosphorylation substrate. Alternatively, the signal transduction by activated Pelle oligomers may occur through the binding of Pellino and other downstream components without any phosphorylation of them by Pelle. Future studies of Pellino and the identification of mutations in the *pellino* gene will allow to better understand the molecular nature of the link between Pelle and the Dorsal/Cactus complex.

4. Experimental procedures

4.1. Flies

Standard methods have been applied in fly experiments including the RNA micro injection procedure. The mutations and strains used have been described elsewhere (Tearle and Nüsslein-Volhard, 1987). We assume for the RNA injection experiments that equal levels of functional

proteins were expressed, since equal amounts of synthetic RNA (at 1 mg/ml) encoding the various fusion constructs were injected, and since the Tor^{WT} and Tor^{4021} alleles are equally stable in eggs (Sprenger and Nüsslein-Volhard, 1993).

4.2. Molecular biology

Preparation and analysis of nucleic acids was performed according to standard protocols (Sambrook et al., 1989). The torso fusion constructs were made by PCR amplification of *tube* and *pelle* cDNA with vent polymerase. The cloning of tor^{4021}pll and tor^{4021}tub has been described previously (Großhans et al., 1994). tor^{WT}pll, tor^{4021}pll3, tor^{WT}pll3, tor^{4021}pll240, tor^{WT}tub, and the constructs with truncated forms were constructed accordingly. tor^{4021}pll3 and tor^{WT}pll3 contained the complete coding sequence and, in addition, the 3′ untranslated sequence of the *pelle* cDNA. The constructs for truncated versions of Tube and Pelle contained fragments starting at the indicated codon or had a stop codon after the indicated codon.

The plasmids lex-pll and JG-tub have been described previously (Großhans et al., 1994). The plasmid lex-pll240 was cloned in a similar way as lex-pll but with the plasmid pSelectpll240 (Shelton and Wasserman, 1993) as a template for the PCR. We could not use a lex-pllΔC215 construct to investigate the Tube-Pelle interaction with the two-hybrid system, because this construct was auto-activating. For lex-Pli408, the *Eco*RI-*Xho*I fragment of JG-Pli408 was cloned into pEG202. The plasmids JG-Pli70, JG-Pli323, JG-Pli408 were isolated in a two-hybrid screen with Pelle as bait. The *Eco*RI-*Xho*I fragments of JG-Pli408 and JG-tube were cloned into pGEX-4T-1 (Pharmacia) to yield the plasmids pGST-Pli and pGST-tube. For QE-Pli the complete coding sequence of *pellino* was amplified by PCR and cloned as a *Sph*I-*Xho*I fragment into pQE30 (Qiagen).

Capped RNA was synthesised with SP6 polymerase and linearised plasmids pNB-pll, pSelect-pllK240R, pSP-pllΔC215, pCS-pllΔN183, pNB-tub, and pCS-Pli as templates (Großhans et al., 1994). The plasmids pCS-Pli and pCS-pllΔN183 (start codon and codons 183 and following) were made by insertion of *Eco*RI-*Xho*I fragments from JG-Pli408 or the *pelle* cDNA into pCS2+ (R. Rupp, Tübingen). pSP-pllΔC215 was made by insertion of a *Nco*I-*Sal*I PCR fragment containing codons 1 to 215 of *pelle* cDNA into pSP64 (Sprenger and Nüsslein-Volhard, 1992).

4.3. Libraries for the two-hybrid system

RNA was extracted from ovaries of OregonR wild-type flies with guanidinium thiocyanate, isolated by caesium chloride centrifugation, and fractionated with 0.25 g oligo dT-cellulose (Boehringer Mannheim). Directional oligo dT primed cDNA was synthesised with reagents from Stratagene (ZAP cDNA synthase kit) and fractionated with sepharose CL-4B (1 ml; Pharmacia) to yield two pools with each about 3 mg cDNA and average sizes of about 900 bp (library *ovo*II) and 1300 bp (library *ovo*I). Samples of the cDNA pools were ligated into vector pJG4-5 (Gyuris et al., 1993) to yield two sets of about 5.5×10^6 and 4×10^6 plasmids. Most of the plasmids contain inserts (41 out of 41 tested), and about one quarter encodes fusion proteins in yeast (11 out of 40 tested).

4.4. Two-hybrid screen

Experiments with yeast were performed according to standard protocols (Rose et al., 1988; Schiestl and Gietz, 1989; Gyuris et al., 1993). For quantitative b-galactosidase assays, three to five measurements were averaged. Plasmids and yeast strains for the two-hybrid experiments were obtained from the laboratory of R. Brent (Boston, MA). The yeast strain EGY48 with pSH18-34 and plex-pll was grown on galactose medium prior to transformation with the library. The transformation mix was plated onto medium requiring activation of the reporter genes for growth (galactose without leucin). After 5–6 days b-galactosidase assays were performed on nitrocellulose replicas. The lex-pll bait weakly activates the *LEU2* reporter gene on its own, but not the lacZ reporter genes. Library plasmids were isolated after retest of the positive clones as recommended and used for transformation of a series of yeast cells with various bait plasmids (lex-cactus, lex-Toll, lex-exu, lex-dl^{QD}, lex-bicoid) to select preys that specifically interact with Pelle. Besides *pellino* clones we identified three other cDNAs (*tube*, *drk*, *dorsal*) that clearly activated and several cDNAs that only weakly but specifically activated the lacZ reporter gene (J.G. unpublished data). The isolated *pellino* clones did not show reporter gene activation with any of the other baits tested. A presumably full length cDNA of *pellino* with the same single open reading frame, was isolated with the *pellino*70 DNA as a probe from an ovarian cDNA library (Stroumbakis et al., 1994). Probing of the northern blot (Fig. 3A) with the complete 3kb *pellino* cDNA detected a third transcript of 800 nt, present in all developmental stages (data not shown).

4.5. Selection of Tube alleles that more strongly bind to Pelle

The *Eco*RI-*Xho*I fragment of mutagenised JG-tube (37°C, 20 h in 1 M hydroxylamin, pH 6–7) was cloned into pJG4-5, yielding the library JG-tub* with about 10^5 clones. The library was used for transformation of the yeast strain EGY48 containing the plasmids plex-pll and the lacZ reporter pJK103 or p1840. Transformants grown on non-selective glucose plates were replica-plated to b-galactosidase indicator plates. With the wild-type allele of Tube and the lacZ reporter p1840 there was no stain and with pJK103, only a weak blue colour developed after 3 days. Plasmids of single blue colonies were isolated. After retest, the *tube* insert was

sequenced. RNA injection of the four *tube* alleles isolated into embryos from *tube* females rescued the dorsoventral pattern and did not induce filzkörper or ventral denticles in embryos from *snake* females.

4.6. Selection of Tube fragments containing the Pelle-binding domain

After sonication of pJG-tub and treatment with *Bal*31 nuclease and T4 DNA polymerase, DNA in the size of 500–750 bp was isolated by gel electrophoresis and cloned first into pUC18/*Sma*I and then as *Eco*RI-*Sal*I fragments into pJG4-5 (Kornau et al., 1995). This library was used for a two-hybrid screen with plex-pll and the lacZ reporter plasmid pSH18-34. The plasmid inserts of positive clones were amplified by PCR and sequenced.

4.7. Protein methods

The Pelle kinase assay was performed as described with His-tagged Tube and GST-Pelo expressed in *E. coli* and Pelle isolated by immuno-precipitation from extracts of wild-type embryos (Großhans et al., 1994). Recombinant GST, GST-Pli, GST-Tube, and Pli-His were expressed and purified as recommended by the manufacturers (Pharmacia, Qiagen). A Pellino specific antibody was induced with purified Pli-His in rabbits and purified with Pli-His coupled to Sepharose (Pharmacia). The antibody is specific for Pellino, since Pellino expressed in bacteria or in reticulocyte lysate is detected by the antibody. In the binding tests, equal amounts of GST proteins bound to GSH Sepharose in 500 ml washing buffer were mixed with 5 ml of reticulocyte lysate (Amersham) containing radiolabelled tester protein and then incubated for 1 h at 4°C. After removal of the supernatant the matrix was washed five times with washing buffer. Analysis of the samples were by SDS-PAGE and fluorography. For signal amplification the gel was incubated in 1 M Na-salicylate before drying. The washing buffer contained 20 mM Tris, pH 7.4, 200 mM NaCl, 10% Glycerin, 0.1% TritonX100, 1 mM PMSF, 2 mg/ml Leupeptin, 2 mg/ml Aprotinin, 1 mg/ml PepstatinA.

Acknowledgements

We thank I. Koch for the help in some of the experiments, S. Anderson, K. Bohmann, C. Brachthäuser, and D. Gilmore for suggestions and corrections of the manuscript. J.G. was supported by a fellowship of the Boehringer Ingelheim Stiftung, Stuttgart, FRG. The *pellino* cDNA sequence is accessible in Genbank with AF09624.

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